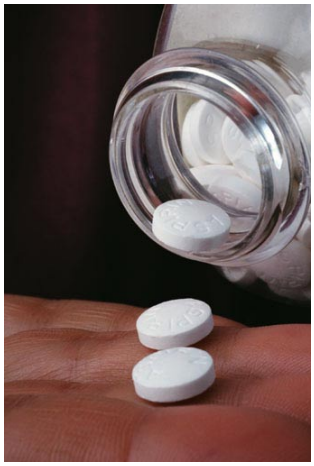
2nd International Conference on Pharmaceuticals and Endocrine Disrupting Chemicals in Water

October 9-11, 2001 _ Hyatt on Nicollet Mall _ Minneapolis, Minnesota



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- **Occurrence of Pharmaceutical Compounds in Ground and Surface Water**
- **Occurrence and Fate of Endocrine Disrupting Chemicals in Water**
- **Analysis of Pharmaceuticals and Endocrine Disrupting Chemicals**
- **Distribution and Effects on Wildlife**
- **Treatment Technologies for Pharmaceuticals and Endocrine Disrupting Chemicals**

Proceedings of the 2nd International Conference on Pharmaceuticals and Endocrine Disrupting Chemicals in Water
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Tuesday, October 9

11:00 a.m. – 5:00 p.m.	**Registration**
	Session I — Occurrence of Pharmaceuticals in Water
	Moderator — Herb Buxton, U.S. Geological Survey, Toxic Substances Hydrology Program
	Keynote Address
1:00 p.m. - 1:30 p.m.	**Pharmaceuticals as New Emerging Environmental Contaminants: A Survey —** Thomas Ternes, ESWE-Institut fuer Wasserforschung und Wassertechnologie Wiesbaden, Germany
1:30 p.m. - 1:55 p.m.	**A National Reconnaissance for Pharmaceuticals and Other Emerging Contaminants in Streams of the United States —** Dana W. Kolpin, Edward T. Furlong, Michael T. Meyer, E. Michael Thurman, Steven D. Zaugg, and Larry B. Barber, U.S. Geological Survey
1:55 p.m. - 2:20 p.m.	**Occurrence and Fate of Pharmaceutical Residues in the Aquatic System of Berlin as an Example for Urban Ecosystems —** Thomas Heberer and K. Reddersen, Institute of Food Chemistry, Technical University of Berlin
2:20 p.m. - 2:45 p.m.	**Pharmaceutical Residues in Ambient Water: Initial Surveillance in New Mexico, USA** Dennis McQuillan and Jennifer Parker, New Mexico Environment Department, David Mills, Ph.D.; Ken Sherrell, and Timothy H. Chapman, New Mexico Department of Health, Scientific Laboratory Division
2:45 p.m. - 3:00 p.m.	*Refreshment Break*
3:00 p.m. - 3:25 p.m.	**Factors Affecting the Concentrations of Pharmaceuticals Released to the Aquatic Environment** David L. Sedlak and Karen E. Pinkston, Department of Civil and Environmental Engineering, University of California, Berkeley

3:25 p.m. - 3:50 p.m.	**Assessment of Potential Antibiotic Contaminants in Water and Preliminary Occurrence Analysis** Ching-Hua Huang, Jay E. Renew, and Kristen L. Smeby, School of Civil and Environmental Engineering, Georgia Institute of Technology; Karen Pinkston and David L. Sedlak, Department of Civil and Environmental Engineering, University of California, Berkeley
3:50 p.m. - 4:15 p.m.	**Reconnaissance of Industrial and Household Use Chemicals and Pharmaceuticals in Selected Surface and Ground Water Resources in Minnesota, October 2000** Kathy E. Lee, Steve D. Zaugg, Jeffery D. Cahill, and Edward T. Furlong U.S. Geological Survey
4:15 p.m. - 4:40 p.m.	**Pharmaceuticals in the Environment: An Industry Perspective —** James P. Hagan, Ph.D., P.E, GlaxoSmithKline Pharmaceuticals and Health Care
4:40 p.m. - 5:05 p.m.	**Presence of Pharmaceuticals in Wastewater and Ground Water in Hawaii and Studies of Their Fate in Soil Following Irrigation with Reclaimed Water** Roger Babcock Jr. and Chittaranjan Ray, Department of Civil Engineering, University of Hawaii, Manoa
5:05 p.m. - 5:30 p.m.	**Occurrence of Antibiotics in Hospital Wastewater, Municipal Wastewater, and Surface Waters** Harald A. Faerber and Dirk Skutlarek, Institute of Hygiene, University of Bonn, Germany
5:30 p.m. – 6:30 p.m.	*Reception* — refreshments provided (cash bar)

Wednesday, October 10

Session I — Occurrence of Pharmaceuticals in Water (continued)

Moderator — James P. Hagan, Ph.D., P.E., GlaxoSmithKline Pharmaceuticals and Health Care

8:05 a.m. - 8:30 a.m.	**Occurrence and Behavior of Iodinated X-ray Contrast Media in Surface and Ground Water** Anke Putschew, Sabine Wischnack, and Martin Jekel - Technical University of Berlin, Department of Water Quality

8:30 a.m. - 8:55 a.m. **Comparison of Pharmaceuticals in Surface Water and Bed Sediments —** Jeffery D. Cahill, Imma Ferrer and Edward T. Furlong, U.S. Geological Survey, National Water Quality Laboratory, Denver

8:55 a.m. - 9:20 a.m. **Presence of Pharmaceuticals in Treated Wastewater Effluent and Surface Water Supply Systems, Metropolitan Atlanta, Georgia, July–September 1999** Deborah M. Moll, Ph.D.[1], Elizabeth A. Frick[2], Alden K. Henderson, Ph.D., M.P.H.[1], Edward T. Furlong, Ph.D. [3], Michael T. Meyer, Ph.D.[4] ([1]Centers for Disease Control and Prevention, National Center for Environmental Health, Atlanta: [2]U.S. Geological Survey, Atlanta; [3]Geological Survey, Denver Federal Center, Denver; [4]Geological Survey, Ocala, Florida)

9:20 a.m. - 9:45 a.m. **Occurrence of Bacteriostatic Sulfonamide Drugs in Surface Waters and Their Behaviour in Bank Filtration** Claudia Hartig and Martin Jekel, Technical University of Berlin, Department of Water Quality Control

9:45 a.m. - 10:00 a.m. *Refreshment Break*

Session II — Analysis of Pharmaceuticals and Endocrine Disrupting Chemicals

Moderator — Ira Adelman, Ph.D., Department of Fisheries and Wildlife, University of Minnesota

10:00 a.m. - 10:25 a.m. **Comparison of Solid Phase Microextraction, Solid Phase Extraction, and Liquid Liquid Extraction Coupled to GC/MS for Trace Analysis of Endocrine Disrupters in Wastewater —**Pamela Wild and Monika Moeder, UFZ, Centre for Environmental Research Leipzig-Halle GmbH, Liepzig, Germany

10:25 a.m. - 10:50 a.m. **On-Line Solid-Phase Extraction and Fluorescence Detection of Selected Endocrine Disrupting Chemicals in Water by High-Performance Liquid Chromatography —** Guang-Guo Ying, Rai Kookana, and Zuliang Chen, CSIRO Land and Water, Adelaide Laboratory, Australia

10:50 a.m. - 11:15 a.m. **Analysis of Selected Antibiotics from Water Associated with Fish Hatcheries —** E.M. Thurman[1], M.E. Lindsey[1] and Hans-Christian Lutzhoft[2] ([1]U.S. Geological Survey; [2]Danish Royal School of Pharmacy, Copenhagen, Denmark)

11:15 a.m. - 11:40 a.m.	**In-Situ Solid-Phase Extraction and Analysis of Ultra-Trace Synthetic Musks in Municipal Sewage Effluent Using Gas Chromatography-Mass Spectrometry, Full-Scan Mode —** L. I. Osemwengie, U.S. Environmental Protection Agency, National Environmental Research Laboratory, Environmental Sciences Division; S. Steinberg, Environmental Chemistry Department. University of Nevada Las Vegas
11:40 a.m. – 12:00 p.m.	**Emerging Approaches for Assessing the Exposure of Children to Endocrine Disruptors —** M.K. Morgan[1], D. A. Vallero[1], J.C. Chuang[2], C. Lyu[3], and N.K. Wilson[3], [1]National Exposure Research Laboratory, U. S. Environmental Protection Agency; [2]Battelle, Columbus, Ohio; [3]Battell, Durham, North Carolina
12:00 p.m. - 1:00 p.m.	*Lunch Provided*

Session III — Endocrine Disrupting Chemicals: Occurrence and Fate

Moderator — Greg Sayles, U.S. EPA National Risk Management Research Laboratory

1:00 p.m. - 1:25 p.m.	**Occurrence and Screening of Treatment Alternatives for PPCPs and EDCs in Waters of Southeastern Louisiana —** Glen R. Boyd, Ph.D., P.E., Civil and Environmental Engineering Department; Deborah A. Grimm, Ph.D., Coordinated Instrumentation Facility, Tulane University
1:25 p.m. - 1:50 p.m.	**Occurrence, Distribution, and Fate of 4-Nonylphenol In Kansas Domestic Wastewater Treatment Plants** Dr. K. Xia, H.L. Keller, A.J. Wagner, and Dr. A. Bhandari, Kansas State University
1:50 p.m. - 2:15 p.m.	**Presence of Wastewater Tracers and Endocrine Disrupting Chemicals in Treated Wastewater Effluent and in Municipal Drinking Water, Metropolitan Atlanta, 1999 —** Alden K. Henderson Ph.D., MPH, and Deborah M. Moll, Ph.D., Centers for Disease Control and Prevention; Elizabeth A. Frick and Steven D. Zaugg, Ph.D., U.S. Geological Survey
2:15 p.m. - 2:40 p.m.	**Processes Affecting the Fate of Nonylphenol During Wastewater Treatment —** Andrea Porter and Dr. Nancy Hayden, University of Vermont

2:40 p.m. - 3:05 p.m.	**Monitoring of Pesticide Residues in Surface and Underground Water by Capillary Gas Chromatography Using C18 Column —** J.K. Dubey and Dr. Y.S. Parmar, University of Horticulture and Forestry, Nauni, Solan, India
3:05 p.m. - 3:20 p.m.	*Refreshment Break*
3:20 p.m. - 3:45 p.m.	**Fate of the Endogenous Hormones 17ß-Estradiol and Testosterone in Composted Manure** — Heldur Hakk, Patricia Millner, and Gerald Larsen, USDA-ARS
3:45 p.m. - 4:10 p.m.	**Sorption, Mobility and Fate of 17ß-Estradiol, Testosterone and Flame Retardant Tetrabromobisphenol A (TBBPA) in Loam Soil and Sand** — G. Larsen, F. Casey, B. Magelky, C. Pfaff, and H. Hakk, USDS-ARS
4:10 p.m. - 4:35 p.m.	**Occurrence of Antibiotics, Pesticides, and Pesticide Degradates in Agricultural Runoff, Stillwater River Basin, Southwest Ohio —** Gary L. Rowe and Michael T. Meyer, U.S. Geological Survey; Kammy D. Johnson, and Carol H. Rubin, Centers for Disease Control and Prevention
4:35 p.m. - 5:00 p.m.	**Occurrence and Fate of Pharmaceuticals During Bank Filtration: Results from German and U.S. Investigations —** Thomas Heberer[1], Ingrid M. Verstraeten[2], Michael T. Meyer[2], A. Mechlinski[1], and K. Reddersen[1] ([1]Institute of Food Chemistry, Technical University of Berlin; [2] U.S. Geological Survey)
5:00 p.m. – 5:25 p.m.	**The Qualitative and Quantitative Evaluation of Estrogen and Estrogen-Mimicking Substances in the South African Water Environment: A 1996-1997 Perspective —** L. Brown, J.L. du Preez, and E. Meintjies
5:25 p.m. - 6:30 p.m.	*Reception* — refreshments provided (cash bar)

Thursday, October 11

Session IV — Distribution and Effects on Wildlife

Moderator — Chris Metcalfe, Trent University

8:00 a.m. – 8:25 a.m.	**The Toxicity of Three Pharmaceutical Agents to Soybean Plants —** Patrick K. Jjemba, Laboratory of Soil Microbiology, Department of Soil, Crop and Atmospheric Sciences, Cornell University

8:25 a.m. - 9:00 a.m.	**Analysis for Environmental Estrogens Using cDNA Microarrays —** Leroy C. Folmar, U.S. EPA National Health and Environmental Effects Research Laboratory
	Altered Serum Sex Steroids and Vitellogenin Induction in Walleye Collected Near a Major Metropolitan Sewage Treatment Plant —Leroy C. Folmar, U.S. EPA National Health and Environmental Effects Research Laboratory
9:00 a.m. - 9:25 a.m.	**Drugs in Canadian Surface Waters: Distribution and Effects on Fish** — Chris Metcalfe, Tom Moon, Vance Trudeau, and John Struger, Environmental and Resource Studies, Trent University
9:25 a.m. - 9:50 a.m.	**Effects of Environmental Estrogens on Reproductive Health of Male Walleye and Carp Near a Major Metropolitan Sewage Treatment Plant —** James T. Levitt, Heiko L. Schoenfuss, and Ira R. Adelman, University of Minnesota, Department of Fisheries, Wildlife, and Conservation Biology
9:50 a.m. - 10:15 a.m.	**Data from an Estrogen Receptor-based Biosensor Correlates with Evidence of Frog Malformation and Demonstrates a Differential Response of hER-α & β to Beneficial and Harmful Estrogenic Compounds —**Judith L. Erb[1], Eric A.E. Garber[2], James G. Downward IV[1], Eric M. Priuska[1], James L. Wittliff[3], and Joe Magner[4] ([1]IA Inc., [2]USDA-ARS Biosciences Research Laboratory, [3]University of Louisville, Kentucky; [4] Minnesota Pollution Control Agency)
10:15 a.m. - 10:30 a.m.	*Refreshment Break*
10:30 a.m. - 10:55 a.m.	**Mineral Deficiency, Estrogenic Activity, and Occurrence of Malformed Frogs in Minnesota** Eric A.E. Garber[1], Judith L. Erb[2], Amy M. McGarvey[1], Gerald L. Larsen[1], Joseph Magner[3] ([1]USDA-ARS; Biosciences Research Laboratory; [2]ThreeFold Sensors, [3]Minnesota Pollution Control Agency)
10:55 a.m. - 11:20 a.m.	**Effects of Water-Borne 17β-Estradiol on Reproductive Fitness in Two Species of Fish** Heiko L. Schoenfuss, Dalma Martinovic, and Peter W. Sorensen, University of Minnesota, Department of Fisheries, Wildlife, and Conservation Biology

11:20 a.m. - 11:45 a.m. *Questions and Answers*

Panel Discussion and Open Microphone

Panelists: Leroy Folmar, U.S. EPA; Chis Metcalfe, Trent University; Dana Kolpin, USGS; James Hagan, GlaxoSmithKline; Thomas Heberer, Technical University of Berlin; Thomas Ternes, Institut fuer Wasserforschung und Wassertechnologie

11:45 a.m. - 1:00 p.m. *Lunch* (on your own)

Session V — Treatment Technologies for Pharmaceuticals and EDCs

Moderator — Thomas Heberer, Technical University of Berlin

1:00 p.m. - 1:25 p.m. **Removal of Pharmaceutical Residues and Other Persistent Organics from Municipal Sewage and Surface Waters Applying Membrane Filtration**
T. Heberer[1], D. Feldmann[2], K. Reddersen[1], H. Altmann[3], T. Zimmermann[4,] ([1]Institute of Food Chemistry, Technical University of Berlin; [2]Central Institute of Bundeswehr Medical Service; [3]Bundeswehr Scientific Institute for Protection, Technologies – NBC – Protection; [4]Federal Ministry of Defence – Medical Service)

1:25 p.m. - 1:50 p.m. **Natural Attenuation of Pharmaceuticals**
Traugott Scheytt[1], Petra Mersamann[1] and Thomas Heberer[2] ([1]Technical University of Berlin, Institute for Applied Geosciences; [2]Technical University of Berlin, Institute for Food Chemistry)

1:50 p.m. – 2:15 p.m. **Role of Subsurface Wetland Treatment in Fate of Endocrine Disrupters in Wastewater**
David M. Quanrud[2], Karen D. Turney[2], Otakuye Conroy[2], Martin M. Karpiscak[3], Kevin E. Lansey[1], and Robert G. Arnold[2] ([1]Department of Civil Engineering and Engineering Mechanics, The University of Arizona; [2]Department of Chemical and Environmental Engineering, The University of Arizona; [3]Office of Arid Lands Studies, The University of Arizona)

2:15 p.m. - 2:40 p.m.	**Removal of Pharmaceuticals During Conventional Wastewater Treatment, Advanced Membrane Treatment and Soil-Aquifer Treatment** Jörg E. Drewes[1], Thomas Heberer[2,] and Kirsten Reddersen[2]([1]National Center for Sustainable Water Supply, Arizona State University, [2]Institute for Food Chemistry, Technical University of Berlin)
2:40 p.m. - 3:05 p.m.	*Refreshment Break*
3:05 p.m. - 3:30 p.m.	**Removal of Endocrine Disrupting Compounds Using Drinking Water Treatment Processes** John L. Cicmanec, DVM, MS, U.S. EPA
3:30 p.m. - 4:05 p.m.	**Routes of Groundwater Contamination with Pharmaceuticals and the Removal of Drug Residues During Water Treatment Processes** Thomas Ternes[1], Martin Meisenheimer[1], Helfried Welsch[1], R.D. Wilken[1], Harald Rückert[2], and Georg Berthold[2] ([1]ESWE-Institut fuer Wasserforschung und Wassertechnologie, [2]Hessische Landesanstalt fuer Umwelt, Wiesbaden, Germany)
4:05 p.m.	*Conference Adjourns*

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Pharmaceuticals as New Emerging Environmental Contaminants: A Survey

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Abstract

Up to date only few investigations have been published about the assessment of the environmental relevance of pharmaceuticals, although tons of those compounds are used every year since several decades. The fate of veterinary and human drugs after urinal or fecal excretion are quite different to each other. In general, excreted human pharmaceuticals have to pass through a STP prior to entering rivers or streams, whereas veterinary drugs are more likely to contaminate soil and groundwater (without previous waste water treatment) when liquid manure is used for top soil dressing. Additionally, after rainfall incidents surface waters can be polluted with human or veterinary drugs by run-off from fields treated with digested sludge or livestock slurries respectively. Industrial wastewater may be another possible source for the contamination of surface waters.
36 of 55 pharmaceuticals and 5 of 9 metabolites were quantified in at least one STP effluent. In general, the removal rates in the investigated municipal STP exceeded 60 %. The highest concentrations of drug residues were measured for the antiepileptic carbamazepine with a maximum of 6.3 µg/L. However, diagnostics were found in concentrations as high as 15 µg/L for iopamidol and 11 µg/L for iopromide. In 40 German rivers and streams 31 pharmaceuticals and five metabolites were quantified in at least one sample. Highest median values were detected for bezafibrate with 0.35 µg/l and carbamazepine with 0.25 µg/L. In ground water samples taken close to the bank of streams sometimes relatively high concentrations of pharmaceuticals up to 2.4 µg/L were detected. In drinking water only 9 of 65 pharmaceuticals analyzed were found without exception in the lower ng/L-range. However, basically the analytes could not be identified in drinking water.
An environmental risk assessment for the drugs detected in river and stream waters is yet not possible, due to the lack of ecotoxicological data. In the European Union as well as in other countries physico-chemical data like Koc or Pow and in particular ecotoxicological data like LC_{50}, EC_{50}, NOEC are not mandatory for the governmental approval of human medicinal products.

Introduction

First results about the occurrence of pharmaceutical residues in the environment were published by *Garrison et al.* (1976) from the Environmental Protection Agency (US-EPA), who found clofibric acid and salicylic acid in a municipal sewage treatment plant (STP) of Athens (United States) with 1-2 µg/L. These two compounds were also found by *Hignite and Azarnoff* (1977) in a STP of Kansas City with relative high concentration levels of 1-95 µg/L. In Canada the pharmaceuticals ibuprofen, clofibric acid and naproxen were identified in wastewater by *Rogers et al.* (1986). However, all these results and publications did not lead to further investigations in the area of the environmental occurrence and relevance of pharmaceuticals in the environment. In Europe, the first comprehensive study about the occurrence of pharmaceuticals in the environment were reported by *Richardson and Bowron* (1986). These investigations in the United Kingdom revealed that drugs were present in the aquatic environment at concentrations up to approximately 1 µg/L, whereas the exact concentrations for the individual drugs were not always determined. Concentration levels up to the µg/L level were also predicted by *Van der Heide* and *Hueck-Van der Pals* (1984). In Germany, clofibric acid has been identified in river and groundwater and even in drinking water with concentration levels ranging up to 0.165 µg/L by *Stan and Linkerhäger* (1972) and *Heberer and Stan* (1994).
Recently, a multitude of pharmaceuticals and metabolites from distinctive medicinal classes such as anti-inflammatory agents, betablockers, β_2-sympathomimetics, antiepileptics, antibiotics, contrast media, lipid regulators, contraceptiva, antineoplastics were identified in wastewater and rivers (Möhle *et al.* 1999; Stumpf *et al.*, 1996, 1998, 1999; Hirsch *et al.*, 1996, 1999; Stan *et al.*, 1994; Ternes, 1998; Ternes *et al.*, 1999a, 1999b; Steger-Hartmann *et al.*, 1996; Belfroid *et al.* 1999; Heberer, 1996, 1997; Sacher *et al.*, 1998; Buser *et al.* 1998, 1999; Wischnack, 1998). The two reviews *Halling-Sørensen et al.* (1998) and *Daughton and Ternes* (1999) summarize the literature in

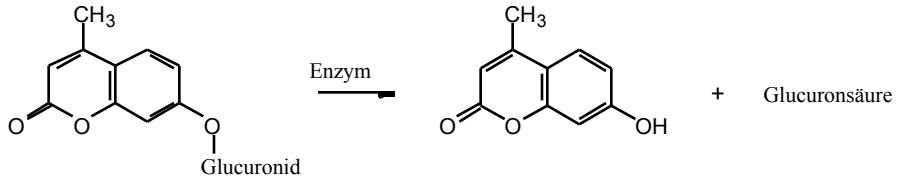
Table 1: Estimation of prescribed quantities of selected pharmaceuticals used in human medicine in Germany

Substance	number of precribed dosis 1994 in Mill	amount of a daily dosis in mg	**precribed annual quantity** **1994** **in tons**	non-metabolized excretion in %	excreted metabolites glucuronide in %	 other in %
Lipid regulator						
Bezafibrate	84,8	400-600	**34-51**	50	22	28
Fenofibrate	60,0	200-300	**12-18**			
Etofibrate	10,8	500-900	**6-10**			principal share
Etofyllinclofibrate	11,7	500	**6**			principal share
Betablocker						
Metoprolol	196,7	100-200	**20-40**	3-10		principal share
Propanolol	59,2	40-80	**2-4**	<1		principal share
Betaxolol	11,3	20-40	**0.3-0.6**	12-19		principal share
Sympathomimetics						
Fenoterol	457,0	1	**0,5**	0-30	--	70-100
Salbutamol	119,0	1-16	**0,1-2**	30-50	--	50
Terbutalin	45,9	1-15	**0.05-0.7**	60-70	--	50
Antiphlogistics						
Diclofenac	514,8	100-150	**51-77**			principal share
Ibuprofen	105,9	600-1200	**64-127**	1-8	+++	14
Indomethacine	51,3	50-200	**3-10**	10-20	80	
Acetylsalicylic acid	137,0	100-500	**14-69**	1-5	< 20	>80
Paracetamol	153,9	500-3500	**77-539**	3-10	55-60	35
Metamizol	42,2	500-2500	**21-106**			principal share

+++: Metabolites are formed in a higher extent

Metabolites

Due to the high application level, detectable concentrations of drugs and their metabolites can be expected in wastewater. The concentrations are dependent on the drugs pharmacokinetical behavior (half life, urinary and fecal excretion, metabolism etc.). However, metabolites formed by conjugation (e.g. with glucuronic acid, sulfate) within phase II reactions are likely cleaved in the environment into the non-metabolized (free) pharmaceuticals, and hence may increase the relevant environmental concentrations (Fig. 1). Many pharmaceuticals are excreted mainly as metabolites, and thus in addition to the non-metabolized compounds principal metabolites have to be analyzed.



4-Methylumbelliferyl-β-Glucuronide 4-Methylumbelliferon

Fig. 1: Cleavage of glucuronides

Analytical Methods

In general, no analytical methods are available for environmental matrices allowing limits of quantification (LOQ) down to the ng/L-range, although tons of pharmaceuticals are used every year since several decades. Therefore, in our laboratory analytical procedures were elaborated for a total of 84 pharmaceuticals and metabolites, enabling the simultaneous determination of polar drug residues belonging to different medicinal classes such as lipid regulators, antibiotics, estrogens and metabolites in sewage and in drinking water.

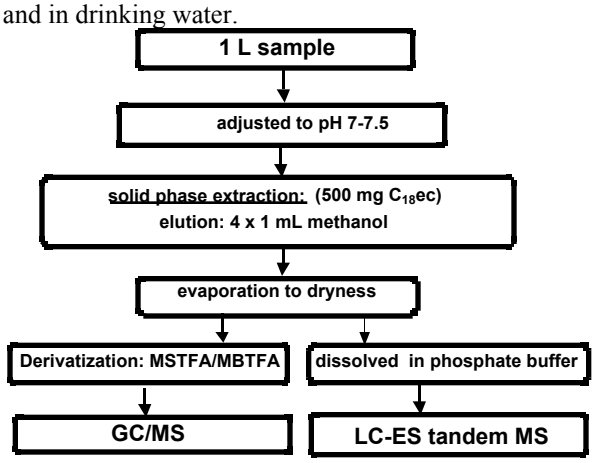


Fig. 2: Scheme of the analytical method to analyse betablockers and other neutral pharmaceuticals

Several methods were applied for the determination of drugs and their metabolites in the lower ng/L-range using solid phase extraction, derivatization, detection and confirmation by GC/MS and GC/MS/MS or especially LC-electrospray/MS/MS. The determination of betablockers and β_2-sympathomimetics as well as neutral drugs like diazepam or carbamazepine at concentrations in the ng/L-range was performed after a joint solid phase extraction using detection by GC/MS or LC-electrospray/MS/MS (Fig. 2, Ternes *et al.*, 1998a). Another multi-method allows the determination of acidic drugs with one carboxylic moiety and additionally in some cases with one or two hydroxy groups down to lower ng/L-range (Ternes *et al.*, 1998b). At least 18 antibiotics can be analyzed simultaneously after lyophilization without any further clean-up step (Hirsch *et al.*, 1998). Contrast media were determined after SPE using LC-electrospray tandem MS detection down to 10 ng/L (Hirsch *et al.*, 2000).

Contamination of wastewater with pharmaceuticals

Composite samples from a German municipal STP were taken daily from the raw influent and the corresponding final effluent over a period of 6 days. Sampling was carried out by a flow proportional automatic sampler, whereby the composite samples of the final effluent were taken time related to the influent. The municipal STP near Frankfurt/Main is connected to about 312,000 population equivalents. It consists of three commonly used main treatment steps: preliminary clarification followed by an aeration tank with the addition of Fe(II)chloride for phosphate elimination and finally end point clarification. All cooled water samples were analyzed within two days in order to keep microbial degradation to a minimum. The elimination rates of the investigated drugs during passage through the STP ranged from 18 % (trimetoprim) to greater than 90 % (sulfamethoxazole) (Ternes 2001). Table 2 exhibits large variations in the elimination efficiency of acidic pharmaceuticals. Obviously, the removal of pharmaceuticals is depending on several STP parameters. One crucial parameter seems to be the temperature (Ternes, 1998). Betablockers exhibited in general relative high removal efficiencies .

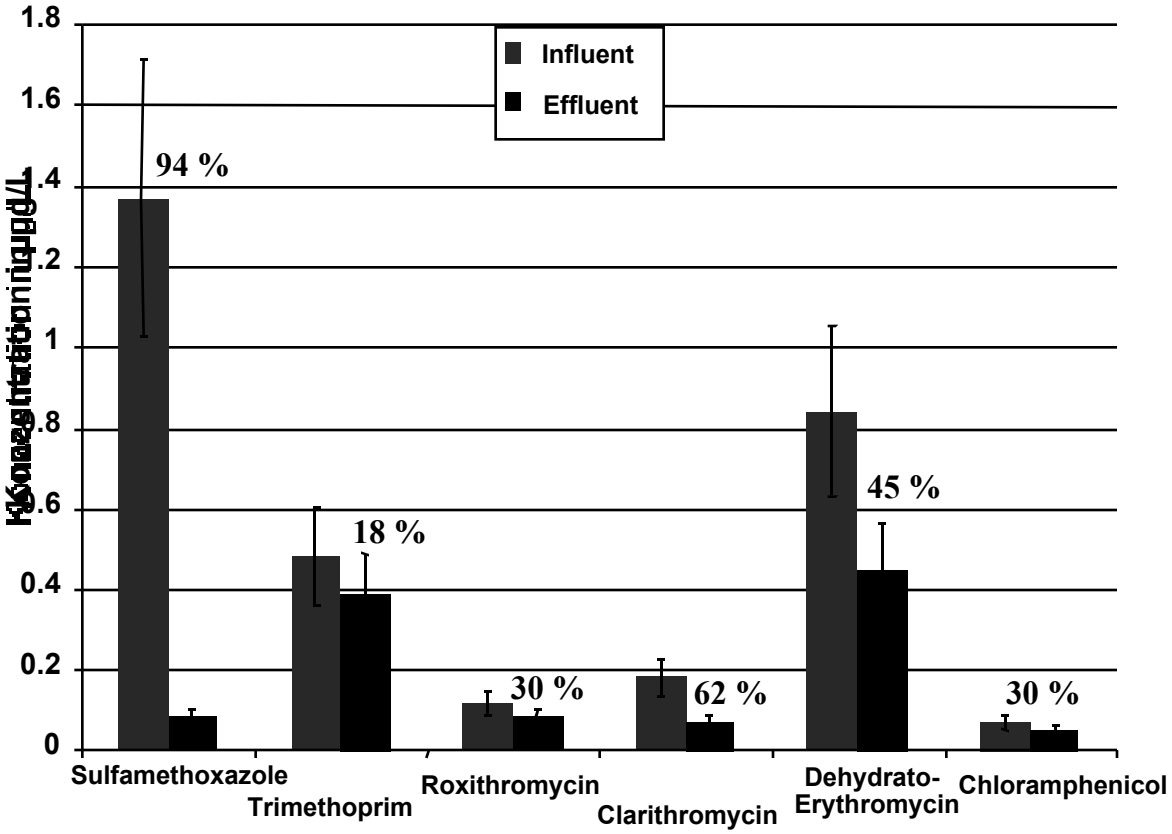


Fig. 2. Elimination of drugs during passage through a municipal sewage treatment plant near Frankfurt/Main over 6 days

Table 2 and 3 show the mean concentrations of the municipal wastewater treatment plant close to Frankfurt/Main. In the influent the concentration levels are ranging up to 10 µg/L for propranolol and in the effluent up to 2.6 µg/L for bezafibrate. In total 36 of 55 pharmaceuticals and 5 of 9 metabolites were quantified in at least one STP effluent of 49 municipal facilities. The highest concentrations of drug residues were measured for the antiepileptic carbamazepine with a maximum of 6.3 µg/L (Ternes, 1998). However, diagnostics were found in concentrations as high as 15 µg/L for iopamidol and 11 µg/L for iopromide (Ternes and Hirsch, 2000). With exception of contrast media, the *lipid regulators* bezafibrate and gemfibrozil, the *anti-inflammatory agents* diclofenac, ibuprofen, indometacine, naproxen, ketoprofen and phenazone, the *betablockers* metoprolol, sotalol and propranolol, and the *antiepileptic* carbamazepine were present with elevated concentrations in the majority of the 49 German municipal STP effluents investigated. Table 2 exhibits variation of elimination efficiencies of a STP at two periods with different outside temperatures. It can be assumed that the temperature is one of the crucial parameters, however further investigations are essential to elucidate those consumptions.

Table 2: Mean concentration and removal efficiencies (1σ, n = 5) of selected acidic pharmaceuticals in the combined influent and the effluent of a municipal wastewater

treatment plant close to Frankfurt/Main. Average flow rate: 28.01.-02.02.1997: 55320 m^3/d; 23.-30.11.1997: 41200 m^3/d (Ternes, 2001)

Substance	Nov. 1997			Feb. 1997		
	Average temperature: -2° C			Average temperature: 5° C		
	influent in µg/L	effluent in µg/L	elimination in %	influent in µg/L	effluent in µg/L	elimination in %
Clofibric acid	0.86 ± 0.17	0.54 ± 0.06	38	1.24 ± 0.21	0.60 ± 0.10	51
Fenofibric acid	0.81 ± 0.14	0.68 ± 0.05	16	1.06 ± 0.19	0.38 ± 0.06	64
Bezafibrate	2.99 ± 0.46	2.65 ± 0.19	12	5.6 ± 1.78	0.96 ± 0.27	83
Gemfibrozil	2.07 ± 0.21	1.22 ± 0.03	41	0.94 ± 0.13	0.29 ± 0.07	69
Ibuprofen	4.31 ± 0.72	1.79 ± 0.12	58	4.36 ± 0.55	0.45 ± 0.14	90
Diclofenac	1.87 ± 0.19	1.29 ± 0.09	31	1.85 ± 0.23	0.58 ± 0.03	69
Ketoprofen	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Indometacine	0.82 ± 0.03	0.52 ± 0.01	38	0.29 ± 0.08	0.07 ± 0.02	75
Naproxen	1.22 ± 0.05	0.80 ± 0.06	35	1.32 ± 0.16	0.45 ± 0.09	66
Acetylsalicylic acid	n.a.	n.a.	n.a.	3.4 ± 0.70	0.64± 0.10	81

n.a.: not analysed (was not in the monitoring program)

Tabelle 3: Mean concentration and removal efficiencies (1σ, n = 5) of betablockers and sympathomimetics in the combined influent and the effluent of a municipal wastewater treatment plant close to Frankfurt/Main. Average flow rate: 24.-30.06.1996: 54300 m^3/d (Ternes, 2001)

Substance	Mean conc. influent in µg/l	Mean conc. effluent in µg/l	load influent in g/d	load effluent in g/d	elimination in %
Propranolol	10.0±2.4	0.41±0.07	520±103	21±5	95±1
Metoprolol	7.2±3.4	2.08±0.36	374±159	108±22	67±11
Bisoprolol	0.41±0.08	0.14±0.02	20±3	5±2	65±9
Betaxolol	0.87±0.15	0.17±0.03	46±6	9±2	80±5
Terbutalin	0.37±0.11	0.11±0.01	19±6	6±1	67±18
Salbutamol	0.21±0.13	< 0.10	9±6	< 1	100±1
Carazolol	0.19±0.03	0.07±0.02	10±1	3±1	66±8
Fenoterol	n.a.	< 0.10	n.a.	< 1	n.a.
Clenbuterol	n.a.	0.027±0.002	n.a.	n.a.	n.a.
Timolol	n.a.	0.036±0.006	n.a.	n.a.	n.a.
Nadolol	n.a.	0.11±0.01	n.a.	n.a.	n.a.

n.a.: not analysed (was not in the monitoring program)

Contamination of Wastewater With Estrogens and Contraceptives

In the German municipal STP close to Frankfurt/Main the raw sewage was contaminated by 17β-estradiol and estrone with average concentrations of 0.015 µg/L and 0.027 µg/L respectively, yielding loads up to 1 g/d (Ternes et al., 1999a). The evaluated removal rates were much lower than those obtained in the Brazilian STP. For instance, the loads of estrone and 17α-ethinylestradiol were not appreciably reduced while passing through the German STP. Considering the standard deviation no elimination rate could be evaluated. However, 16α-hydroxyestrone and 17β-estradiol

fact that the efficiency of a STP for the elimination of drugs is influenced by several parameters such as microbial activity or rain events (Ternes, 1998), only a long term study can reveal, whether the differences are as a result of increased temperatures or whether other contributory factors were responsible. However, 17β-estradiol and 16α-hydroxyestrone were basically eliminated with a higher efficiency than 17α-ethinylestradiol and estrone.

Aerobic batch experiments revealed that in contact with activated sludge the natural estrogen 17β-estradiol was oxidized to estrone, which was eliminated in a nearly linear time dependence (Ternes *et al.*, 1999b). Degradation products of estrone could not be observed. The contraceptive 17α-ethinylestradiol was principally persistent under the selected aerobic conditions. Two glucuronides of 17β-estradiol (17β-estradiol-17-glucuronide and 17β-estradiol-3-glucuronide) were cleaved in contact with the diluted activated sludge solution and 17β-estradiol was released. Hence, it is very likely that the glucuronic acid moiety of 17β-estradiol glucuronides and also of other glucuronides are cleaved in a real municipal STP, thus the concentrations of the free estrogens are increased.

In STP discharges mainly the natural estrogens estrone, 17β-estradiol, 16α-hydroxyestrone as well as the synthetically altered contraceptive 17α-ethinylestradiol could be found in the lower ng/L-range (Ternes *et al.*, 1999b). Mestranol was only present in two samples and 17β-estradiol-17-valerate could not be detected at all.

In German STP predominantly estrone was determined with a maximum concentration of 0.070 µg/L and a median value of 0.001 µg/L. Additionally, median values could be evaluated for 16α-hydroxyestrone with 0.001 µg/L (quantification limit). The contamination of STP discharges are within the concentration ranges found by Aherne and Briggs (1989), Wegener *et al.* (1999), Belfroid *et al.* (1999), Desbrow *et al.* (1998) and Routledge *et al.* (1998). In UK Desbrow *et al.* (1998) reported also that natural estrogens like 17β-estradiol or estrone were dominant in STP effluents compared to the synthetic 17α-ethinylestradiol and mestranol.

Contamination of Rivers and Streams with Pharmaceuticals

In 40 German rivers and streams 31 pharmaceuticals and 5 metabolites were quantified in at least one sample (Ternes, 1998). Highest median values were detected for bezafibrate with 0.35 µg/L and carbamazepine with 0.25 µg/L. Acidic drugs and the betablockers metoprolol and propranolol were found in small rivers and streams at higher concentrations than in the larger rivers, Rhine and Main. In general residues of acidic drugs in the river Rhine (*Mainz)* and Main (*Bischofsheim)* ranged from 0.05 µg/L to 0.3 µg/L (Fig. 3). With the exception of phenazone and dimethylaminophenazone the maximum and 90 percentile concentrations were always observed in streams and not in rivers. Phenazone and dimethylaminophenazone were present in the river Main up to 0.95 µg/L, presumably due to an industrial STP discharge. Additionally, a multitude of drugs (up to 20) were detectable in several investigated streams up to concentration higher than 6 µg/L (Fig. 3). These streams, located in the Hessian Ried (center of Germany) contain a high percentage of STP discharges, as indicated by their relatively high boron concentrations, ranging from 0.25 to 1.0 mg/L (Fooken *et al.*, 1996). Due to the fact that the STP discharges are highly contaminated by those drugs which are present in these streams, it can be concluded, that the enhanced percentage of municipal STP effluents are responsible for the elevated concentrations of pharmaceuticals.

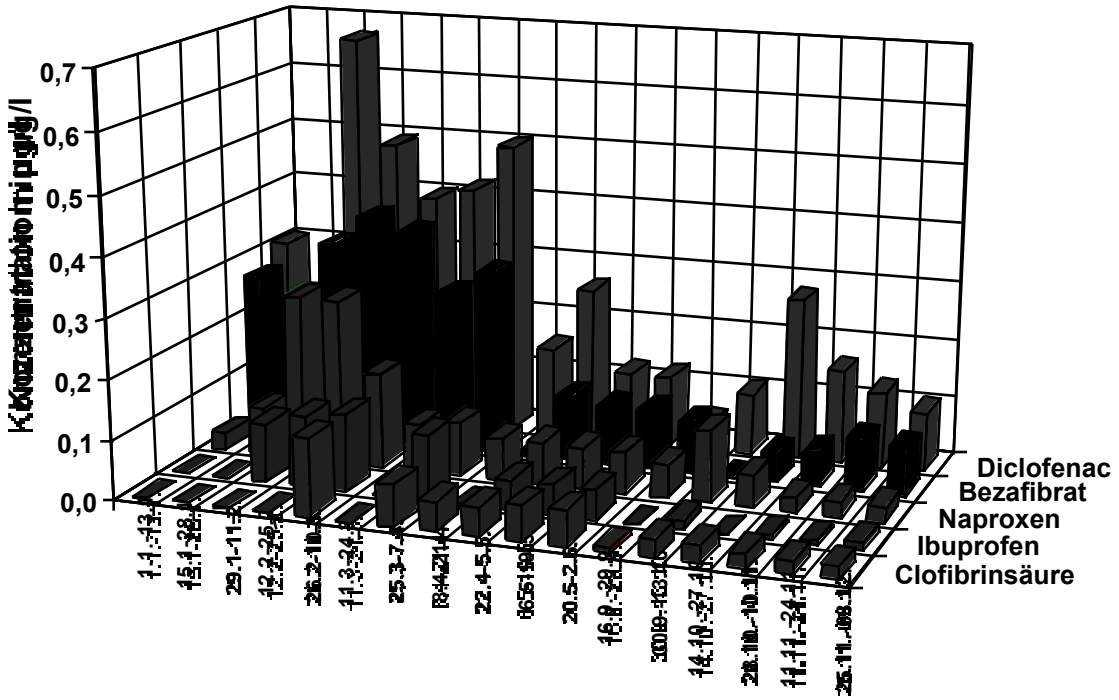


Fig. 3: Contamination of the Rhine/Mainz with pharmaceuticals

Table 4: Concentration ranges of pharmaceuticals, phenolic antiseptics and X-ray contrast media in German surface waters (1996-1998)

Median > 0,05 µg/L	**Median 0,05-0,01 µg/l**	**Median < LOQ; 90-Perzentile > LOQ**	**90-Perzentile < LOQ; Maximum > LOQ**
Lipid regulator	*Lipid regulator metab.*	*Antiphlogistics*	*Antiphlogistics*
Bezafibrate	Fenofibric acid	Ketoprofen	Dimethylaminophenazonee
Gemfibrozil	*Antiphlogistics*	Acetylsalicylic acid	*Betablocker*
Lipid regulator -metabolite	Indometacine	*ASA metabolite*	Betaxolol
Clofibric acid	Phenazone	Salicylic acid	Timolol
Antiphlogistics	*Betablockers*	Gentisic acid	*sympathomimetics*
Diclofenac	Metoprolol	*Betablocker*	Salbutamol
Ibuprofen	Propranolol	Bisoprolol	Fenoterol
Naproxen	*Antibiotic*	Carazolol	Clenbuterol
Antiepileptic	Sulfamethoxazole	*Antibiotics*	*Antibiotikum*
Carbamazepine	*X-ray contrast media*	Clarithromycin	Chloramphenicol
Antibiotic metabolite	Ioxithalamic acid	Roxithromycin	*Antiseptics*
Dehydrato-Erythromycine	*Antiseptics*	Trimethoprim	Tetrabromocresol
X-ray contrast media	Clorophene	*X-ray contrast media*	4-Chlorxylenol
Diatrizoate	Biphenylol	Iothalamic acid	
Iopamidol			
Iopromide			
Iomeprol			

LOQ: Limit of Quantification

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A National Reconnaissance for Pharmaceuticals and Other Emerging Contaminants in Streams of the United States

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Abstract

To provide the first nationwide investigation of the occurrence of pharmaceuticals, hormones and other organic wastewater contaminants (OWCs) in streams of the United States, the U.S. Geological Survey's Toxic Substances Hydrology Program conducted a reconnaissance study of 142 streams in 32 states during 1999 and 2000. Sampling sites were biased toward streams susceptible to contamination (i.e. downstream of intense urbanization and livestock production). Samples were analyzed for 95 OWCs (during the 1999 sampling not all target compounds were analyzed at all streams sampled). Almost 90% of the streams sampled had a detection of one or more of these compounds. The compounds detected represent a wide range of residential, industrial, and agricultural origins and uses with 77 of the 95 OWCs being found. Detergent metabolites, plasticizers, and fecal sterols contributed almost 80% of the total measured concentration (sum concentration of detected compounds in any given sample) for this study. The detection of multiple OWCs was common, with a median of 14 compounds detected per stream sample (maximum of 31 compounds). Little is known about the potential interactive effects (synergistic or antagonistic toxicity) that may occur from complex mixtures of these compounds in the environment.

The results of this study are not only intended to determine the extent to which OWCs are present in potentially susceptible streams, but to also provide a focal point for the development and testing of new laboratory methods for measuring OWCs in environmental samples at trace levels, an interpretive context for future assessments of OWCs, and a means for establishing research priorities and future monitoring strategies.

Biographical Sketch

Dana W. Kolpin is a research hydrologist and has worked for the USGS since 1984. His research interests include the occurrence of pesticides, pharmaceuticals, and other emerging contaminants in the environment.

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Occurrence and Fate of Pharmaceutical Residues in the Aquatic System of Berlin as an Example for Urban Ecosystems

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Abstract

In recent years, the occurrence and fate of pharmaceutically active compounds (PhACs) in the aquatic environment was recognized as one of the emerging issues in environmental chemistry and as a matter of public concern. Residues of PhACs have been found as contaminants in sewage, surface and groundwater samples. The results obtained from several occurrence studies provided some important information on the origin, distribution, and fate of these contaminants in the aquatic environment. However, a continuous study on the occurrence and fate of these compounds in all compartments of a water cycle has, until today, not been carried out. Often only random samples were analyzed and sampling was not repeated.

Since June 2000, a new long-term monitoring program of sewage, surface, and drinking water is carried out in Berlin, Germany. The city of Berlin represents an excellent location to study the occurrence and fate of PhAC residues in the water cycle of an urban environment. Berlin is among those areas with the highest population densities in Europe, the surface waters, where the municipal sewage effluents are being discharged, have relatively low flow rates and carry high proportions of purified sewage, and the drinking water is mainly produced by groundwater recharge. In the course of the monitoring program, samples collected periodically from selected sites in the Berlin area are investigated for residues of PhACs and some other polar contaminants. The purpose of this monitoring, carried out in co-operation with the Senate of Berlin and the Berlin water works (BWB), is to investigate these compounds over a long time period to get more reliable data on their occurrence and fate in the different aquatic compartments. Moreover, the surface water investigations allow the calculation of season dependent contaminant loads in the Berlin waters. The monitoring is accompanied and supported by several other investigations such as laboratory column experiments and studies on bank filtration and drinking water treatment using conventional or membrane filtration techniques. This paper presents first results from the monitoring program for PhACs in the Berlin water cycle.

1. Introduction

About ten years ago, clofibric acid, the pharmacologically active metabolite of drugs used as blood lipid regulators in human medical care, was detected in groundwater and drinking water samples in Berlin (Stan & Linkerhägner, 1992; Stan et al., 1994; Heberer & Stan, 1996; 1997). In the following years, the occurrence of the so-called pharmaceutically active compounds (PhACs) has been investigated and confirmed in sewage, surface and groundwater, especially in Germany and in several other European countries (Stan & Heberer, 1997; Halling-Sørensen et al., 1998; Heberer & Stan, 1998; Daughton & Ternes, 1999). Meanwhile, more than 50 PhACs from various prescription classes such as analgesics, antibiotics, anti-epileptics, anti-rheumatics, beta blockers, chemotherapeutics, steroid hormones, and X-ray contrast media have been detected in the aquatic environment (Stan & Heberer, 1997; Ternes, 1998; Halling-Sørensen et al., 1998; Daughton & Ternes, 1999; Möhle et al., 1999; Heberer, in press). The studies show that some PhACs originating from human therapy are not eliminated completely in the municipal sewage treatment works and are, thus, discharged as contaminants into the receiving waters. Especially in urban areas such as Berlin (Germany), with high municipal sewage water outputs and low surface water flows, there is a potential risk of drinking water contamination by some polar PhACs when groundwater recharge is used in drinking water production (Heberer & Stan, 1997; Heberer et al., 1997; Heberer, in press).

In terms of two monitoring studies carried out in 1996 and 1999, PhACs such as clofibric acid, diclofenac, ibuprofen, propyphenazone, primidone and carbamazepine were detected at individual concentrations up to the

µg/L-level in the Berlin surface waters (Heberer et al., 1998; Heberer, in press). Additional investigations have also shown a relation between the municipal sewage discharges and the contamination of Berlin surface and groundwater samples by persistent drug residues (Heberer & Stan, 1997; Heberer, in press). Thus, several compounds were also found at individual concentrations up to 7.3 µg/L in groundwater aquifers near to contaminated water courses (Heberer et al., 1997), whenever bank filtration and/or artificial ground water enrichment are used in drinking water production. A few of these compounds also occur at trace level concentrations at the low ng/L-level in Berlin tap water samples (Heberer & Stan, 1997; Heberer, in press). The results obtained from these and all other occurrence studies on PhACs provided some important information on the origin, the distribution, and the fate of these contaminants in the aquatic environment. A continuous investigation of the occurrence and fate of these compounds in all compartments of a water cycle has, however, not been carried out. Often only random samples were analyzed and sampling was not repeated.

Thus, a new long-term monitoring program of sewage, surface, and drinking water is currently carried out in Berlin, Germany. The city of Berlin represents an excellent location to study the occurrence and fate of PhAC residues in the water cycle of an urban environment. Berlin is among those areas with the highest population densities in Europe. The surface waters, where the municipal sewage effluents are being discharged, have relatively low flow rates and therefore they carry high proportions of purified sewage. This is also demonstrated in table 1 by the comparison of the flow rates of the Rhine river and the Oder river with Berlins most important waterways the Havel river and the Spree river.

Table 1: Average flow rates of selected German rivers and municipal wasterwater discharges in Berlin, Germany. (Weigert, TU Berlin, personal commun.)

	Flow rate in m^3/s (MQ, 1995)
Rhine river	2,280
Oder river	542
Havel river (Berlin)	35
Spree river (Berlin)	13
Municipal wastewater discharges (Berlin)	7

Additionally, the drinking water is mainly produced by groundwater recharge. In the course of the monitoring program, samples collected periodically from selected sites in the Berlin area are investigated for residues of PhACs and some other polar contaminants. The purpose of this monitoring, carried out in co-operation with the Senate of Berlin and the Berlin water works (BWB), is to investigate these compounds over a long time period to get more reliable data on their occurrence and fate in the different aquatic compartments. Moreover, the surface water investigations allow the calculation of season-dependent contaminant loads in the Berlin waters. The monitoring is accompanied and supported by several other investigations such as laboratory column experiments (Scheytt et al., 2001) and studies on bank filtration (Heberer et al., 2001a) and drinking water treatment using conventional or membrane filtration techniques (Heberer et al., 2001b; Drewes et al., 2001). This paper presents first results from the monitoring program for PhACs in the Berlin water cycle which started in June 2000.

2 Description of the Berlin (Germany) water cycle

The contamination of the surface waters by municipal waste water discharges is of great relevance in the Berlin area. High concentrations of sewage contaminants are found in the receiving waters due to the high proportions of municipal sewage effluents in the surface waters. These result from low surface water flows and from the large amounts of raw sewage produced by its population of around four million people. Being situated in a densely populated area and considering the associated burden of waste contaminants, the intensive use of the waterways and their high nutrient levels, they are subject to high ecological pressure. Due to the high proportions of bank filtrate and artificial ground water enrichment of approximately 75% used in drinking water production in Berlin (SenSUT, 1997) polar organic compounds such as residues from pharmaceutically active

compounds (PhACs) and pesticides may under unfavorable conditions leach into the ground water aquifers (Heberer et al., 1998; 2001a). Thus, polar organic contaminants have been detected at concentrations up to the low µg/L-level in ground water wells near to the contaminated surface waters (Heberer & Stan, 1997; Heberer, in press).

Figure 1 shows a map of Berlin and its important lakes, rivers and canals. This map also shows the locations and drainages of the sewage treatment plants (STPs), the sampling locations of the surface water monitoring carried out since June 2000, and the two newly constructed transects at the Havel river (Heberer et al., 2001b). In total, the municipal area of Berlin includes an area of 889 km^2. 59 km^2 are used for agriculture, 155 km^2 are forests and a total water area of 57 km^2 consists of several rivers, approximately 60 lakes (>1 ha) and about 500 natural ponds (SenSUT, 1997; 1999; Berlin Fishery Board, 1997). Berlin's most important rivers the Spree and the Havel are typical shallow and slow flowing lowland rivers with a mean water depth of 7 meters (max. depth: 16 m) (Berlin Fishery Board, 1997). The Spree river enters Berlin from the south-east, passes through the whole city and discharges into the Havel river in the north-western districts of Berlin. The Havel river enters Berlin from the north-west and leaves the town in south-west direction.

The municipal sewage produced in the Berlin area is purified by six (formerly seven) STPs. With regard to their capacities, Berlins most important STPs are located south of Berlin in Waßmannsdorf and in the north-west of Berlin in Ruhleben. During autumn and winter time, the sewage effluents from the STP in Ruhleben are discharged into the Spree river which merges with the Upper Havel river. From April to September (bathing season), the Teltowkanal, a canal in the south of Berlin (shown in figure 1), is fed via a force main by additional effluents from Berlin's largest STPs in Ruhleben. In several small brooks located in inner city districts, downstream of STPs, the mean proportions of sewage in the surface waters are very high. Thus, the brooks Erpe, Panke and Wuhle show mean proportions of 58, 64 and 93% of sewage in the surface water, respectively (SenSUT, 1999).

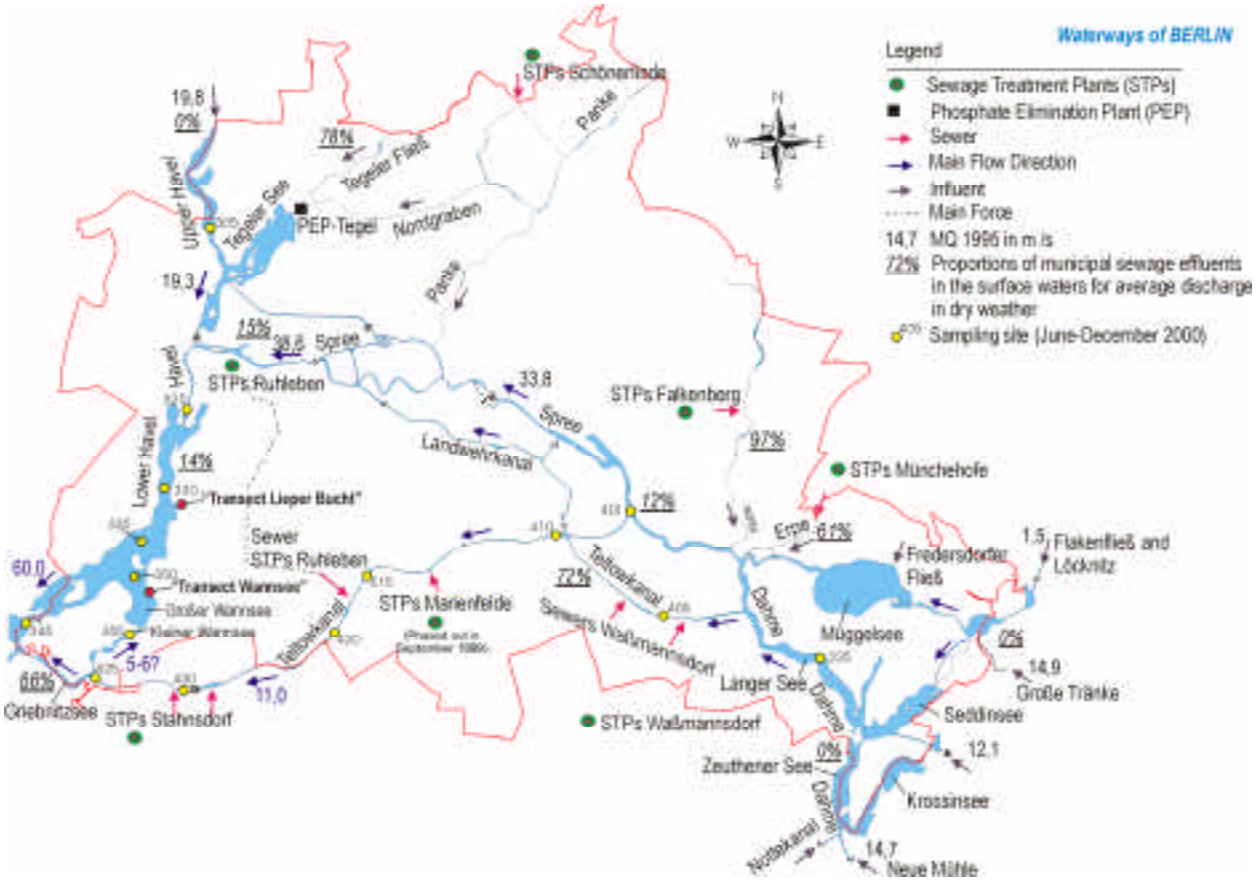

Figure 1: Map of Berlin, Germany, showing the waterways, the medium flow rates (in m^3/s), the sewage treatment plants (STPs) and their sewers, the proportions of sewage in the waterways (for average discharge in dry water), the sampling sites of the new surface water monitoring program, and the locations of the two newly constructed transects at the Havel river.

The above mentioned Teltowkanal carries the highest loads of sewage discharges. The Teltowkanal was build between 1901 and 1906 and used as drainage for rainwater and industrial waste water from districts formerly located outside of Berlin (SenStadtUm, 1983). Additionally, it was used as a shipping canal for industrial supply and as a short cut for the shipping routes between the Oder and Elbe rivers. The canal has a total length of approximately 35 kilometers and connects the rivers Dahme and Havel (SenStadtUm, 1983). Today, it is characterized by high proportions of sewage effluents discharged into the canal by Berlin's two largest STPs (Waßmannsdorf and during spring and summertime Ruhleben) and by the STPs in Stahnsdorf. Until September 1999, the STPs in Marienfelde also discharged their effluents into this canal. At several locations in the canal, municipal sewage effluents account for up to 40% of the average surface water flow (SenSUT, 1999). Under extreme conditions (dry periods with low surface water flows), the proportions of municipal sewage may reach up to 84% (SenSUT, 1999). With regard to contaminations from municipal sewage discharges, the canal represents a scenario that may be called a "worst-case situation". This is also the reason why many of the sampling sites selected for the surface water monitoring studies are located in the Teltowkanal (figure 1). The other sampling sites are located in the Havel river to consider the loads of PhACs entering Berlin (locations 225 and 305) and the loads discharged into the Spree River and the Upper Havel river (locations 325 and 330).

3 Results from the investigation of PhACs in the aquatic system of Berlin, Germany

3.1 PhACs in municipal sewage effluents

Several PhACs were observed in repeated investigations of 24h composite samples from different STPs in Berlin (Heberer, in press). Table 2 compiles recent occurrence data of some PhACs and several similar compounds detected in several investigations of STPs in Berlin. Residues of pharmaceuticals from various prescription classes such as blood lipid regulators, analgesics, bacteriostatics, and anticonvulsants were detected up to the µg/L-level in the effluents of the sewage treatment plants in Berlin (Heberer, in press). However, synthetic estrogens such as 17_-ethinylestradiole and mestranole were only detected at trace-level concentrations in the sewage effluents of the Berlin STPs because of their low total amounts in annual prescription. On the other hand, the "phytoestrogen" _-sitosterole was found at average concentrations of 1.9 µg/L in the effluents from Berlins STPs (table 2). The estrogenic potential of this compound is, however, very low compared with the above mentioned compounds.

Table 3. Average effluent concentrations of various PhACs in different STPs in Berlin. Results from different investigations partly compiled by Heberer (in press).

Analyte	Use / Origin	Average effluent concentration in µg/L
Aminophenazone	analgesic / antiinflammatory	0.1
Anonymous	not identified because of potential legal ramifications	0.72
Carbamazepine	anticonvulsant	1.63
Clofibric acid	active metabolite of lipid regulators	0.48
Diclofenac	analgesic / antiinflammatory	2.51
Gemfibrozil	blood lipid regulator	0.07
Ibuprofen	analgesic / antiinflammatory	0.1
Indomethacine	analgesic / antiinflammatory	0.8
Ketoprofen	analgesic / antiinflammatory	0.23
Naproxen	analgesic / antiinflammatory	0.10
Oxazepam	neuroleptics	0.25
Phenazone	analgesic	0.52
Phenobarbital	barbiturate, anticonvulsant, major metabolite of primidone	0.03
Phenytoine	anticonvulsant	0.1
Primidone	anticonvulsant	0.14
Propyphenazone	analgesic / antiinflammatory	0.39
Sulphadiazine*	sulphonamide	0.05
Sulphamethizole*	sulphonamide	<0.01
Sulphamethoxazole*	sulphonamide	0.90
Cholesterol	fecal sterol	6.75
Coprostanol	fecal sterol metabolite of cholesterol	3.2
Sitosterol	„phytoestrogen“	1.9
Salicyclic acid	primary metabolite of acetylsalicylic acid, keratolytic, dermatrice, preservative of foods	0.04
Caffeine	beverages, drugs	0.18

* data from Hartig et al. (1999)

As shown exemplarily in table 3, PhACs such as clofibric acid, the anti-epileptic drug carbamazepine and the analgesic drug diclofenac were not or only to a very small extent removed during sewage purification. On the other hand, caffeine, also originating from PhACs but to a larger extent from beverages and often used as indicator compound for the presence of municipal sewage in the aquatic system, was found to be readily biodegradable and removed by more than 99.9% in Berlins STPs. Some PhACs were found in the sewage effluents at concentrations similar or even larger than those of compounds often described as markers for municipal sewage in surface waters, e.g. caffeine or coprostanol (metabolite of cholesterol). The studies of the PhACs showed that some of them are much better suited as marker compounds for municipal sewage than the classical organics because of their persistence in the STPs and in the aquatic environment (Heberer, in press).

Table 3. Concentrations and removal rates for three drug residues and for caffeine detected in composite samples (24h) of influents (n=10…20) and effluents (n=20…27) from different STPs in Berlin* (Heberer, in press).

Analyte	Average influent concentration in µg/l	Average effluent concentration in µg/l	Removal rate in %
Carbamazepine	1.78	1.63	8
Clofibric acid	0.46	0.48	- **
Diclofenac	3.02	2.51	17
Caffeine	230	0.18	< 99.9

* STPs in Berlin: Ruhleben, Schönerlinde, Waßmannsdorf (mixed samples: 24 hours, sampling series in May-December 1999). ** - : no removal was observed.

3.2 PhACs in Berlins water ways (new results from the surface water monitoring started in June 2000)

Residues of PhACs and several other organic compounds such as synthetic musks widely used as fragrances in personal care products have been identified as important pollutants in a monitoring study carried out in Berlin, Germany, in 1996 (Heberer et al. 1998; 1999). Samples were collected from 30 sampling locations upstream and downstream from municipal sewers. The impact and the extend of contamination by the sewage discharges showed the need for further investigations to get more reliable data on the occurrence and fate of these residues in the aquatic system. In 1999, further studies on the occurrence of synthetic musks (Heberer et al., in press) and several PhACs (Heberer, in press) were carried out. Additionally, a new long-term monitoring program of sewage, surface, and drinking water for PhACs and several other emerging organic contaminants was initiated in June 2000. In terms of this monitoring program, samples are collected periodically from selected sites in the Berlin area (figures 1 and 3).

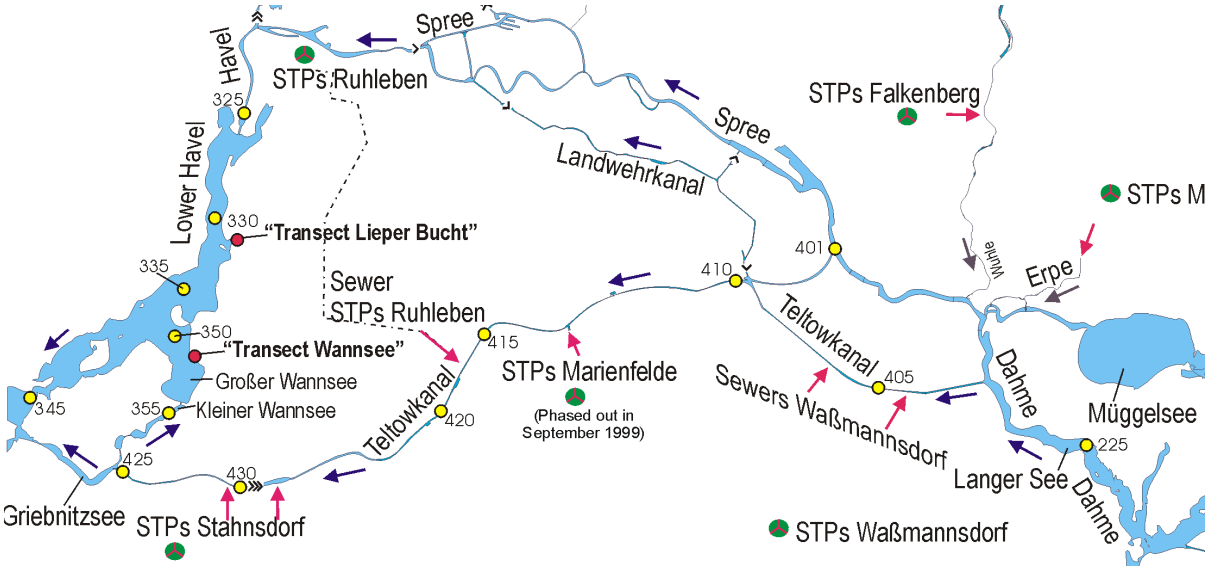

Figure 2: Cut from the map in figure 1, showing the sampling sites (except of location 305) of the new surface water monitoring program, and the locations of the two newly constructed transects at the Havel river.

The purpose of this monitoring carried out in co-operation with the Senate of Berlin and the Berlin water works (BWB) is to investigate these compounds over a long time period to get more reliable data on their occurrence and fate in the different aquatic compartments. In the course of the study, seasonal loads will be calculated for the contaminants detected in the Berlin waterways. This is possible because the individual flow rates of the

Berlin waterways are determined parallel to the sampling. Additionally, some conventional parameters such as nitrate, nitrite, ammonia, phosphate, and boron are determined more frequently and may also be used for confirmation or correction of the calculations.

Some positive findings of PhACs obtained from the first three sampling series in June, September, and December 2000 are compiled in table 4. Several PhACs have been detected at individual concentrations up to 2 µg/L in Berlins waterways.

Table 4: Concentrations of PhACs and caffeine detected in terms of the surface water monitoring in Berlin, Germany. Results from the sampling series carried out in June, September, and December 2000. n.d.: not detected.

Compound	Concentrations in ng/l
Anonymous*	n.d. – 830
Carbamazepine	25 – 1075
Clofibric acid	n.d. – 450
Caffeine	80 – 265
Diclofenac	n.d. – 1030
Gemfibrozil	n.d. – 35
Ibuprofen	n.d. – 55
Ketoprofen	n.d. – 65
Mefenamic acid	n.d. – 20
Naproxen	n.d. – 95
Oxazepam	n.d. – 70
Pentoxifylline	n.d. – 30
Primidone	n.d. – 635
Propiphenazone	n.d. – 1970
Tolfenamic acid	n.d. – 20

* This compound cannot be identified because of potential legal ramifications but a publication is planned (Reddersen et al., in prep.).

More interesting than the total figures are the concentration profiles of the individual compounds measured at the different sampling locations in the Teltowkanal, the Havel river, and the connecting waterways. Therefore, the concentration profiles for clofibric acid, diclofenac, and carbamazepine in the Teltowkanal, are exemplarily shown for the three sampling series in figures 3 to 5. As expected from the preliminary studies in 1996 and 1999, the highest concentrations for the PhACs were found in the Teltowkanal. Nevertheless, the total loads in the Havel river may be similar or even higher due to the higher flow rates. This question will be clarified when the loads are calculated. But some other interesting aspects can already be drawn from these figures. At sampling location no. 225 in the Dahme river (figures 1 and 2) PhACs were only detected at very low concentrations (carbamazepine) or not at all. This confirms that the high concentrations of PhACs detected in the Teltowkanal almost totally come from discharges of sewage effluents into the canal. Peak concentrations were observed where purified municipal sewage was discharged by the STPs. Generally, the impact of sewage effluents on the surface water quality was found to be much more significant for the PhACs than for any of the common chemical parameters (Heberer et al., 1998).

Another very interesting question is the fate of the drain from the Teltowkanal. For many years, it was assumed that the main flow direction of the Teltowkanal is into the lake Griebnitzsee and finally into the Lower Havel River (figures 1 and 2). Today, it is clear that the main flow direction is into lake “Kleiner Wannsee” which connects the Teltowkanal with lake “Großer Wannsee” (Havel river). As can be seen by the results in figures 3 to 5, the concentrations of the PhACs in lake “Kleiner Wannsee” (no. 355, figure 2) are almost as high as those detected at the end of the Teltowkanal (location no. 425) and much higher than those detected in the Havel river. Thus, the surface water from lake “Kleiner Wannsee” consists mainly of water from the Teltowkanal which is discharged into the lake “Großer Wannsee”. This effect is very important as it shows that the surface water quality of lake “Großer Wannsee” is not only affected by the Havel river but also to a high extent by the Teltowkanal. On the other hand, the surface water quality of lake “Großer Wannsee” is of recreational

importance (e.g. open air bath, water sports) and also important for public drinking water supply as the waterworks in Beelitzhof produce more than 60% of their drinking water from bank filtrate (Heberer et al., 2001a).

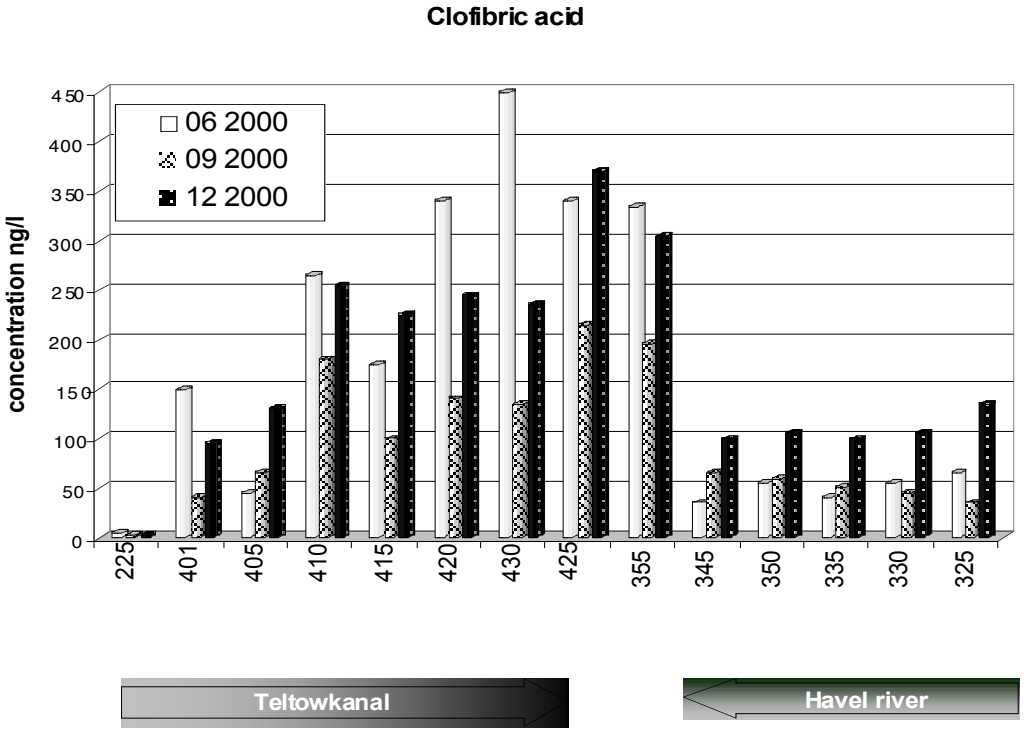

Figure 3. Concentration profile of clofibric acid in the Teltowkanal, the Havel river, and some connecting waterways determined at three different sampling series in June, September, and December 2000 (06, 09, 12 2000). The arrows labeled with Teltowkanal and Havel rivers show the flow direction of the waterways. Sampling location 355 (figure 2) is in lake "Kleiner Wannsee" which connects the Teltowkanal with lake "Großer Wannsee" (Havel river).

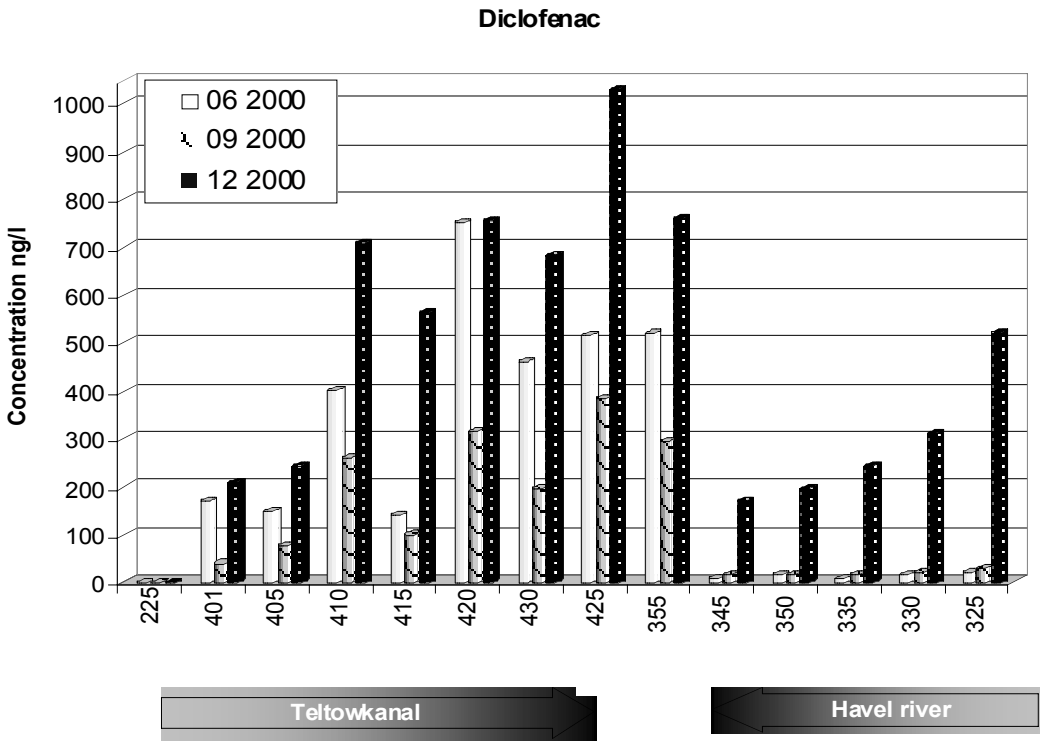

Figure 4. Concentration profile of diclofenac in the Teltowkanal, the Havel river, and some connecting waterways determined at three different sampling series in June, September, and December 2000 (06, 09, 12 2000). The arrows labeled with Teltowkanal and Havel rivers show the flow direction of the waterways. Sampling location 355 (figure 2) is in lake “Kleiner Wannsee” which connects the Teltowkanal with lake “Großer Wannsee” (Havel river).

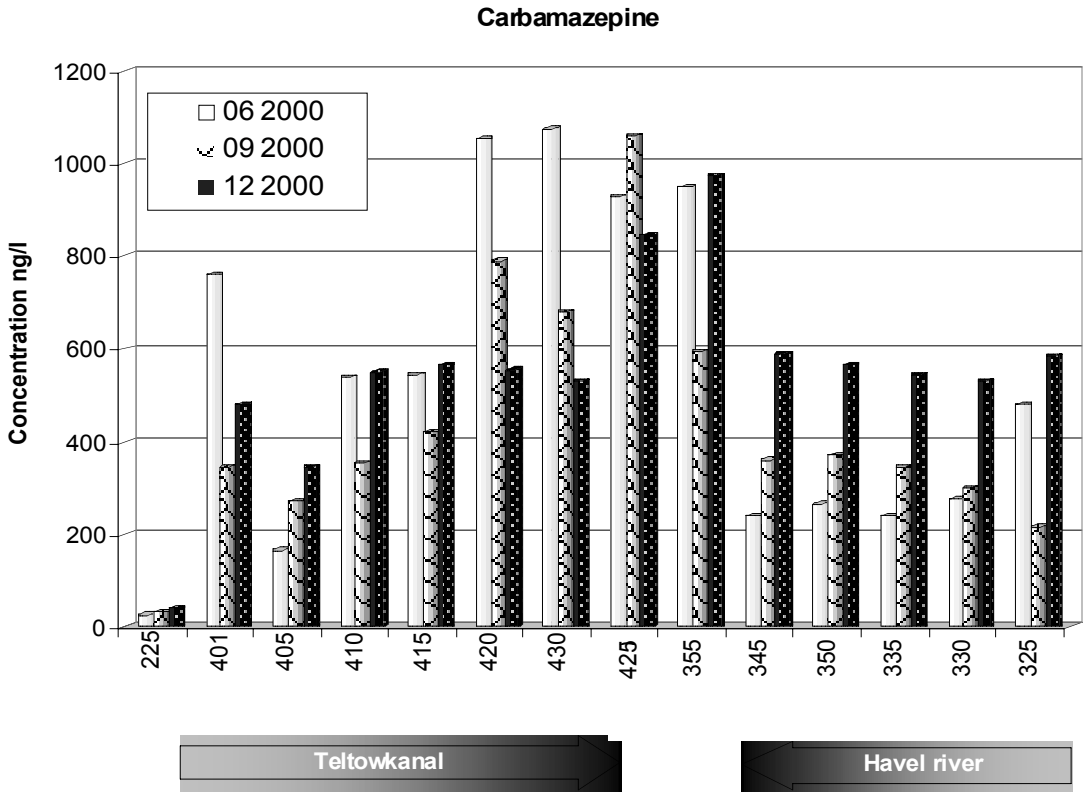

Figure 5. Concentration profile of carbamazepine in the Teltowkanal, the Havel river, and some connecting waterways determined at three different sampling series in June, September, and December 2000 (06, 09, 12 2000). The arrows labeled with Teltowkanal and Havel rivers show the flow direction of the waterways. Sampling location 355 (figure 2) is in lake "Kleiner Wannsee" which connects the Teltowkanal with lake "Großer Wannsee" (Havel river). river).

Figures 3 to 5 also show some seasonal differences, especially, between the sampling series in June and December 2000. As already described before, during autumn and winter time, the sewage effluents from the STP in Ruhleben are discharged into the Spree river which merges with the Upper Havel river. From April to September (bathing season), the Teltowkanal is fed via a force main by additional effluents from the STPs in Ruhleben. The switching of the force main can be seen very clearly when looking at the concentrations of the PhACs at locations no. 415 and 420 in figures 3 to 5. Thus, the increase of the concentrations that was observed in the sampling series in June 2000 was missing in December. On the other hand, the concentrations of the PhACs detected in the Havel river are increasing in December. The concentration profiles of the PhACs are looking very similar. Nevertheless, there are several differences, especially, between figure 4 and figure 3 and 5. Thus, the total amounts of diclofenac detected in December are much higher than those detected in June or September. This may be caused by an enhanced photodegradation of diclofenac described by Buser et al. (1998). This may, hover, more likely be due to a higher consumption of the analgesic and anti-rheumatic drug diclofenac during the winter time! This assumption is also supported by the results of several other analgesics which show a similar distribution pattern and also higher winter loads. This and several other questions will be answered in the following sampling series.

3.3 PhACs in Berlins groundwater aquifers and in drinking water

In 1990, after the reunification of East and West Germany, the Senate of Berlin commissioned monitoring of water collected from wells near the former Berlin wall. The purpose of this monitoring was to identify and quantify pesticide residues caused by the intensive use of these compounds by the border troops of the former German Democratic Republic to see clearly and have a free field of fire. As a result of this survey, the pharma-

cologically active drug metabolite clofibric acid (2-(4)-chlorophenoxy-2-methyl propionic acid) was first detected in Berlin groundwater samples in 1991 (Stan and Linkerhägner, 1992). In 1993, 64 tap water samples randomly collected from different households in Berlin were investigated. Clofibric acid was found in all samples at concentrations between 10 and 165 ng/L (Stan et al., 1994). In 1994, by analyzing drinking-water samples collected from 14 utilities in Berlin (Heberer and Stan, 1996 and 1997), a relation was found between bank filtration, artificial groundwater enrichment, and the level of drinking-water contamination by the drug clofibric acid. Exemplarily, the results obtained for four of the investigated waterworks shall be discussed. As shown in table 5, the highest concentrations of clofibric acid were found in the tap water samples from the waterworks in Beelitzhof and Johannisthal. Both waterworks are characterized by high proportions of bank filtrate in their drinking water and both are located near waterways highly contaminated by municipal sewage effluents. On the other hand, the drinking water from the waterworks in Spandau and Kaulsdorf did not contain any measurable concentrations of clofibric acid. In the case of the waterworks in Spandau, this is due it's location at the Upper Havel river that is not contaminated by this compound and consequently, it is also not found in the drinking water mainly produced by bank filtration and groundwater infiltration. The drinking water produced by the waterworks in Kaulsdorf was not polluted because this waterworks does not use any groundwater enrichment. Thus, the contaminants which are present in the surface water could not leach into the groundwater aquifers of this waterworks. The determining factors for the appearance and the concentration levels of clofibric acid in the drinking-water samples were the proportions of drinking water produced from bank filtration and groundwater infiltration, the geographic location of the waterworks, and the degree of contamination of the neighboring waterway used as source for groundwater recharge by the particular waterworks (Heberer and Stan 1997).

Table 5. Concentrations of clofibric acid and N-(phenylsulfonyl)-sarcosine in drinking-water samples from the Berlin waterworks, Germany, 1994. [BF, bank filtration; GWE, artificial groundwater enrichment; SWC, degree of contamination of the neighboring watercourse; -, below the limit of detection of 1 ng/L for clofibric acid; ++, high level of contamination by municipal sewage effluents; +, moderate level of contamination; o, low or even very low level of contamination]

Waterworks	BF (%)	GWE (%)	Clofibric acid (ng/L)	SWC
Beelitzhof	66	7	60	+
Johannisthal	62	0	170	++
Kaulsdorf	0	0	-	++
Spandau	30	48	-	o

Meanwhile, several additional PhACs such as diclofenac, ibuprofen, propyphenazone, gemfibrozil, or primidone also have been detected in groundwater wells from different bank-filtration sites in Berlin (Heberer in press). A compilation of some results is shown in table 6.

Table 6. Concentrations of pharmaceutical residues in wells located near contaminated surface water in Berlin, Germany. [Data from Heberer et al. 1997 and Heberer in press; PhACs, pharmaceutically active compounds; ng/L, nanograms per liter; n.d., not detected, detection level was between 1 and 10 ng/L; E, concentrations were estimated because standards were not available commercially]

Residues of PhACs	Concentration range (ng/L)
Clofibric acid	n.d. - 7 300
Diclofenac	n.d. – 380
Fenofibrate	n.d. – 45
Gentisic acid	n.d. – 540
Gemfibrozil	n.d. – 340
Ibuprofen	n.d. – 200
Ketoprofen	n.d. – 30
Phenazone	n.d. - 1 250
Primidone	n.d. – 690
Propyphenazone	n.d. - 1 465
Salicylic acid	n.d. - 1 225
Clofibric acid derivative	n.d. - E2 900
N-methylphenacetin	n.d. - E470

In 2001, a new scientific project has been initiated to investigate the natural attenuation of pharmaceutical residues and several other environmentally relevant contaminants systematically under natural conditions at different bank-filtration sites in Berlin (Heberer et al., 2001a). The bank filtration research is carried out in cooperation with the Senate of Berlin (Senate Department of Urban Development, Environmental Protection and Technology) and the Hydrogeology Research Team of the Free University Berlin. Both transects ("Lieper Bucht" and "Wannsee") are along the Havel River (figures 1 and 2). Some preliminary results obtained for samples collected in April 2001 from the "Wannsee" transect are presented by Heberer at al. (2001a). A small compilation of results is also shown in table 7.

Table 7: Concentrations/concentration ranges of some PhACs detected in the surface water of lake Wannsee and in the shallow wells and deep wells from the bank filtration site "transect Wannsee" (Heberer et al., 2001a), Berlin, Germany, April 18, 2001.[n.d.: not detected; detection level, 1 to 10 nanograms per liter; data are reported in nanograms per liter]

Analyte	Surface water (lake Wannsee)	Shallow wells	Deep wells	Water supply well
Anonymous*	200	135 – 350	n.d.	100
Bezafibrate	170	n.d. - 20	n.d.	n.d.
Carbamazepine	235	160 - 360	n.d.	20
Clofibric acid	50	n.d. – 60	n.d.	70
Diclofenac	50	n.d. – 40	n.d.	n.d.
Primidone	105	195 – 535	n.d.	15
Propyphenazone	280	10 – 170	n.d.	50

* This compound has been identified as a metabolite of a pharmaceutical substance originating from a source at the Upper Havel River outside of Berlin (Reddersen et al., in prep.). This compound cannot be identified because of potential legal ramifications (Reddersen et al., in prep.).

Seven PhACs, namely anonymous (cannot be identified because of potential legal ramifications), bezafibrate, carbamazepine, clofibric acid, diclofenac, primidone, and propyphenazone, were detected only in the shallow wells, not in the deep wells. Several compounds, such as bezafibrate and diclofenac seem to be removed effectively during bank filtration (Heberer et al, 2001a). But several other compounds namely carbamazepine, clofibric acid, primidone, propyphenazone, and anonymous were present at concentrations of as much as 100 ng/L in the water-supply wells.

Conclusions

PhACs are found as very persistent residues at the µg/L-level in the effluents of Berlins municipal STPs. These residues are discharged into the surface waters where they are also detected at concentrations up to the µg/L-level in samples collected from several canals, lakes, and rivers. Especially, several of the polar PhACs were identified as excellent markers for sewage contamination in surface and ground waters because of their persistence in the aquatic environment. Whenever bank filtration or other methods for groundwater recharge are used in drinking water production, these compounds can leach from the contaminated watercourses into the groundwater aquifers and may also appear at trace-level concentrations in drinking water.

Outlook

The monitoring study of Berlin's surface water already revealed some important and indispensable information on the occurrence, seasonal distribution, and fate of sewage contaminants such as PhACs in the aquatic environment. Depending on further funding, the study will be continued until the end of 2003 to obtain some long-term results, to recognize certain trends, and to get much more reliable data on the loads of the PhACs in the watercycle. Additionally, the study will be combined with and supported by several other investigations such as laboratory column experiments (Scheytt et al., 2001), studies on bank filtration (Heberer et al., 2001a), and investigations of drinking water treatment using conventional or membrane filtration techniques (Heberer et al., 2001b; Drewes et al., 2001).

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Drug Residues in Ambient Water: Initial Surveillance in New Mexico, USA

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Abstract

Pharmaceutical residues, excreted by humans into sewage, have been detected in surface and ground water in other states and countries. Detected drugs include painkillers, lipid (cholesterol) regulators, antiseptics, chemotherapy agents, antibiotics, and hormones. Some drug residues have caused ecological impacts such as the widespread sexual disruption of male fish exposed to estrogenic hormones, and the development of antibiotic-resistant pathogenic bacteria in rivers and birds, including Salmonella in the Rio Grande.

The State of New Mexico is testing water where drug residues would most likely occur. Grab samples of treated sewage effluent, surface water receiving treated sewage, and ground water contaminated by sewage (both septic-tank and treatment-plant plumes) are being collected. We are able to detect some analgesic, anti-convulsant, anti-depressant, anti-imflammatory, and hormonal drugs in water at the ng/L level. Samples are extracted with dichloromethane, concentrated, and analyzed by GCMS. HPLC-MS analytical capability is being developed for antibiotic, cholesterol-regulating and cardiovascular drugs.

Drug Residue Testing Results for Ambient Water in New Mexico.

Sample Media	Number of Samples	Results (ng/L)
Treated Sewage Effluent	5	amitriptyline "Elavil" (30), caffeine (1000), phenytoin "Dilantin" (250-300), propoxyphene "Darvon" (231-820)
Surface Water	15	amitriptyline (30), caffeine (1500), estrone (140), ethynyl estradiol (10)
Ground Water Contaminated by Sewage	7	residues not detected
Public Drinking Water	2	residues not detected

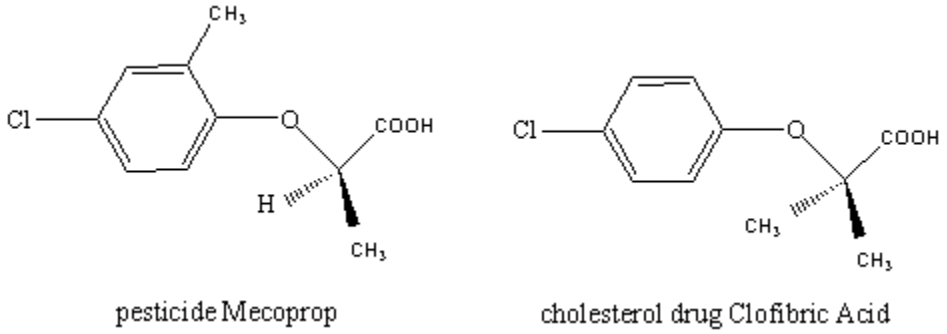
All sewage effluents contained at least one drug residue, but did not contain a complex variety of them. Four different drug residues were detected in four of 15 surface water samples. Estrogenic hormones, frequently detected in studies outside of New Mexico, were not detected in any sewage effluent sample and were detected in only two of 15 surface water samples. Drugs were not detected in any ground-water samples, or in any public drinking-water samples.

Other areas of New Mexico are scheduled to be sampled. We have not yet developed the analytical capability to test for many drugs of major clinical volume and public-health importance such as antibiotics. There is no evidence, however, that the drugs tested for thus far widely occur in ambient water throughout New Mexico. It is hypothesized that the intense sunlight in New Mexico causes rapid photolytic degradation of drug residues discharged into surface water.

Introduction

Swiss scientists studying pesticides in water serendipitously discovered Clofibric Acid (CA), the excretory metabolite of a cholesterol-lowering medication, in ambient surface water (Buser, et al., 1998a). The discovery was made because of the structural similarity between CA and the pesticide Mecoprop (Figure 1). Other studies have found that pharmaceutical residues, originating in treated sewage effluent, occur widely in European surface, ground and drinking water (Belfroid, et al., 1999, Heberer, et al., 1998, Heberer and Stan, 1996, Heberer and Stan, 1997, Hirsch, et al., 1999, Richardson and Bowron, 1985, Stumpf, et al., 1996, Stumpf, et al., 1999, Ternes, 1998, Ternes, et al., 1998, Ternes et al., 1999a, b). Drug residues were detected in all 64 drinking-water samples collected in a Berlin, Germany study (Stan, et al., 1994), and it is estimated that 48 to 96 tons of CA are dissolved in the North Sea (Buser, et al., 1998a). Studies in the United States and Brazil have also detected drug residues in ambient water (Sieler, et al., 1999, Stumpf, et al., 1999). Drugs detected in Europe and/or the Americas include painkillers, lipid (cholesterol) regulators, antiseptics, chemotherapy agents, antibiotics, and hormones. The presence of caffeine and pharmaceuticals, in ground water containing elevated nitrate, has been used as an indicator of septic-tank contamination (Sieler, et al., 1999).

Figure 1. Molecular Structures of Mecoprop and Clofibric Acid. The two compounds are isomers, differing only by the location of a methyl group, CH_3.



Conventional sewage-treatment technologies vary greatly in their ability to eliminate drug residues and, in certain instances, have even been shown to increase the bioactivity of the pharmaceutical residues passing through them (Ternes et al., 1999a). Some polar drug residues readily leach through the vadose zone into ground water (Heberer and Stan, 1997, Heberer, et al., 1998). Activated carbon filtration has been shown to remove drug residues from drinking water (Hirsch, et al., 1996).

Documented ecological impacts of these residues in ambient water include the development of antibiotic resistant bacteria in rivers and birds, and sexual disruption of fish exposed to estrogenic hormones. Antibiotic-resistant pathogenic bacteria have been detected in United States rivers, including Salmonella species in the Rio Grande and in Canadian geese near Chicago (Raloff, 1999). Male fish in rivers receiving sewage effluent produce the female egg-yolk protein, vitellogenin, resulting from exposure to estrogenic compounds (both natural and synthetic) in the low part-per-trillion (ppt, ng/L) range. The presence of vitellogenin, a yolk sac protein normally found only in females, in male fish has been related to the widespread occurrence of intersexuality, the appearance of female characteristics and the progressive disappearance of male characteristics, in United Kingdom fish. Intersexuality can be a serious threat to the survival of affected species. Similar sexual disruption caused sterility and the extinction of certain mollusk species that were exposed to tributyltin (TBT). (Jobling, et al., 1998) Estrogenic hormones and TBT are among a complex variety of environmental contaminants known as endocrine disrupting chemicals (EDCs). EDCs can bind with a hormone receptor and either mimic a hormone, triggering an identical response, or block a hormone from triggering the normal response. EDCs also can interfere with hormonal activity without bonding to the receptor.

In response to the growing body of evidence in the research literature of the ubiquitous nature of this problem, the New Mexico Environment Department (NMED) and the Scientific Laboratory Division (SLD) of the New Mexico Department of Health are currently conducting initial surveillance to determine what drug residues are present in ambient water in the state, and at what concentrations. Some drugs can be detected with analytical methods used to detect other classes of organic compounds, such as pesticides. Analytical technology has pushed aqueous-phase drug detection limits into low ppt, the range where many drugs have been detected. The most commonly prescribed drugs in the United States are listed in Table 1. SLD is currently able to detect 28 of these drugs at the ppt level in water, including a number of anti-depressants, and hormones. Testing for antibiotics, and lipid-regulating and cardiovascular pharmaceuticals will begin after SLD becomes proficient with a High-Performance Liquid Chromatograph, Mass Spectrometer (HPLC-MS), which it recently acquired with funding provided by the Centers for Disease Control.

Table 1. Most Commonly Prescribed Drugs in the United States (The Top 200 Prescriptions, http://www.rxlist.com).

Drug Class	Specific Drugs
Analgesics	hydrocodone, ibuprofen, propoxyphene (Darvon), acetaminophen
Antibiotics	amoxicillin, azithromycin, cephalexin, ciprofloxacin, clarithromycin, penicillin VK
Anti-Convulsants	diazepam, phenytoin (Dilantin)
Anti-Depressants	fluoxetine (Prozac), paroxetine, setraline, amitriptyline
Cardiovascular	amlodipine, digoxin, enalapril, lisinopril, furosemide, diltiazem
Hormones	thyroxine, estrogen hormones
Lipid Lowering Agents	atorvastatin, lovastatin, simvastatin

Methods

Surveillance Strategy

Initial surveillance is focusing on the pharmaceuticals that are heavily prescribed in the United States, and are within SLD's analytical capability, along with other commonly prescribed pharmaceuticals including tricyclic antidepressants. All drugs included in the analyses are listed in Table 2.

Table 2. Drugs and drug metabolites included in the analysis.

Drug Class	Specific Drugs
Analgesics	propoxyphene (Darvon)
Anti-Convulsants	phenytoin (Dilantin)
Anti-Depressants	fluoxetine (Prozac), sertraline, amitriptyline, protriptyline, trimipramine maleate, nortriptyline, desipramine, imipramine, doxepin, nordoxepin, paroxetine
Anti-Inflammatory	methyprednisolone, prednisone
Hormones	estradiol, equilin, estrone, ethynyl estradiol, medroxyprogesterone, megestrol acetate, mestranol, progestrorone, norethindrone, norethynodrel, norgestrel
Other	caffeine, tamoxifen

Samples are being collected at locations where drug residues would most likely occur. NMED staff are sampling treated sewage effluent outfalls, surface water receiving sewage, ground water contaminated by sewage (nitrate and anoxic; municipal, military, and septic tank sites), and public drinking-water systems served

by surface water or by groundwater that may contain treated sewage. Locations that have been sampled to date are listed in Table 3, and identified on Figure 2.

Sewage effluents from Espanola and Las Cruces are discharged to the Rio Grande. Farmington's effluent is discharged to the San Juan River. Santa Fe's effluent is discharged to the Santa Fe River.

Ground water at Carnuel and Western Terrace was sampled from private domestic wells containing high nitrate attributed to household septic-tank discharges. The Kirtland and White Sands samples were collected from monitoring wells containing high nitrate attributed to military base sewage discharges. Ground-water samples at La Cieneguilla were collected from private domestic wells containing elevated nitrate attributed to discharges from a municipal sewage treatment plant. To date, samples of anoxic ground water contaminated by septic tank discharges have not been collected.

Farmington's public drinking-water system is supplied by an intake on the Animas River. The Walking Sands Rest Area public water system is supplied by a well containing high nitrate.

Sampling Protocol

An eight L sample, consisting of two 4 L bottles, was collected at each site. Samples were stored immediately in an ice bath, and delivered to SLD for extraction within 24 hours of collection. Two trip blanks were carried into the field and analyzed during the first sampling run, and this process was discontinued after no residues were detected in the trip blanks. Blind duplicates were submitted periodically.

Analytical Methods

Two liters of each sample were extracted with 60 mL of HPLC-grade dichloromethane (DCM) within 24 hours of collection. The 2 L separatory funnels, with teflon stopcocks and either teflon or ground-glass stoppers, were shaken for two minutes. The water and DCM were allowed to separate for about 5-10 minutes, and the DCM was drained into a 250 mL flask. Another 60 mL of DCM was added to the separatory funnel, and the liquid-liquid extraction process was repeated twice, draining the DCM into the flask after each shake. Sodium Sulfate (Na_2SO_4) was baked at 400^0 C and kept in the drying oven until needed to remove water from the DCM. Approximately 10 grams of the cooled Na_2SO_4 were added to each flask, swirling the solvent while adding, and the flasks were allowed to stand for at least 30 minutes. The DCM extracts were then evaporated to 0.5 mL using a Zymark Turbo-Vap II at 38^0 to 40^0 C. The concentrates were analyzed using Electron Ionization, then Selected Ion Storage, and also Tandem MS (MS/MS) for some of the compounds. The instrument used was a Saturn 2000 Ion Trap Mass Spectrometer (Varian, Walnut Creek, CA) with a 3800 Gas Chromatograph (Varian, Walnut Creek, CA) with a 1079 Temperature Programmable injector (Varian, Walnut Creek, CA). Every sample batch was analyzed with a lab reagent blank, two lab fortified blanks, and a lab fortified matrix that were extracted and concentrated just like the samples. All positive results were quantitated using freshly-prepared standards. Chemical standards were obtained from the following manufactures: Sigma (St.louis .MO), Aldrich (Milwaukee,WI) and Alltech (Deerfield,IL). The solvents used were purchased from Burdick and Jackson.

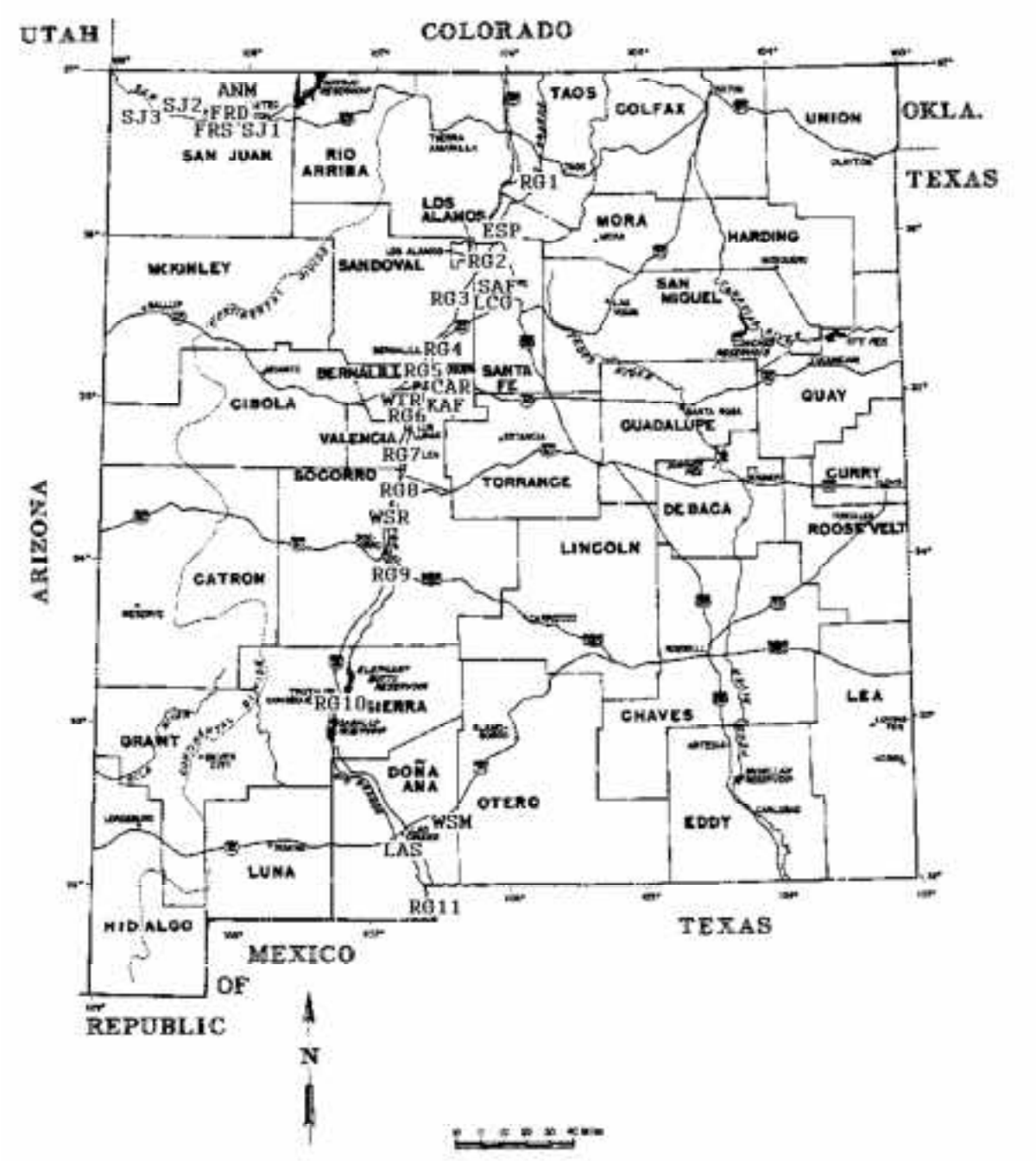
Results

Samples were collected in August and September of 2000, and in April 2001. Analytical results for the samples collected to date are listed in Table 3. Sampling locations are identified on Figure 2 by their location codes. No drug residues were detected in the trip blanks.

Table 3. Sample Locations and Analytical Results for Drug Residues in Water.
Sampling locations are mapped by their location codes in Figure 2. Detection limits are about 10 ng/L.
Sample Location (Location Code)

Sample Medium	**Analytical Results (ng/L)**
Treated Sewage	
Espanola effluent outfall (ESP)	propoxyphene (500), phenytoin (300)
Espanola effluent ditch (ESP)	propoxyphene (400), phenytoin (250)
Farmington effluent outfall (FRS)	caffeine (1000), propoxyphene (820)
Las Cruces effluent outfall (LAS)	propoxyphene (231)
Santa Fe River below WWTP (SAF)	amitriptyline (30)
Surface Water	
Animas at Aztec (ANM)	residues not detected
Rio Grande at Pilar (RG1)	residues not detected
Rio Grande at Buckman Crossing (RG2)	amitriptyline (30)
Rio Grande below Cochiti Lake (RG3)	residues not detected
Rio Grande at Bernalillo, 2000 (RG4)	residues not detected
Rio Grande at Bernalillo, 2001 (RG4)	residues not detected
Rio Grande at Paseo del Norte Bridge (RG5)	residues not detected
Rio Grande in Albuquerque South Valley (RG6)	caffeine (1500), estrone (140)
Rio Grande at Belen (RG7)	residues not detected
Rio Grande at Bernardo (RG8)	residues not detected
Rio Grande at San Antonio (RG9)	residues not detected
Rio Grande below Elephant Butte Lake (RG10)	residues not detected
Rio Grande at Sunland Park (RG11)	caffeine (200)
San Juan at Bloomfield (SJ1)	ethynyl estradiol (10)
San Juan at the Hogback (SJ2)	residues not detected
San Juan at the Hogback, blind dup.	residues not detected
San Juan at Shiprock (SJ3)	residues not detected
Ground Water Contaminated by Sewage	
Carnuel private well (CAR)	residues not detected
Kirtland Air Force Base (KAF)	residues not detected
La Cieneguilla private well #1 (LCG)	residues not detected
La Cieneguilla private well #2 (LCG)	residues not detected
La Cieneguilla private well #3 (LCG)	residues not detected
Western Terrace private well (WTR)	residues not detected
White Sands Missile Range (WSM)	residues not detected
White Sands Missile Range, duplicate	residues not detected
Public Drinking Water	
Farmington drinking water; Animas River source (FRD)	residues not detected
Walking Sands Rest Area; ground water source (WSR)	residues not detected

Figure 2. Drug Residue Sampling Locations.
Sample-location codes are identified in Table 2.



Sewage Effluents

All sewage effluents contained at least one drug residue, but did not contain a complex variety of them. Propoxyphene (Darvon) was found in four of the five samples thus far. Additionally, phenytoin (Dilantin) was detected in Espanola, caffeine was detected in Farmington, and amitriptyline (Elavil, Endep) was detected in Santa Fe. Estrogenic hormones were not detected in any sewage sample.

San Juan Watershed

The San Juan River at Bloomfield was the only sample that contained a detectable drug residue, ethynyl estradiol, a synthetic estrogen hormone used in birth-control pills. Neither ethynyl estradiol nor the two drugs detected in Farmington sewage effluent, caffeine and propoxyphene, were detected in the two downstream river samples (Hogback and Shiprock).

Rio Grande Watershed

Drug residues were not detected in the Rio Grande at Pilar, Cochiti Lake, Bernalillo, Paseo del Norte bridge, Belen, Bernardo, San Antonio, or at Elephant Butte Lake. Amitriptyline was detected at Buckman Crossing. Caffiene and estrone were detected in the Albuquerque South Valley, about 3 Km downstream from the municipal sewage effluent outfall. Caffeine was detected at Sunland Park, immediately downstream from the sewage effluent outfall.

Ground Water Contaminated by Sewage

Drug residues were not detected in any of the seven sewage-contaminated ground-water samples.

Public Drinking Water

Drug residues were not detected in either of the two public drinking-water samples, one supplied by surface water, the other supplied by nitrate-laden ground water.

Discussion and Conclusions

This study was designed to test for drug residues at locations where they would most likely occur (ie. treated sewage effluent outfalls, rivers that receive effluent, and ground water contaminated by sewage. Test results thus far provide no evidence that the drug residues for which testing was completed widely occur in ambient water in New Mexico. It should be noted, however, that many of the drugs of major clinical volume and public-health importance were not included in this study (eg. antibiotics, and lipid-regulating and cardiovascular drugs).

Estrogenic hormones, frequently detected in studies outside of New Mexico, were not detected in any of the five sewage-effluent samples, and were detected in only two of 15 surface-water samples. The concentrations detected, however, are of potential concern because they are within the range to which Jobling, et al. (1998) have attributed sexual disruption of wild fish. The San Juan-Bloomfield site is a trout fishery. The Rio Grande-South Valley site is within the area of concern over the Silvery Minnow, an endangered species that has caused much local controversy. It should be noted, however, that most samples thus far have been one-time grabs, with the exception being the two negative samples at the Rio Grande-Bernalillo site. Some of these sites, particularly in the middle Rio Grande valley, will be re-sampled in the future.

No drug residues, whatsoever, were detected in any of the eight ground-water samples. The presence of caffiene and pharmaceutical residues in ground water, therefore, was not an indicator of nitrate contamination by septic tanks at the two sites that were sampled (Carnuel and Western Terrace).

The decrease in drug concentrations between the Espanola sewage outfall and the effluent ditch located approximately 100 meters downstream from the outfall could be the result of photolysis, the destruction of drug compounds by sunlight. The samples were collected around 12:00 pm on a hot August day.

Drug residues were not detected in any of the three private domestic wells in La Cieneguilla. These wells all have elevated nitrate attributed to historical discharges of treated sewage effluent, containing approximately 30 mg/L of total nitrogen, from the Santa Fe waste-water treatment plant. Treated sewage is discharged to the Santa Fe River, a losing stream in this area, and infiltrates into the aquifer tapped by La Cieneguilla wells. Total nitrogen in the effluent was greatly reduced around 1985 after improvements were made to the treatment

plant, and nitrate levels in wells closest to the plant have since decreased to less than detectable. The three wells that were sampled are located in a different area of the nitrate plume that has not yet been displaced by post-1985 sewage effluent. It is postulated that any drug residues were either destroyed by photolysis prior to infiltration, or were naturally attenuated in the subsurface. Future sampling will include wells closest to the plant that are pumping ground water containing post-1985 sewage effluent.

Buser, et al., (1998b) demonstrated that sunlight can rapidly degrade pharmaceutical residues in water. New Mexico has an arid, often sunny, climate, a high elevation, and sparce populations relative to many areas in Europe, for example. Additionally, our rivers are relatively wide and shallow, enhancing the exposure of river water to sunlight. It is hypothesized that the intense sunlight in New Mexico causes rapid photolytic degradation of drug residues discharged into surface water. The decreasing concentrations in Espanola, the absence of drug residues in the Rio Grande downstream of Albuquerque (Belen, Bernardo, San Antonio, and Elephant Butte), and the absence of drug residues in the San Juan downstream of Farmington (Hogback and Shiprock) provide strong evidence that this is the case.

Acknowledgments

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Factors Affecting the Concentrations of Pharmaceuticals Released to the Aquatic Environment

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Abstract

Although recent research has demonstrated that pharmaceuticals are widely distributed in the aquatic environment, it is difficult to assess the threat that they pose to drinking water supplies or their rate of attenuation in natural systems without an adequate understanding of the sources of contamination. To identify pharmaceutical compounds of significance to water supplies in the United States, we have reviewed available data on the use of prescription drugs. Results of our analysis indicate that approximately 40 compounds could be present in municipal wastewater effluent at concentrations above 1,000 ng/L and at least 120 compounds could be present at concentrations above 1 ng/L. Important classes of prescription drugs include analgesics, beta-blockers and antibiotics.

Analysis of a group of the most commonly used pharmaceuticals in the United States indicates that they are ubiquitous in wastewater effluents. We have detected concentrations ranging from approximately 10-3,000 ng/L for high use pharmaceuticals such as beta-blockers (*e.g.,* metoprolol, propranolol) and acidic drugs (*e.g.,* gemfibrozil, ibuprofen). The concentration of pharmaceuticals in effluent from conventional wastewater treatment plants is similar. Advanced wastewater treatment plants equipped with reverse osmosis systems reduce concentrations of pharmaceuticals below detection limits. In addition to removal during biological wastewater treatment, pharmaceuticals also are attenuated in engineered natural systems (*i.e.,* treatment wetlands, groundwater infiltration basins). Preliminary evidence suggests limited removal of pharmaceuticals in engineered treatment wetlands and nearly complete removal of pharmaceuticals during groundwater infiltration.

Introduction

During the 1990s, researchers in Germany and Switzerland reported the occurrence of numerous pharmaceuticals in municipal wastewater and in surface waters that receive wastewater effluent (Stan *et al.* 1994, Hirsch *et al.* 1996, Stan and Heberer 1996, Stumpf *et al.* 1996, Buser *et al.* 1998a, Buser *et al.* 1998b, Hartmann *et al.* 1998, Ternes 1998, Buser *et al.* 1999, Hartig *et al.* 1999, Hirsch *et al.* 1999,Ternes and Hirsch 2000). Preliminary results from studies conducted in North America have confirmed the presence of many of the same compounds (Metcalfe and Koenig 2000, Sedlak *et al.* 2000, USGS 2001). Despite these findings, our ability to predict the concentrations of pharmaceuticals in receiving waters and in potable water supplies is limited by a lack of understanding of the attenuation mechanisms of pharmaceuticals. To predict the concentrations of pharmaceuticals in the aquatic environment and to design strategies to minimize exposure to pharmaceuticals, scientists and engineers must understand the processes that result in attenuation of pharmaceuticals both in wastewater treatment plants and in engineered receiving waters.

Organic contaminants present in municipal wastewater, such as pharmaceuticals, may be removed or transformed by a variety of mechanisms. In conventional wastewater treatment plants, pharmaceuticals can be removed by sorption to particles or by biotransformation. In advanced wastewater treatment plants, such as those employed prior to indirect potable reuse, pharmaceuticals may be removed by physical separation processes (*e.g.,* reverse osmosis). Finally, when wastewater effluent is discharged to engineered receiving waters, attenuation of pharmaceuticals also could occur through a combination of physical, biological and photochemical processes.

To develop a better understanding of the factors controlling concentrations of pharmaceuticals in the aquatic environment in the United States, we have studied the sources of pharmaceuticals in municipal wastewater and the effect of different treatment processes on concentrations of some of the most common pharmaceuticals present in municipal wastewater. Results of our research suggest that it may be possible to design cost-effective approaches for minimizing concentrations of pharmaceuticals in the aquatic environment. However, further research will be required to optimize these treatment systems.

Estimation of Pharmaceutical Concentrations in Municipal Wastewater

As mentioned previously, most available data on concentrations of pharmaceuticals have been collected in Europe. To assess the occurrence of pharmaceuticals in the United States, it is necessary to identify those compounds that are most likely to be present. To accomplish this goal, we have reviewed data on pharmaceutical use in the United States and studies on the occurrence of pharmaceuticals in Europe. The purpose of our review is to identify compounds likely to be present at relatively high concentrations that can serve as indicators for the larger suite of pharmaceuticals.

The first step in identifying pharmaceuticals meriting further study requires us to predict the concentrations of compounds present in water discharged by municipal and agricultural sources. To achieve this goal, we have estimated concentrations in untreated wastewater for the most popular U.S. pharmaceuticals. Estimates were not made for less popular drugs, non-prescription drugs, and drugs used mainly in hospitals (*e.g.,* X-ray contrast media, cancer chemotherapy drugs) because sales data were unavailable.

Our approach for estimating the concentrations of prescription drugs involved dividing the mass of drug excreted by patients by the volume of wastewater discharged to municipal wastewater treatment plants. Calculations were performed for the top 200 prescription drugs listed in a 1998 survey conducted by IMS Health (1999). The top 200 prescription drugs include 136 compounds, because some of the drugs contain the same active ingredient. Because numerous assumptions are needed to convert the number of prescriptions administered to the concentration of pharmaceuticals in municipal wastewater, considerable uncertainty is associated with these estimates. Despite the uncertainties, the estimates are useful in identifying pharmaceuticals that are candidates for further study.

Estimation of the concentrations of pharmaceuticals in municipal wastewater required the conversion of the number of prescriptions administered into the mass of compound discharged. Because several formulations are available for each prescription drug, the mass of active ingredient in a dose varies between prescriptions. For example, the β-blocker timolol is prescribed at 40 mg/prescription in an oral formulation and 6 mg/prescription in an eye ointment. To estimate the mass of active drug associated with each dose, we consulted medical reference books (Katzung 1998, PDR 1999) and interviewed a practicing pharmacist who provided information on the most popular form of each prescription of interest. After estimating the mass of active ingredient in each dose of the most popular form of the drug, we estimated the number of doses per prescription. Estimates were made for the maximum and minimum masses per prescription assuming the most common drug formulations. For drugs that were given on a one-time basis (*e.g.,* antibiotics), we assumed that each prescription included a sufficient number of doses to treat the ailment (typically 10 days). For drugs administered on a continuing basis (*e.g.,* beta-blockers, birth control pills) we assumed that each prescription was renewed monthly. The basis for this assumption was the current practices of many health maintenance organizations (HMOs) to refill prescriptions once per month.

After estimating the mass of each drug prescribed, we estimated the concentration present in untreated wastewater (results for those compounds predicted to be present at concentrations above 1,000 ng/L are included in Table 1). The values given in Table 1 represent the geometric mean of estimates based on the upper and lower bound estimates. When excretion data were readily available, we estimated the fraction of the dose excreted in its original form. However, excretion data were not readily available for many drugs, or when the

Table 1: Estimated concentrations of popular prescription drugs in untreated wastewater in the United States.

		Excluding Metabolism	Including Metabolism	
Name	**Classification**	**Predicted Wastewater Conc. (1) (ng/L)**	**Predicted Wastewater Conc. (1) (ng/L)**	**Excretion (2)**
acetominophen	analgesic	61,000	53,000	C
ibuprofen	analgesic, anti-inflammitory	37,000		B
amoxicillin	antibiotic	27,000	16,000	D
metformin	antidiabetic	24,000	21,000	D
cephalexin	antibiotic	14,000	12,000	D
nabumetone	analgesic, anti-inflammitory	12,000		F
azithromycin	antibiotics	9,200		J
oxaprozin	analgesic, anti-inflammitory	6,600		B
sodium valproate	anticonvulsant	6,000	2,400	H
gabapentin	anticonvulsant	5,400	5,400	D
carisoprodol	skeletal muscle relaxant	5,100		J
penicillin	antibiotic	4,000		J
sulfamethoxazole	antibiotic	3,800	3,200	G
gemfibrozil	cholesterol lowering	3,400		D
metoprolol	β-blocker	3,100	160	B
ciprofloxacin	antibiotic	3,100	1,400	G
ranitidine	H_2-receptor antagonist	3,000		J
mupirocin	antibiotic	2,800		A
clarithromycin	antibiotic	2,800	680	F
phenytoin	anticonvulsant	2,700		A
diltiazem	calcium channel blocker	2,600	79	A
naproxen	analgesic, anti-inflammitory	2,400		C
verapamil	calcium channel blocker	2,400	85	J
ipratropium	bronchiodiolater	2,400		E
trimethoprim	antibiotic	2,200	1,500	J
tramadol	analgesic	2,200	640	E
cimetidine	H_2-receptor antagonist	2,200	1,000	D
clavulanic acid	antibiotic	2,100	680	E
propoxyphene	opiod analgesic	2,100		J
bupropion	antidepressant	2,100		F
hydrochlorothiazide	diuretic	1,900	1,200	D
troglitazone	antidiabetic	1,800		B
cefprozil	antibiotic	1,700	1,000	J
pseudoephedrine	decongestant	1,600		J
erythromycin	antibiotic	1,500	75	I
atenolol	β-blocker	1,500	1,500	D
sertraline	antidepressant	1,400	180	F
triamterene	diuretic	1,400		J
nefazodone	antidepressant	1,300	13	F
tetracycline	antibiotic	1,200		J
allopurinol	antigout	1,000		F

(1) This calculation was made assuming that the population of the U.S. is 250 million, that each person produces 320 L of wastewater per da and that the excreted pharmaceuticals are evenly distributed among all wastewater in the U.S.

(2) (A) Extensive metabolism to inactive metabolites
(B) Extensive metabolism, possibly to conjugates
(C) Excreted mostly as conjugates
(D) Excreted mostly in original form (>50%)
(E) Excreted partially in original form (25-50%)
(F) Extensive metabolism to active metabolites
(G) Excreted as mixture of conjugates/original form
(H) Excreted partially as conjugates (25-50%)
(I) Little excreted in urine
(J) Data on metabolism not obtained

References for doses and metabolism:
Katzung, B.G. 1998. *Basic and Clinical Pharmacology.* Stamford, CT: Appleton and Lange.

Physicians' Desk Reference. 1999. Montvale, NJ: Medical Economics Company, Inc.

data were available, it was unclear if glucuronide or sulfate conjugates were considered to be transformation products. Since the conjugates appear to be converted back into their original, unconjugated forms prior to, or during, wastewater treatment, conjugated forms of drugs should be included with the parent compound. As a result of missing or ambiguous data, information on metabolism was only available for 30% of the pharmaceuticals in the top 200 list. Therefore, comparisons between estimated concentrations of PhACs are made without consideration of metabolism. No attempts were made to quantify active metabolites.

Estimated concentrations of prescription drugs in untreated wastewater (only the top 49 drugs are included in Table 1) range from less than 1 ng/L to approximately 133,000 ng/L. The estimated concentrations are distributed over a wide range, with the majority of compounds estimated to be present at concentrations between 100 and 1,000 ng/L. In general, the compounds expected to be present at the highest concentrations consisted of analgesics (*e.g.,* acetominophen, ibuprofen) and antibiotics (*e.g.,* amoxicillin, cephalexin). Because some of the analgesics on the list also are available as over-the-counter products, their concentrations in wastewater could be considerably higher. Compounds estimated to be present at the lowest concentrations tended to be potent drugs such as hormones (*e.g.,* medroxyprogresterone, equilin). Therefore, compounds estimated to be present at low concentrations should not be eliminated from further consideration without considering issues related to potency. However, such compounds will be extremely difficult to detect using conventional analytical techniques.

After considering the predicted concentrations of pharmaceuticals, the availability of suitable analytical techniques and data on the occurrence of pharmaceuticals in Europe, we identified a sub-set of compounds to serve as indicators for pharmaceuticals in wastewater effluent. In several cases, compounds were included even though they were not expected to be present at high concentrations because they could be analyzed readily with the selected analytical methods. Excluding antibiotics, which are be discussed elsewhere (Huang *et al.,* 2001), our list included six acidic drugs (*i.e.,* diclofenac, gemfibrozil, ibuprofen, indometacine, ketoprofen and naproxen) and three beta-blockers (*i.e.,* metaprolol, nadolol and propranolol).

Materials and Methods

Samples collected from wastewater treatment plants and engineered receiving waters were subjected to solid-phase extraction and were analyzed for pharmaceuticals using gas chromatography/tandem mass spectrometry (*i.e.,* GC/MS/MS) and immunoassays. Details of the analytical methods have been, or will be, reported elsewhere. Therefore, we have only provided a brief description of our approach.

Between 1 to 4 liters of samples were filtered through 0.45-μm glass fiber filters followed by solid phase extraction. For the acidic drugs, the sample pH was adjusted to pH less than 2 with sulfuric acid followed by extraction using endcapped C-18 resin. For neutral drugs and beta-blockers, the solid phase consisted of C-18 resin. For hormones, a C-18 Empore disc was used for solid phase extraction.

Samples to be analyzed for acidic drugs or beta-blockers were eluted from the resins using methanol followed by derivitization. For the acidic drugs, we used a diazomethane/diethylether mixture for derivitization. The beta-blockers were derivitized using MSTFA followed by MBTFA. Samples were analyzed by GC/MS/MS using a 30-meter DB-5 column. Internal standards were added to each sample to monitor extraction and derivitization efficiency as well as GC performance. Typical detection limits for pharmaceuticals in wastewater effluent were around 10 ng/L.

Analysis of hormones was performed by modification of a previously published immunoassay approach (Huang and Sedlak 2000). Samples were eluted from the Empore disc using a methanol water mixture followed by dual column cleanup using gel permeation chromatography followed by reverse-phase chromatography. The detection limit for 17β-estradiol and 17α-ethinyl estradiol was approximately 0.02 ng/L. Confirmatory analysis was conducted using GC/MS/MS.

Attenuation of Pharmaceuticals

To assess factors controlling the concentrations of pharmaceuticals in receiving waters, we measured concentrations of pharmaceuticals present after different treatment processes. Additional analyses are underway and we plan on publishing complete results of these studies during the coming year. Therefore, the results presented here should be considered preliminary. However, they do illustrate the relative significance of different mechanisms that can be pursued in more detail as part of future investigations. In the following sections, we describe the attenuation mechanisms and available data for each of the different treatment processes.

Conventional Wastewater Treatment Plants

Two attenuation mechanisms are potentially important for the removal of pharmaceuticals in conventional wastewater treatment plants: (1) sorption to particles followed by settling and removal; and, (2) biotransformation. The relative importance of these mechanisms will be controlled by the configuration of the treatment plant, the physical and chemical properties of the compounds and the concentrations of the compounds.

During primary and secondary wastewater treatment, particles are efficiently removed. Therefore, any pharmaceutical with a high affinity for particles also will be removed in the sludge. (If these compounds are not transformed prior to sludge disposal, they could lead to an additional exposure pathway that is not considered further in this paper.) Sorption of pharmaceuticals to particles present in wastewater treatment plants can occur either via hydrophobic or electrostatic interactions (*e.g.,* ion exchange, surface complexation).

For hydrophobic interactions, the octanol/water partition coefficient is a good predictor of the affinity of the compound for the solid phase. Under the conditions encountered in conventional wastewater treatment plants, only those compounds with octanol/water partition coefficients greater than approximately 100 will be removed to an appreciable degree (Sedlak *et al.,* 2000). With the exception of the steroid hormones, which have octanol/water partition coefficients of approximately 10,000, few pharmaceuticals meet this criterion. Therefore, we do not expect this mechanism to result in substantial removal of pharmaceuticals.

Sorption of pharmaceuticals via other interactions usually requires the presence acidic, phenolic or amino functional groups. Although many of the pharmaceuticals contain such functional groups, removal via these mechanisms is not expected to be significant under the conditions encountered in municipal wastewater. However, some antibiotics appear to undergo sorption via ion exchange reactions.

Removal of pharmaceuticals also can occur via biotransformation. Predicting the relative importance of biotransformation from chemical structure is notoriously difficult. Furthermore, compounds that are metabolized by microorganisms at relatively high concentrations may not be transformed to an appreciable degree when present at the extremely low concentrations encountered in wastewater. Available data from full-scale wastewater treatment plants suggest that certain compounds, such as ibuprofen, are readily degraded while other compounds, such as and carbamazepine, are removed to a much smaller extent (Ternes 1998, Buser *et al.* 1999).

Preliminary results from our survey of wastewater treatment plants are consistent with these findings (Figure 1). The horizontal lines included in the figure, which represent our estimate of pharmaceutical concentrations in untreated wastewater (*i.e.,* Table 1), allow us to compare expected influent concentration with observed effluent concentrations. These results indicate that more than 99% of the ibuprofen is removed during secondary wastewater treatment while less than half of the gemfibrozil and propranolol are removed during secondary treatment.

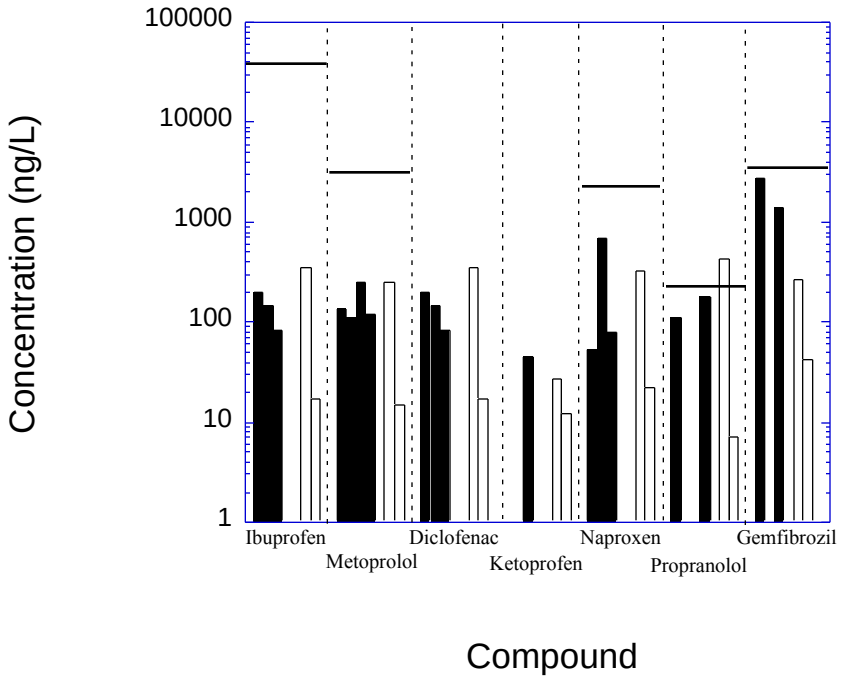

Figure 1: Concentrations of selected pharmaceuticals detected in final effluent collected from secondary (filled bars) and tertiary (hollow bars) municipal wastewater treatment plants. Horizontal lines indicate estimated concentrations in untreated wastewater.

The six pharmaceuticals depicted in Figure 1 were detected in wastewater effluent samples at concentrations ranging from approximately 10 to 3,000 ng/L. Concentrations detected in secondary effluent samples (filled bars) were similar for all four sites. For the two sites that also employed nitification (hollow bars) concentrations at the first site, which is a trickling filter followed by a nitrification tower, were comparable to those detected in the secondary effluent samples while concentrations detected at the second site, which employed activated sludge followed by nitrification, exhibited significantly lower concentrations.

Advanced Wastewater Treatment Plants

Indirect potable reuse projects equipped with state-of-the-art advanced wastewater treatment plants have been operated in several locations in the United States for over twenty-five years (NAS 1998, Sedlak *et al.* 2000). To remove pathogens and chemical contaminants, many of these facilities employ membrane treatment. Currently, the most common configuration for these systems involves the use of lime coagulation or microfiltration to remove colloids followed by reverse osmosis. Because the polar pharmaceuticals should not be associated with particles, we expect little removal to occur during lime clarification or microfiltration. In contrast, reverse osmosis should remove most of the pharmaceuticals that have molecular weights above approximately 200 gm/mole.

Measurements of the concentrations of pharmaceuticals at the West Central Basin Groundwater Replenishment Project, an advanced wastewater treatment plant that uses microfiltration and thin-film composite reverse osmosis membranes, are consistent with these expectations (Figure 2). Concentrations of pharmaceuticals measured before and after microfiltration are nearly identical. Concentrations of pharmaceuticals decrease to levels below detection limits after reverse osmosis treatment. These results suggest that full-scale reverse osmosis systems effectively remove these polar pharmaceuticals.

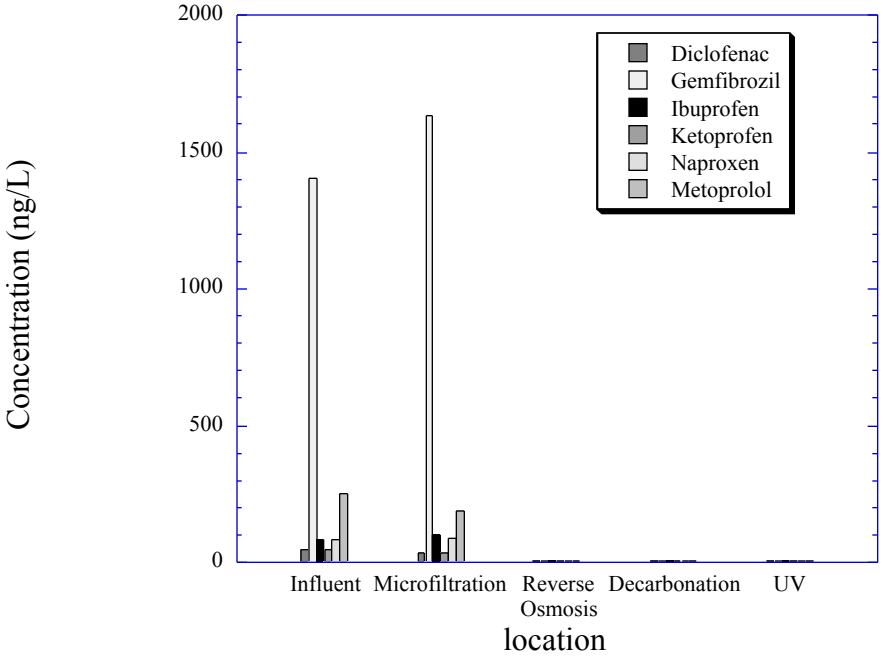

Figure 2: Concentrations of pharmaceutical measured after different unit processes at the West Central Basin Groundwater Replenishment Project. After reverse osmosis treatment concentrations of all pharmaceuticals are below 10 ng/L.

Engineered Receiving Waters

After conventional or advanced wastewater treatment, engineered receiving waters may be used to further attenuate contaminants, especially when water is being reused or discharged into a sensitive aquatic habitat. In the United States, the most common types of engineered receiving waters are surface engineered treatment wetlands and groundwater infiltration basins. Although most of the attenuation mechanisms found in wastewater treatment plants also will occur in these systems, changes in environmental conditions (*e.g.,* less organic matter available for metabolism) could alter the rates of transformation reactions. Furthermore, new attenuation mechanisms may also be important in these systems (*e.g.,* phototransformation in wetlands). To assess these processes, we have analyzed concentrations of pharmaceutical in samples from engineered receiving waters.

In groundwater infiltration basins, the microbial community metabolizes many organic compounds (Drewes and Fox 1999). The biological processes occurring during infiltration, which are sometimes referred to as soil aquifer treatment, could provide a means of removing pharmaceuticals that pass through conventional wastewater treatment systems. To assess the efficacy of soil aquifer treatment systems, we collected samples from the Sweetwater Groundwater Recharge Facility, located in Tuscon, Arizona. The facility consists of an infiltration basin that receives secondary wastewater effluent that replenishes a deep aquifer. Samples collected from the recharge pond, a shallow well (screened at 5 meters below the basin) and a deep well (screened at 40 meters below the basin) indicate that little removal occurs during the initial stages of infiltration and that all of the compounds are removed between the shallow and deep well. Because previous studies have indicated that almost all of the water sampled by the deep well originated in the infiltration basin, these data suggest that the pharmaceuticals are attenuated during passage through the aquifer. However, it should be noted that previous sampling of the deep well indicated the presence of several pharmaceuticals at concentrations as high as 50 ng/L (data not shown).

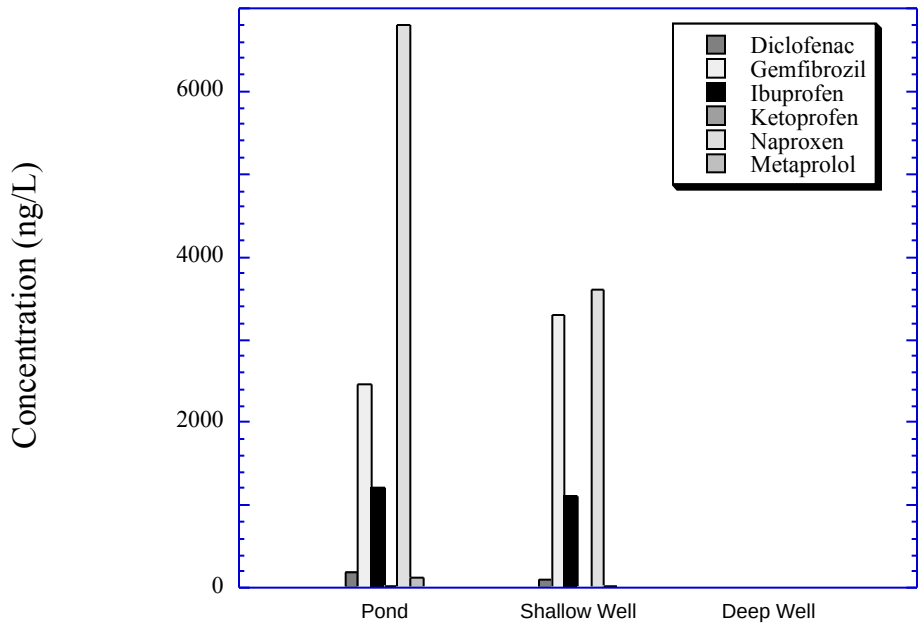

Figure 3: Concentrations of pharmaceuticals measured after groundwater infiltration at the Sweetwater Groundwater Recharge Site in Tuscon, Arizona. Concentrations of all compounds measured in the deep groundwater well were below detection limits.

Pharmaceuticals also could be removed during passage through engineered treatment wetlands. Attenuation mechanisms of potential importance in engineered treatment wetlands include biotransformation, photolysis and hydrolysis. To assess losses of contaminants during passage through treatment wetlands, we have measured

concentrations of pharmaceuticals at locations throughout an engineered treatment wetland that receives nitrified wastewater effluent. Samples were collected at the exit weirs from several wetland cells located along the flow path of the water. Previous tracer studies at the wetland indicate a hydraulic residence time of approximately seven days. Little or no loss of pharmaceuticals was observed during passage through the treatment wetland (data not shown). In contrast, significant losses of the estrogenic hormone, 17α-estradiol were observed: influent concentrations decreased from approximately 2 ng/L in the first pond to less than 0.5 ng/L in the final pond. Because ethinyl estradiol is resistant to biotransformation in activated sludge treatment systems we hypothesize that the observed transformation is attributable to some other process, such as indirect photolysis.

Acknowledgments

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Assessment of Potential Antibiotic Contaminants in Water and Preliminary Occurrence Analysis

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Abstract

Antibiotics are among the emerging microcontaminants in water because of concerns of their potential adverse effects on the ecosystem and possibly on human health. Antibiotics are likely to be released into the aquatic environment via wastewater effluent and agricultural runoff as results of incomplete metabolism, ineffective treatment removal or improper disposal because large quantities of antibiotics are used annually in human therapy and in agriculture. Despite large quantities of use, published data on the amounts and use patterns of antibiotics are scarce. To assess the magnitude of the potential risks associated with antibiotics, a comprehensive literature review was conducted on the usage, occurrence and behavior of antibiotics.

To identify antibiotics that are likely to be present in water sources, concentrations of antibiotics in municipal wastewater and animal waste in the US were estimated and were classified according to chemical properties. The estimation of human health antibiotics was based upon the number of prescriptions administered. The estimated concentrations of antibiotics in untreated wastewater range from 3.9 ng/L to approximately 27,000 ng/L. The estimation of animal health antibiotics was based upon the sub-therapeutic usage in feed for growth promoting. Considerable variation in antibiotic usage exists among different animal species. Reported data on the occurrence of antibiotics in the aquatic environment confirm the persistence of certain antibiotics. Although information is limited, studies on the transformation and sorption of antibiotics indicate that these processes significantly affect the fate of most classes of antibiotics. By combining information on environmental fate with the predicted concentrations, we identify that antibiotics of sulfonamides and fluoroquinolones are the most likely water contaminants, followed by macrolides. Among sulfonamide and fluoroquinolone antibiotics, sulfamethoxazole and ciprofloxacin are most likely to be present in municipal wastewater effluent and sulfamethazine is most likely to be present in agricultural runoff. Azithromycin and tylosin are the most likely macrolides present in municipal wastewater effluent and in agricultural runoff respectively.

An occurrence study, which is currently underway, focuses on three of the potential antibiotic contaminants, ciprofloxacin, sulfamethoxazole and sulfamethazine, identified by literature review. Solid phase extraction methods were developed. Recoveries ranged from approximately 45 to 106 %. Analysis of the three antibiotics were conducted by liquid chromatography mass spectrometry (LC-MS). Additionally, high performance liquid chromatography (HPLC) with fluorescence detection was employed for ciprofloxacin analysis. Preliminary results indicated the presence of ciprofloxacin in secondary wastewater effluent at approximately 80 to 150 ng/L. Sulfamethoxazole was detected in one of the wastewater samples. Sulfamethazine was not detected. Concentrations of antibiotics were found to be much lower or below the detection limits in the effluent of advanced treatment processes including granular activated carbon and ozonation, indicating significant removal by those processes.

Introduction

Large quantities of antibiotics are administered to humans and animals to treat diseases and infection every year. Antibiotics are also commonly used at sub-therapeutic levels to livestock to prevent diseases and promote growth. Despite the long history of antibiotic usage, information regarding antibiotic production and use patterns in the US is severely limited due to the lack of coordinated and comprehensive monitoring and documenting efforts. The large amounts of antibiotic usage may result in their presence in environmental waters because up to 90% of the administered antibiotics can be excreted without undergoing metabolism.

Results of recent studies indicate the presence of low concentrations of antibiotics in municipal wastewater effluent and surface water (Hartmann et al., 1998; Hirsch et al., 1999; Hartig et al., 1999; Meyer et al., 2000; Alder et al., 2000; Nipales et al., 2000; Frick et al., 2001). Despite the detection of antibiotics, little is known about their distribution in the environment, their mobility and persistence in natural and engineered water systems. The presence of antibiotics in environmental waters is a concern because antibiotic contaminants could perturb microbial ecology, increase the proliferation of antibiotic-resistant pathogens, and could pose threats to human health (Daughton and Ternes, 1999). The contamination of antibiotics in waters also presents challenges for the water industry on the issues of water reuse and water resource planning.

To assess the magnitude of the potential risks associated with antibiotics as water contaminants, a better understanding of the occurrence and environmental fate of these compounds is strongly needed. Based upon this need, we conducted a comprehensive review on scientific literature and available data regarding the usage, occurrence and behavior of antibiotics. Since a large number of antibiotic compounds are currently in use, conducting studies on the occurrence and fate of all compounds can be time-consuming and costly. Our objective is to review the commonly used antibiotics systematically to identify compounds that are likely to be water contaminants. Results of this review can facilitate prioritizing future research efforts.

Based upon the information on antibiotic usage, concentrations of antibiotics in municipal wastewater, animal manure and liquid animal waste were estimated and were classified according to chemical properties. Although information is limited, studies on the transformation and sorption of antibiotics indicate that these processes significantly affect the fate of most classes of antibiotics. By combining information on environmental fate with the predicted concentrations, antibiotics that are likely to be present in water sources can be identified. After the potential antibiotic contaminants in waters have been identified, occurrence analysis for these compounds has been initiated. Our field measurements focus on three of the potential antibiotics, ciprofloxacin, sulfamethoxazole and sulfamethazine, identified by literature review. Robust and sensitive analytical techniques have been developed to extract, concentrate and detect these compounds in complex water matrices. Some of the preliminary results of the field measurements are reported.

Methodology

Literature Review

The methodology for literature review involved several tasks in the following order: (1) identification of popular antibiotics commonly used in human therapy and in animal husbandry, (2) estimation of concentrations of antibiotics in untreated municipal wastewater and in liquid waste from confined animal feeding operations, (3) review of occurrence data of antibiotics in the aquatic environments, (4) review of information on sorption and transformation of antibiotics, (5) comparisons between human and veterinary antibiotics, and (6) assessment of potential antibiotic contaminants in water.

Antibiotics were categorized according to their chemical and structural properties. Members of the same class of antibiotics have similar structures, act by similar mechanisms, and likely to behave similarly in the environment. Such an approach will yield results that can be used to predict structurally-related compounds currently in use or being developed. More than ten antibiotic classes (aminoglycoside, ionophore, β-lactam, macrolide, polypeptide, quinolones, sulfonamide, tetracycline, streptogramin and other) are currently in use. Among the antibiotic classes, six are important in both human medicine and animal husbandry (aminoglycoside, β-lactam, macrolide, quinolone, sulfonamide and tetracycline). Our literature review focused on these six antibiotic classes because of the higher risks for human health if they are present in water as contaminants. The cross use of these antibiotic classes both in human and animals may lead to a more rapid development of bacterial resistance toward these drugs.

For antibiotics used in animal husbandry, we focused on beef, swine and poultry production. Antibiotic usage in other food animal species and aquaculture was not included in the literature review because of the relatively small production quantities in the US and thus less amounts of antibiotic usage. Our literature review also did not include antibiotic usage on pet animals.

Analytical Methods for Occurrence Survey

Effluent samples from local municipal wastewater treatment plants were collected in fluorinated high-density polyethylene bottles and kept in a cooler with ice during transportation. After being brought back to the lab, the samples were immediately filtered by 0.5 μm glass fiber filter and stored at 4°C until extraction, which occurred within two to three days. After acidification to approx. pH 3-4 with phosphoric acid, 1 L of effluent sample was extracted using a solid-phase extraction apparatus (Supelco). A combination of an anion-exchange cartridge (LC-SAX) (Supelco) and an Oasis HLB cartridge (Waters) was used in the solid-phase extraction. Prior to extraction , the cartridges were conditioned with methanol and deionized water (pH 4). The sample was pulled through the cartridges under a vacuum at a flow rate of less than 5 ml/min. Wastewater organics were eluted with 10 ml acidified methanol. The collected eluents were blow down to dryness under a gentle stream of nitrogen gas and redissolved in 1 ml acidified methanol/water mixture (1:4). For each wastewater sample, duplicate analyses were conducted for both unspiked and spiked samples. For spiked recovery analyses, 200 ng/L to 20 μg/L of antibiotics were added.

A reverse-phase HPLC system with a fluorescence detector (1100, Aligent Technology) was used to analyze ciprofloxacin. Detection of ciprofloxacin was conducted at an excitation wavelength of 278 nm and an emission wavelength of 450 nm. The mobile phases include a solution containing 20 mM H_3PO_4 and 20 mM NaH_2PO_4 (eluent A, pH ≈ 2.4), acetonitrile (eluent B) and methanol (eluent C). The mobile phase begins with 0.5 minute isocratic 98% A (2% B), followed by a gradient decrease to 90%A in 0.5 minute, then a gradient decrease to 75% A in 9 minutes, followed by 5 minutes isocratic 75% A. The mobile phase is then switched to 15% A, and the column was flushed under these conditions for 5 minutes. The mobile phase then shifts to isocratic 100% C for 10 minutes to flush the column thoroughly and finally switches back to 98% A and 2% B for the final 6 minutes of the run.

A HPLC/UV/MS system (Hewlett-Packard, Series 100 MSD G1946A) was used to analyze ciprofloxacin, sulfamethoxazole and sulfamethazine. The employed mobile phases include a solution containing 0.2% acetic acid with 10% acetonitrile (eluent A) and acetonitrile (eluent B). The flow rate was set at 0.2 ml/min with an injection volume of 20 μL. The column temperature was maintained at 30°C. Detection at 265 nm was used on the UV detector. MS analysis was conducted using electrospray ionization at positive ion mode. Both total-ion and single-ion monitoring analyses (SIM) were conducted. SIM was conducted for the molecular ions and some of the fragment ions of the antibiotics.

Results and Discussion

Estimation of Introduction Concentrations

A. Antibiotics Used in Human Therapy

Antibiotics that are likely to be present in discharged municipal wastewater are primarily antibiotics used in human therapy. To identify the most important antibiotics present in US municipal wastewaters, quantitative estimates on the concentrations of antibiotics in municipal wastewater were made. The estimation was focused on the most popular antibiotics in the U.S, i.e., the antibiotics (18 of them) that were among the top 200 prescription drugs listed in a 1998 survey conducted by IMS Health (Health, 1999).

Our approach for estimating the concentrations of antibiotics involved dividing the mass of antibiotics excreted by patients by the volume of wastewater discharged to municipal wastewater treatment plants. Because numerous assumptions are needed to convert the number of prescriptions administered to the concentration of antibiotics in municipal wastewater, considerable uncertainties are associated with these estimates. Despite the uncertainties, the estimates should be useful in identifying antibiotics that are candidates for further study.

The mass of antibiotics excreted was estimated based upon the number of prescriptions administered, the number of doses per prescription and the mass of antibiotic ingredient in each dose. To improve the accuracy of the estimation, we consulted medical reference books (Katzung, 1998; PDR, 1999) and interviewed a practicing pharmacist (Field, 1999). Antibiotics are normally given on a one-time basis. It was assumed that each prescription included a

sufficient number of doses (typically 10 days) to treat the ailment. When excretion data are readily available, we estimated the fraction of the dose excreted in its original form. However, excretion data were not readily available for all antibiotics, or when the data were available, it was unclear if conjugates (e.g., glucuronide or sulfate) were considered to be transformation products. Since the conjugates appear to be converted back into their original unconjugated forms prior to or during wastewater treatment, conjugated forms of antibiotics should be included with the original forms. As a result, estimated concentrations of antibiotics were made both with and without consideration of metabolism.

After estimating the mass of each antibiotic prescribed, we estimated the concentration present in untreated wastewater. For this calculation, it is assumed that a total amount of 8×10^{10} L of wastewater was produced daily (a population of 250 million and each person produces 320 L of wastewater per day) and that the excreted antibiotics are evenly distributed among all wastewater in the US. Estimated concentrations of antibiotics in untreated municipal wastewater range from 3.9 ng/L to approximately 27,000 ng/L.

Among the 18 most popular antibiotics, there are six β-lactams (amoxicillin, cephalexin, penicillin, cefprozil, cefuroxime, and loracarbef), three macrolides (azithromycin, clarithromycin and erythromycin), two fluoroquinolones (ciprofloxacin and levofloxacin), two aminoglycosides (neomycin and tobramycin), one sulfonamide (sulfamethoxazole), one tetracycline (tetracycline), and three others. The β-lactam antibiotics account for the most antibiotic usage in human therapy followed by macrolide, sulfonamide and fluoroquinolone antibiotics. Tetracycline, aminoglycoside and other antibiotics account for smaller quantities of usage compared to the above four antibiotic classes. Among the β-lactam, macrolide, sulfonamide and fluoroquinolone classes, our estimation indicates that the most important antibiotics are amoxicillin, azithromycin, sulfamethoxazole and ciprofloxacin respectively. The estimated concentrations for these antibiotics in untreated municipal wastewater are listed in Table 1.

Table 1. Predicted concentrations of antibiotics in untreated municipal wastewater.

Antibiotic	**Class**	**Predicted Wastewater Conc. (ng/L) – excluding metabolism**	**Predicted Wastewater Conc. (ng/L) – including metabolism**
amoxicillin	β-lactam	27,000	16,000
azithromycin	macrolide	9,200	NA
sulfamethoxazole	sulfonamide	3,800	3,200
ciprofloxacin	fluoroquinolone	3,100	1,400

NA = not available.

B. Antibiotics Used in Livestock

Information on the use of antibiotics in livestock is severely limited. The lack of data in this field is worse than in human therapy as a result of animal antibiotic use not being documented. Currently, publicly available information on the antibiotic usage or use patterns in livestock is not available. Recently, the Union of Concerned Scientists (UCS) (Mellon et al., 2001) issued a report on the estimates of antibiotic use in beef cattle, swine and poultry production in the United States. Their estimates focused on nontherapeutic uses of antibiotics in livestock for growth promotion and disease prevention. Many assumptions were employed in these calculations due to information limitations. Despite the uncertainties associated with these assumptions, the UCS estimates were carefully based on a wide range of information (direct and indirect) related to the usage of antibiotics in livestock. The Animal Health Institute (AHI) (2001) also issued a report on antibiotic usage in livestock. This report was based upon surveys among their industry members. The information released by the AHI is difficult to utilize because information on specific antibiotic compounds was not provided. Therefore, we relied upon the UCS report to facilitate the identification of important antibiotic compounds used in agriculture. The information in the UCS report allows us to identify which antibiotics are used in the greatest quantities in livestock operations in the US.

Based upon the UCS estimates, considerable differences in antibiotic usage exist among different food animal species (beef vs. swine vs. poultry). Therefore, the types of antibiotic compounds that are likely to be found in

surface water will strongly depend upon the types of livestock operations within the watershed. In considering all three animal productions, generally high quantities of tetracycline, macrolide, sulfonamide, aminoglycoside, and β-

Antibiotics	Class	Antibiotic Concentration (_g of antibiotic/kg of manure)
chlortetracycline sulfamethazine	tetracycline sulfonamide	3973
tylosin	macrolide	1946
bacitracin	polypeptide	1703
oxytetracycline	tetracycline	1064
chlortetracycline	tetracycline	993
erythromycin thiocyanate	macrolide	730

lactam antibiotics are commonly used. Chlortetracycline and oxytetracycline are the most commonly used tetracycline antibiotics. Among macrolides, tylosin is used in greatest quantity, followed by erythromycin and oleandomycin. Sulfamethazine is the most frequently used sulfonamide antibiotic. Among aminoglycosides, lincomycin is used in greatest quantity followed by apramycin. Penicillin is the most commonly used β-lactam antibiotic. Fluoroquinolone antibiotics are not used in livestock as growth promoters and are not included in the UCS estimates. Fluoroquinolones (enrofloxacin and sarafloxacin) have been used in poultry to prevent and treat diseases. However their use is expected to decrease as the USDA moves toward banning the use of these antibiotics in agriculture due to increases in bacterial resistance to fluoroquinolone drugs.

Other classes of antibiotics also play an important role in agricultural usage. These antibiotics include polypeptides (e.g., barcitracin), ionophores (e.g., monensin and lasalocid), arsenicals (e.g., roxarsone), and others (e.g., virginiamycin and bambermycin). These classes of antibiotics are used almost exclusively for livestock and thus may pose less threat to human health in contributing to antibiotic resistance, although exceptions exist.

Land application of animal waste provides routes for agricultural antibiotics to enter the aquatic environments, which may eventually reach drinking water supplies. To assess the input of antibiotics from agricultural sources, concentrations of antibiotics in manure are desirable. The concentration of antibiotics in the manure of both beef cattle and swine have been estimated and selected results are shown in Table 2 and Table 3. The concentrations were calculated by dividing the amount of antibiotic excreted by the amount of manure produced by the animal. The amount of manure produced by the animal is estimated based upon data found in the literature (Miner et al., 2000). The amount of antibiotic excreted by the animal is estimated based upon the antibiotic dosage reported by the UCS and in the Feed Additive Compendium (2000), assuming up to 80 % of the administered antibiotic is excreted unmetabolized.

The antibiotic concentrations in beef manure range from 0.73 mg/kg for erythromycin to 4.87 mg/kg for both of the two ionophores, lasalocid and monensin. The antibiotic concentrations in swine manure range from 1 mg/kg for bambermycin to 133 mg/kg for the mixture of chlortetracycline, sulfathiazole and penicillin. Note that not all of the antibiotics listed in the tables will be administered to the animals at one feeding operation. The selection of antibiotics and use pattern could vary considerably among different operations; such information is particularly limited and difficult to obtain. In general, use of two to four antibiotics is a common practice according to results of surveys (Mellon et al., 2001). Our estimates represent the expected concentrations in manure when a particular antibiotic(s) is in use and are useful in assessing the magnitude of the source for particular antibiotic compounds from manure.

Table 2. Estimates of concentrations of antibiotics in manure of beef cattle in a confined feeding operation. Cattle are in the 700-1200 pound (ave. weight of 1000 pounds) feedlot stage.

Table 3. Estimates of concentrations of antibiotics in manure of swine in a confined feeding operation. Swine are in the 100-260 pound (ave. weight of 150 pounds) finishing stage.

Antibiotics	Class	Antibiotic Concentration (mg of antibiotic/kg of manure)
chlortetracycline sulfathiazole penicillin	tetracycline sulfonamide β-lactam	133
tylosin sulfamethazine	macrolide sulfonamide	89
chlortetracycline	tetracycline	37
bacitracin	polypeptide	27
oxytetracycline	tetracycline	21
lincomycin	aminoglycoside	11
tylosin	macrolide	11
oleandomycin	macrolide	6

Furthermore, the concentrations of antibiotics in the liquid waste generated by a confined animal feeding operation (CAFO) were estimated. Our calculation focused on swine operations because CAFO is common for swine production and the facilities usually consist cement-based grounds, yielding limited on-site soil infiltration. The estimation involved dividing the mass of antibiotics excreted by animals by the volume of liquid waste generated. The calculation was conducted assuming a typical CAFO of 2500 head of swine (approx. 100-260 pound in the finishing-stage).

The liquid waste from a CAFO is comprised of (i) the waste quantities being generated by animals, and (ii) the water added to the waste from sources such as flushwater to remove manure from alleys and barns, water for cleaning, rainfall runoff from roofs and open lots and direct rainfall on pretreatment facilities (Overcash et al., 1983). The volume of (i) is usually insignificant compared to that of (ii). Water uses varies considerably from one operation to another, depending on such factors as type of buildings, methods of flushing, and type of management. Based upon the suggestions by Overcash et al. (1983), the estimated flushwater flow is approx. 9000 gallons per minute (gpm). In addition, it is assumed that one thirty minute flushing period takes place per day.

The results of the calculation range from 12 _g/L for bambermycin to 1.4 g/L for the chlortetracycline-sulfathiazole-penicillin mixture. Selected results are shown in Table 4. Similar to the previous discussion, only two to three of the antibiotics will be used simultaneously on swine in this stage and the estimates represent the concentrations expected in the swine CAFO wastewater when the particular antibiotic(s) is in use.

Table 4. Estimates of concentrations of antibiotics in the liquid waste of a swine confined feeding operation.

Antibiotics	Class	Ave. Antimicrobial (mg/day-animal)[1]	Concentration in Flushwater (ug/L)[2]
Chlortetracycline Sulfathiazole Penicillin	tetracycline sulfonamide β-lactam	741	1449
Tylosin Sulfamethazine	macrolide sulfonamide	496	971
Chlortetracycline	tetracycline	207	406
Oxytetracycline	tetracycline	118	232
Tylosin	macrolide	59	116
Lincomycin	aminoglycoside	59	116

(1) Based on data from the report issued by the Union of Concerned Scientist (UCS) (Mellon et al., 2001).
(2) Assumptions for the swine confined feeding operation: 2500 head in the 100-260 pounds (average weight of 180 pounds, finishing stage) and one thirty minute flushing period daily.
(3) It is assumed that up to 80 % of antibiotics are excreted unmetabolized.

Occurrence Data

Occurrence information of antibiotics is limited and most previous studies were conducted in Europe where antibiotic use could be different from that of the US. However, the previous studies provide guidance for identifying the classes of antibiotics that are more persistent in the environment.

The previous studies indicate that β-lactam antibiotics (many are widely used in Europe) were not detected in most environmental waters (Hirsch et al., 1999). Other classes such as fluoroquinolones (Hartmann et al., 1998), macrolides (Hirsch et al., 1999) and sulfonamides (Hirsch et al., 1999; Hartig et al., 1999) have been detected in wastewater effluent and surface water. Tetracyclines have been detected in the liquid hog lagoon waste (Meyer et al., 2000) and in general have not been found in municipal wastewater effluent, surface water or ground water (Hirsch et al., 1999; Meyer et al., 2000). A few other antibiotics including chloramphenical and trimethoprim have been detected in limited number of municipal wastewater effluent and surface water (Hirsch et al., 1999). More recent studies on the detection of fluoroquinolones and macrolides were reported at the spring 2000 national meeting of the American Chemical Society in San Francisco (Alder et al., 2000; Nipales et al., 2000). The U.S. Geological Survey (USGS) has recently conducted occurrence survey on pharmaceutical compounds including antibiotics in surface waters collected from sites across the US (USGS) and detection of antibiotics has been reported at the 2001 Georgia Water Resources Conference (Frick et al., 2001). The detected antibiotics including sulfonamide, macrolide, aminoglycoside, fluoroquinolone and others.

Environment Fate of Antibiotics

Once antibiotics are excreted from the dosed animals, they are subject to various processes such as sorption, abiotic transformation and biotic transformation in natural and engineered aquatic environments. These processes directly influence the fate and transport of these compounds in the environment as well as their biological activities. At present, published literature regarding the fate and transport of antibiotics in the aquatic environment is very limited, although more studies are being reported recently due to the increasing concerns over these compounds. We reviewed previous studies particularly focusing on the six antibiotic classes (aminoglycoside, β-lactam, macrolide, quinolone, sulfonamide and tetracycline) that are important in both human medicine and animal husbandry. Results are discussed in the following paragraphs.

A. Sorption

Previous studies indicate strong adsorption of tetracyclines (tetracycline, chlortetracycline and oxytetracycline) to clay materials (Sithole and Guy, 1987), soil and sediments (Rabolle and Spliid, 2000; Pouliquen and Lebris, 1996) throughout a wide range of environmental conditions. Although not as strong as that of tetracyclines, significant sorption of some macrolide (e.g., tylosin and avermectin B_{1a}) (Gruber et al., 1990; Rabolle and Spliid, 2000) and fluoroquinolone (Nowara et al., 1997) antibiotics to soil clay minerals have been reported. Sulfonamides exhibit weak adsorption to soil (Thiele, 2000; Fontaine et al., 1991) and activated sludge (Ingerslev and Halling-Sorensen, 2000). Information is scarce for the sorption of aminoglycoside and β-lactam antibiotics. Aminoglycosides are typically comprised by two or more sugars or amino sugars attached to an aminocyclitol ring, resulting in high polarity of the compounds. The amino groups of aminoglycosides can be positively charged by protonation under acidic conditions. The positive charge may facilitate adsorption to soil clay minerals that typically posses negative charge. β-Lactam antibiotics are highly polar compounds. Sorption of β-lactams to soil is expected to be weak due to their high polarity and carboxylic acid functional groups.

B. Abiotic Transformation

Hydrolysis is an important degradation pathway for organic pollutants in the aquatic environment. Among the six antibiotic classes, β-lactams, macrolides and sulfonamides are susceptible to hydrolysis. However, hydrolysis of macrolides and sulfonamides at neutral pH range is very slow and can be considered negligible (Volmer and Hui, 1998). β-Lactams generally undergo hydrolysis fairly quickly under mild acidic and base conditions (Hou and Poole, 1969). Photodegradation is another abiotic transformation that can affect organic pollutant persistence in the surface layers of water bodies that receives appreciable amount of sunlight. Quinolones and tetracyclines are susceptible to photodegradation (Torniainen et al., 1996; Davies et al., 1979). Currently little information is available for abiotic reductive or oxidative transformation of antibiotics in the aquatic environments.

C. Biotic Transformation

Gavalchin and Katz (1994) examined the stability of a number of agricultural antibiotics in soil. The observed persistence of antibiotics followed the trend of chlortetracycline > bacitracin > erythromycin > bambermycin > tylosin > penicillin and streptomycin. The loss of antibiotic was attributed to biodegradation although other reactions are also possible. Oxytetracycline, quinolone derivatives, and sulfonamide antibiotics were found to be persistent in model marine aquaculture sediment (Samuelsen et al., 1994). In liquid manure, considerable degradation of tetracycline was reported (Kuhne et al., 2000). In manure-containing systems, rapid loss of tylosin from the aqueous phase was observed under both aerobic and methanogenic conditions (Loke et al., 2000). The loss of tylosin was caused by a combination of sorption, abiotic transformation and biodegradation, however no further details were examined. Low biodegradability of β-lactam (penicillin G, cefotiam and meropenem), fluoroquinolone (ciprofloxacin) and sulfonamide (sulfamethoxazole) antibiotics was reported by Al-Almad et al. (1999). Partial biodegradation was observed with the β-lactam antibiotics and no biodegradation was observed with ciprofloxacin and sulfamethoxazole. Marengo et al. (1997) reported very slow aerobic biodegradation of sarafloxacin. Ingerslev and Halling-Sorensen (2000) examined biodegradation of several sulfonamides in activated sludge. Significant biodegradation occurred only after a considerable lag time and the employment of unusually high sulfonamide concentrations to stimulate particular degraders. It was suggested that biodegradation of sulfonamide in sewage treatment systems may be negligible. Based upon the previous studies, aminoglycosides, β-lactams and some macrolides are likely to biodegrade to a greater extent than quinolones, sulfonamides and tetracyclines.

Potential Antibiotic Contaminants in Water

The routes for entering the aquatic environment are different for human and agricultural antibiotics. For antibiotics used in human therapy, the excreted antibiotics undergo the treatment processes in the wastewater treatment facilities before entering surface water. After entering surface water, they are subject to various fate and transport processes in the environment. The routes of agricultural antibiotics entering the aquatic environment may be similar to those of many pesticides. Soil infiltration and biodegradation are likely to play an important role.

In general, antibiotics that degrade slowly and adsorb less strongly to soil and sediments are more likely to be emitted into surface and ground water flows and be transported to greater distance. Compounds with such properties also are more likely to pass through wastewater treatment. However, it is possible that persistent antibiotics with strong affinity toward particulate matters can be transported in the aquatic environment with particles and colloids.

As far as compound stability in the environment is concerned, sulfonamide antibiotics appear to be least susceptible to any transformation. Degradation of quinolone and tetracycline antibiotics is expected to be slow when exposure to sunlight is limited. Some macrolide antibiotics may be degraded to some extent. Aminoglycoside and β-lactam antibiotics are readily degraded in the environment and are unlikely to be persistent. As far as compound sorption to soil and sediments is concerned, tetracyclines adsorb most strongly followed by quinolones and macrolides. Sulfonamides exhibit weak to moderate adsorption to soils. Sorption of aminoglycosides and β-lactams to soils are likely to be weak. As a result, sulfonamide and fluoroquinolone antibiotics are the most likely water contaminants followed by the macrolides. Due to its strong affinity to soils and sediments, tetracyclines are likely to be found in water and soils near the antibiotic sources. Furthermore, the occurrence data on antibiotics generally correlate well with the above conclusion.

Comparison between the antibiotics commonly used in human therapy and animal husbandry indicate that, in general, different compounds within each antibiotic class are used separately for humans and for food animals. Among sulfonamide and fluoroquinolone antibiotics, sulfamethoxazole and ciprofloxacin are most likely to be present in municipal wastewater effluent and sulfamethazine is most likely to be present in agricultural runoff. Azithromycin and tylosin are the most likely macrolides present in municipal wastewater effluent and in agricultural runoff respectively.

Preliminary Results of Occurrence Analysis

After identifying the potential antibiotic contaminants in water, field measurements focusing on three compounds, ciprofloxacin, sulfamethoxazole and sulfamethazine were conducted. Robust and sensitive analytical techniques were developed for detection of these antibiotics in complicated matrices such as wastewater and surface water. The developed analytical methods yield good recoveries, ranging from approximately 45 to 106 % for ciprofloxacin and 45 to 95 % for the two sulfonamides.

Preliminary results indicated the presence of ciprofloxacin in secondary wastewater effluent (after activated sludge treatment) at approximately 80 to 150 ng/L. Utilization of advanced treatment such as granular activated carbon and ozonation reduced the concentrations of ciprofloxacin to around 10 ng/L and less. The presence of ciprofloxacin was also confirmed by LC/MS. The presence of sulfamethoxazole was detected in one of the secondary effluent samples. Sulfamethoxazole was not detected in effluent after granular activated carbon and ozonation treatment processes. In general, sulfamethazine was not found in any of the municipal wastewater effluent samples. Since the studied municipal wastewater treatment plants receive little wastewater from livestock operations, this observation is consistent with our expectation since sulfamethoxazole is used primarily in human medicine while sulfamethazine is used primarily in livestock, thus less likely to be present in municipal wastewater. The field measurements are currently in progress. Future occurrence study will include additional wastewater effluent from conventional and advanced treatment processes, surface water and wastewater from animal feeding operations.

Conclusions

Six classes of antibiotics including aminoglycoside, β-lactam, macrolide, fluoroquinolone, sulfonamide and tetracycline are commonly used in human therapy and in animal husbandry. The presence of these antibiotics in water as contaminants may pose risks to human health. A systematic literature review was conducted on antibiotic usage and environmental fate. The concentrations of antibiotics in municipal wastewater, animal manure and liquid animal waste were also estimated.

The literature review indicates that sulfonamides and fluoroquinolones followed by macrolides are more likely to persist and transport in the aquatic environments. Tetracyclines may persist for a significant period of time but less mobile. Aminoglycosides and β-lactams are least likely to persist in the environment. Among sulfonamide and fluoroquinolone antibiotics, sulfamethoxazole and ciprofloxacin are most likely to be present in municipal wastewater effluent and sulfamethazine is most likely to be present in agricultural runoff. Azithromycin and tylosin are the most likely macrolides present in municipal wastewater effluent and in agricultural runoff respectively.

Preliminary occurrence study using robust and sensitive analytical techniques was conducted for ciprofloxacin, sulfamethoxazole and sulfamethazine. Field measurement of these three antibiotics are currently underway. Presently, the presence of ciprofloxacin in conventional municipal wastewater effluent has been detected at approx. 80 to 150 ng/L. The presence of sulfamethoxazole was detected in one sample. In general, sulfamethazine was not found in the municipal wastewater effluent. Concentrations of antibiotics are much lower in the effluent of advanced treatment processes such as granular activated carbon and ozonation, indicating significant removal by these processes.

As analyses of antibiotic contaminants in complex water matrices such as wastewater and surface water can be time-consuming and costly. The antibiotics identified by this literature review may serve as "indicators" in analytical survey for antibiotic contamination. Furthermore, the antibiotic use patterns change rapidly and new compounds are being developed and introduced continually. To assess the potential risks associated with antibiotics in water sources, consideration of chemical and structural properties of antibiotics is critical and allows predictions for structurally-related compounds that are currently in use or being developed. Results of this literature review can also facilitate prioritizing future studies on the fate and transport of antibiotics in natural and engineered water systems.

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Biographical Sketches:

Ching-Hua Huang: Dr. Ching-Hua Huang is an assistant professor of Civil and Environmental Engineering at Georgia Institute of Technology. She received her B.S. in chemistry from National Taiwan University, M.S. and Ph.D. in Environmental Engineering from Johns Hopkins University. She joined Georgia Tech in January 2000 after two years of postdoctoral study at the University of California-Berkeley. Her research and teaching interests focus on the fate of organic contaminants in natural and engineered water systems. Specific areas include the fate of pharmaceuticals and agrochemicals in the environment, novel analytical method development, and kinetics and mechanisms of chemical transformation.

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Reconnaissance of Industrial and Household Use Chemicals and Pharmaceuticals in Selected Surface and Ground Water Resources in Minnesota, October 2000

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Abstract

Industrial and household use chemicals (surfactants, plasticizers, flame retardants, solvents, fragrances, food additives, disinfectants), prescription and non prescription pharmaceuticals (caffeine, antimicrobials, antibiotics, antiasthamatics, antacids, antihypertensives, and psychiatric drugs), and sex and steroidal hormones are newly recognized classes of contaminants (emerging contaminants) that have high usage rates, potential health effects, and are continuously released into the environment through human activities. These products enter the environment through transport pathways such as direct disposal in sewage systems and landfills, as well as runoff or infiltration from fields following application of wastewater treatment sludge or animal manure. The continual introduction of these chemicals into the environment may have undesirable effects on non-target organisms, including endocrine disruption and development of antibiotic resistance or toxicity.

Because of these concerns, a reconnaissance study was initiated to document the occurrence of emerging contaminants in selected surface and ground-water resources in Minnesota. During the first collection period in October 2000, water samples were collected from 4 ground-water locations, 15 stream locations, from the intake and finished water at 6 drinking-water facilities, and from the effluent of 2 wastewater treatment facilities.

Solvents, disinfectants, flame-retardants, and insecticides were the most common industrial and household-use chemicals detected in surface and ground-water samples. Among all samples, 44 industrial and household-use chemicals were detected with an average of five chemicals detected per sample. Isophorone, phenol, d-limonene, bromoform, tris(2-butoxyethyl) phosphate, and N,N-diethyltoluamide (DEET) were the most frequently detected compounds.

Caffeine compounds, analgesics, antibiotics, antihypertensives, and cotinine (nicotine metabolite) were among the most common pharmaceuticals detected in surface water samples. No pharmaceuticals were detected in ground-water samples. Among all surface water sites, ten pharmaceuticals were detected with an average of 2.6 pharmaceuticals detected per sample. The most frequently detected pharmaceutical was caffeine, followed by 1,7-dimethylxanthine (caffeine metabolite), acetaminophen, sulfamethoxazole, diphenhydramine, trimethoprim, ibuprofen, diltiazem, codeine, and cotinine. Among all samples, three sterols (cholesterol, beta-sisterol and 3-beta-coprostanol) and one hormone (17*B*-estradiol) were detected.

Biographical Sketch

Kathy Lee began her career as a hydrologist at the U.S. Geological Survey in 1992. She has worked on a variety of studies involving the fate of contaminants in the aquatic environment and potential effects on biota. Her current work has focused on identification of biomarkers of endocrine disruption, and detection of potential endocrine disrupting chemicals and pharmaceuticals in the aquatic environment. She received her B.S. and M.S. degrees in Environmental Science and Aquatic Ecology at the University of Kansas.
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Pharmaceuticals in the Environment

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Abstract

The presence of pharmaceuticals in the environment from use has been estimated and reported to the appropriate regulatory agencies as appropriate. This paper will review the past and present regulatory review of this issue as well as providing an overview of the pathways, occurrence and behavior of pharmaceuticals in the environment. An understanding of risk evaluations of pharmaceuticals in the environment will conclude this review.

Introduction

Though the earliest published research on pharmaceuticals in the environment dates back to the 1970s, recent efforts have refocused attention to their presence at low levels in the aquatic environment and piqued academic interest as to their environmental fate and effects.

The research published to date describes the sampling and analysis of surface water, groundwater, drinking water, and sewage treatment plant (STP) effluent, and the detection of pharmaceutical active ingredients and their degradation products, usually at concentrations much less than 1 µg/L. The pharmaceuticals reported in surface water include hormones (*e.g.*, synthetic and natural estrogens), antibiotics, blood lipid regulators, non-steroid analgesics and anti-inflammatory agents, beta-blockers, antiepileptics, antineoplastics, tranquilizers, and diagnostic contrast media. The pharmaceuticals detected in surface water and STP effluent are reported to be those that are complex molecules that have high solubility in water.

Most of the published research papers documenting the presence of pharmaceuticals in surface water, drinking water and STP effluent are European, although US researchers have reported pharmaceuticals in surface water, groundwater, and STP effluent as well. This research, most of which has been published between 1990-2000, has identified and quantified the presence of pharmaceuticals in these environmental media, but has not investigated the human health-related or environmental significance of their presence.

Summary of Relevant Surface Water and Effluent Sampling Programs in the US and Europe

United States

Until 1999, there had been no nationwide program in the US to sample either surface water or STP effluent for pharmaceutical chemicals. In 1999, the US Geological Survey (USGS) initiated sampling at 100 stream stations for its National Reconnaissance of Emerging Contaminants in Surface Waters program. Twenty-four prescription and non-prescription pharmaceuticals (including steroids and hormones) have been targeted. The stream monitoring stations are located at sites which are identified as "susceptible" to the presence of these chemicals, *i.e.*, areas impacted by urban and agricultural discharges. Although no published results are currently available, additional sampling was conducted in 2000 to confirm the results from the 1999 study and to add additional sampling stations.

There has been enhanced interest by state agencies in the issue as well. For example, the *Albuquerque Journal* recently reported the detection of two drugs in New Mexico rivers in a statewide study of drug residues in water conducted by the state environmental and health departments. New Mexico plans to continue to collect and analyze STP effluent, surface water, ground water and drinking water. No reports on the detected concentrations

of pharmaceuticals are available.

Finally, the EPA and state environmental agencies have routine surface water monitoring programs and all point source dischargers, including STPs, must monitor their effluent in compliance with their National Pollutant Standard Elimination System (NPDES) permit requirements. However, to date, none of these programs include pharmaceuticals in their lists of analytes. EPA has been requested to evaluate the need to regulate the discharge of pharmaceuticals from feedlots during the development of the Effluent Guidelines, Pretreatment Standards, and New Source Performance Standards for that industry.

Europe

Recently, the Federal Environmental Agency of Austria published a report, *Drug Residues in the Environment.* The report identifies the need to investigate ecological "compartments" for pharmaceutical substances and lists 50 priority human pharmaceutical substances and categories of veterinary drugs. It recommended that influents and discharges from wastewater treatment plants be screened for these compounds. If detected, proposed alternatives to eliminate their release include, among other things, control of point sources, imposition of requirements for enhanced environmental data for new pharmaceutical authorizations, and the development of new forms of drug administration.

The Environment Agency in England issued a similar report in 2000 prepared by WRc-NSF's National Center for Environmental Toxicology (NCET)[1] which reviewed information available on the occurrence, fate and effect of human pharmaceutical compounds in the environment and made recommendations for future actions. Among the report's recommendations is the suggestion that a guidance note on the environmental assessment of pharmaceuticals be expedited in the European Union and that efforts be undertaken to coordinate studies among researchers, regulators, manufacturers and the water industry. The UK Environment Agency has recently reviewed the issue and has recommended a number of actions, including measurement of concentrations of pharmaceuticals in effluents and receiving waters and assessing potential chronic environmental effects on a mode of action basis.

Evaluating Pharmaceutical Environmental Safety – the Regulatory Framework

Overview

Pharmaceuticals approved in the US and Europe are subject to a regulatory review of the environmental issues pertaining to the manufacture, use and disposal of each new drug product. The manner in which this review occurs has varied over time both in the US and Europe. In order to understand the current situation, it is important to review the history and evolution of these regulations.

United States

The 1969 National Environmental Policy Act (NEPA) specified that any major action by a federal agency required an environmental review. This environmental review was initiated by a Environmental Assessment (EA) submitted by the applicant that provided sufficient information for the federal agency to determine any potential for a significant environmental impact. If no such potential impact was determined, no further action was required. However, if a significant impact was anticipated, a comprehensive environmental impact statement was required for the project in question.

In 1975, the Council for Environmental Quality notified the US Food and Drug Administration (FDA) that the approval of a New Drug Application (NDA) constitutes a major federal action as identified in NEPA. The FDA was therefore mandated to issue regulations to require an environmental review program for NDAs.

In 1977, the FDA published regulations (amended in 1985) that required EAs for new drugs as part of an NDA

submission. Companies provided summary documentation with early NDAs. However, as time went on, the FDA requested that the industry provide more extensive information in their EAs (or in support of the categorical exclusions allowed by regulation) throughout the late 1980s and early 1990s.

As a result, the Pharmaceutical Manufacturers Association (currently P*h*RMA), in conjunction with the FDA, proposed guidance as to how to comply with the regulations and address the FDA's heightened concern in this area. This guidance recognized that while many of the industry's manufacturing activities were already regulated under other existing programs, the impacts from use and disposal by patients were not. Consequently, the guidance provided a detailed approach for evaluating new pharmaceuticals for potential environmental hazards and risks from patient use and disposal. This risk-based approach recognized that certain minute quantities of a drug substance could be present in human waste and that there was a potential for these materials to be present in municipal STP effluent. To provide assurance of the environmental safety of these compounds, this risk-based methodology was used by the industry to determine whether new drug substances posed any potential environmental risk. From 1990 to approximately 1995, over three hundred NDAs with corresponding EAs were submitted to the FDA.

As part of the FDA's efforts under the Reinventing Government initiative, the FDA determined that only one EA out of the 300 submitted warranted a further environmental impact statement (which was initiated due to environmental concerns about the sourcing of raw material). Based on this information, the FDA issued new regulations in 1997 requiring an EA in two situations only. The first is where the average concentration of the drug substance in the environment from use is estimated to equal or exceed 1 part per billion. In general, this corresponds to production greater than 44,000 kilograms per year. The second instance is in a situation that would be defined as an "extraordinary circumstance." This would include such examples as harvesting natural materials or where there may be adverse impacts on endangered species.

This revised EA program has been underway since the new regulations were issued in 1997. Companies conduct initial reviews that allow a categorical exemption to be claimed where it is warranted and for EAs to be submitted where required.

Europe

Although an Environmental Risk Assessment (ERA) is not yet a codified regulatory requirement in the EU, approval of a European Medicines Approval Application (MAA) is generally not provided unless an ERA is submitted. For MAA submissions, potential releases to air, water and soil must be evaluated. Air emissions are generally not relevant for pharmaceuticals. For aquatic impact, calculations are made to determine predicted environmental concentration (PEC) for each EU country (population divided by expected sales volume). The one with the highest PEC is selected for further evaluation and the PEC:PNEC (predicted no effect concentration) ratio is calculated. If this ratio does not exceed 1, no further evaluation is necessary. The allowable soil PEC is 10 parts per billion (μg/kg). The specified calculations take into account binding to sludge and the Koc (adsorption coefficient) of the pharmaceutical being evaluated. As in the USA, removal by metabolism, biodegradation, hydrolysis, etc., may be factored into the impact evaluation.

The fundamental difference between US requirements and EU requirements is that the dilution factor of 10 used to reflect the dilution of effluent that occurs in receiving waters, which is allowed in US requirements, is not allowed in EU submissions. This effectively puts the threshold limit at 0.1 part per billion; therefore, additional environmental fate and effects information may be required in Europe for drug substances with anticipated peak production of 4,000 kilograms or more per year.

At the present time, European regulatory officials are continuing to examine the requirements and the final format for the ERAs that will need to be implemented by member countries.

Understanding the Pathways, Occurrence and Behavior of Pharmaceuticals in the Environment

Pathways to the Environment

Since most of the pharmaceuticals detected in the environment are water- soluble, understanding water as the means of transport is key to understanding the pathways to the environment. Pharmaceuticals can be introduced into the environment through their intended use, manufacture or disposal.

The most significant pathway, in terms of the quantity of pharmaceuticals introduced, is *via* the therapeutic use of pharmaceuticals and the subsequent excretion of the active ingredients or their metabolites in urine and feces. The excreted chemicals generally enter sewage treatment systems and then enter surface water and groundwater when the treated wastes are discharged.

Disposal of sewage sludge on land, either as a fertilizer/soil amendment or in landfills, has been cited as a potential source for pharmaceuticals in the environment. The pathways would be to surface water by runoff that contacted the sludge or to ground water by precipitation that percolates through the sludge into an underlying aquifer. There is no available research that supports sewage sludge disposal as an important source and pathway for pharmaceuticals into the environment.

Disposal of unused pharmaceuticals in landfills has been linked to the presence of pharmaceuticals in ground water. There are several published papers reporting the detection of pharmaceuticals in landfill leachate and the resulting entry of the leachate into groundwater. To date, disposal of unused pharmaceuticals in landfills has not been identified as a major pathway to ground water; although such disposal probably occurs commonly among individual consumers via their household trash, it is not generally the method of disposal used by the US pharmaceutical industry for unused product. "Returned Goods" programs sponsored by pharmaceutical firms that encourage pharmacies, wholesalers and distributors to return expired or damaged pharmaceutical stocks to the manufacturer. These programs help assure the proper disposal of unused pharmaceuticals.

Although there are some indications in the literature that manufacturing is a source of pharmaceuticals in the environment, waste waters and wastes generated at pharmaceutical manufacturing facilities are not thought to be major sources of pharmaceuticals and their metabolites in the overall environment. Manufacturing facilities make every effort to assure that product is not lost to the waste stream. Manufacturing releases would be localized and likely a minor contributor to the environment.

Certain pharmacologically active chemicals occur naturally or are found in certain food. These chemicals of "natural" origin have also been identified in STP effluent and surface water. The most significant of these are human estrogen (17ß-estradiol) and caffeine. Treated effluent has been known for many years as the principal source of these chemicals in the aquatic environment. There are likely other "natural" chemicals with pharmacologically active properties that may be found in surface waters if sufficiently selective and sensitive analytical methods are developed and the chemicals are targeted by research efforts.

Occurrence in the Environment

Increased Analytical Capability and Sensitivity

Pharmaceuticals in STP effluents were first identified in the late 1970s. It wasn't until the early 1990s that there were more frequent reports of pharmaceuticals being positively identified in the environment. The principal reason for this is that the analytical methods used prior to the 1990s were generally incapable of detecting, identifying, and quantifying pharmaceutical chemicals at concentrations below 1 µg/L. Specifically, sensitive analytical methods for organic chemicals with polar properties, which many pharmaceuticals exhibit, were largely unavailable.

In the late 1980s, new, highly sensitive analytical methods for organic chemicals with polar and nonpolar properties were developed. These new analytical methods could detect and quantitate organic chemicals, including pharmaceuticals, at concentrations ranging from 1-100 nanograms/litre (ng/L). Some pharmaceutical chemicals can now be identified and quantified at sub-ng/L concentrations, i.e., down to 100 parts per

quadrillion (to 0.1 ng/L).

Efforts to improve the sensitivity of analytical methods for trace analytes in water will continue. As an example of the sensitivity of analytical methods for trace organics in water, the currently approved analytical method for 2,3,7,8-tetrachloro-p-dioxin can detect this chemical at less than 1 picogram/litre (pg/L) - 1000 times lower than 1 ng/L, nearly approaching the molecular level. As the sensitivity of analytical methods increases, it is likely that additional chemicals will be identified in ambient waters and that chemicals already found may prove to be more widespread.

The Relationship of Water Reuse Patterns to Concentrations

The research on pharmaceuticals in surface waters shows a strong correlation between the surface water concentrations of these chemicals and the fraction of treated sewage effluent in the surface water when the sample was collected. This correlation is not surprising once one understands the natural water cycle.

Water used by humans is sometimes altered by its use. Industrial and domestic uses add a variety of chemicals to water. Treatment removes some, but not all, of these chemicals. The treated wastewater is then discharged to a receiving stream where it is diluted with the natural flows generated by precipitation and/or ground water and by treated wastes discharged by upstream users. The next downstream user receives the water and chemicals from all upstream users and repeats the process.

Eventually, the water in rivers and streams reaches the oceans (except in completely closed watersheds). Evaporation from the oceans, lakes, and other bodies form precipitation and the water is cycled back into the terrestrial watersheds where the process then repeats itself. Ultimately, the oceans are the receptors for all contaminants that are not destroyed by physical, chemical, or biological processes. In some closed watersheds, *e.g.*, the Great Salt Lake, the final receptor is a lake and/or wetland.

How this plays out is illustrated as follows. New Orleans, for example, uses Mississippi River water as its principal supply of drinking water and thus is using treated sewage effluent generated by tens of millions of people in the Mississippi River watershed (albeit highly diluted with natural stream flow). This type of water use is thought to be "unplanned" reuse so as to distinguish it from direct reuse. However, because the water laws in many western states require water users to discharge specified amounts of treated sewage to surface waters to maintain stream flows, this reuse is considered integral to available water supplies and is a planned reuse in the broadest sense.

In watersheds with high population densities situated in arid regions, treated sewage may constitute a large fraction of the available water supply during certain periods of the year. In the US, one example of significant unplanned reuse is the Trinity River where the typical summer stream flow is almost 100% treated sewage from the Dallas-Fort Worth metropolitan area. Downstream, Houston also obtains a portion of its drinking water supply from the Trinity River. Germany also has a high population density that results in the use of high fractions of treated wastewater as the drinking water supply. An estimated 100 tons of pharmaceuticals are prescribed annually to Germany's 80 million people.

Planned reuse of treated wastewater is important in water-short areas of the US and many other countries where water resources are limited relative to need. Planned reuse can be divided into two categories: (1) use that does not involve human consumption of the water such as irrigation of golf courses and landscaping; and (2) uses that result in human exposure, including drinking water supply. Planned reuse is currently always indirect rather than direct (*i.e.*, using treated sewage effluent as drinking water supply influent). Indirect reuse includes recharging an aquifer used for a ground water supply with treated effluent and discharging treated effluent to a water supply reservoir. Sewage treatment in conjunction with planned water reuse is always much more rigorous than that used for discharges to surface waters and typically includes processes such as activated carbon adsorption and membrane filtration to remove trace organic substances, metals, and sometimes mineral salts.

Direct reuse has been evaluated by some political entities. The City of San Diego actually put a proposed direct reuse plan to the voters in 1999, but the plan was defeated. Direct reuse is still highly controversial and is not likely to happen in the US and Europe for a number of years but will be considered as populations grow.

Behavior in the Environment

Aquatic transport and transformation processes in the environment include sorption, ionization, volatilization, hydrolysis, oxidation-reduction, photolysis, biological transformation-degradation and precipitation-dissolution. These processes occur continuously in the environment and influence the presence and bioavailability of pharmaceuticals in aquatic ecosystems. Response of drugs to any of these pathways for partitioning, degradation or change in the environment could reduce their concentrations in the environment or remove them entirely and thereby reduce their potential to impact human health. Pharmaceutical compounds that are marketed in large quantities and are soluble or slightly soluble yet resistant to degradation through biological or chemical processes have the greatest potential to reach steady-state levels in the environment and be detected in surface and ground waters and potable water supplies.

Understanding whether there is Risk to Human Health

The active ingredients in pharmaceutical products are specifically designed to modify biological processes in order to treat diseases. Increasingly these compounds are developed from a complete mechanistic understanding of the disease or biological process. As part of the drug development process a complete pre-clinical assessment is performed to determine whether the drug is safe in humans. Pre-clinical safety assessment studies include: acute toxicity, pharmacology, genetic toxicity, developmental and reproductive toxicity, chronic toxicity and carcinogenicity studies. If shown to be safe and efficacious, the drug is studied in phased clinical trials. Once complete, all studies undergo a thorough regulatory review prior to marketing approval. Once approved, pharmaceuticals continue to be studied using post-marketing surveillance of patients undergoing therapeutic treatment. The pre-clinical, clinical and post-marketing data takes, on average, 10 to 12 years to obtain, however it results in as complete an understanding of the potential impacts of human health as can be determined for a pharmaceutical active ingredient.

Modeling as One Approach

Whether there is a potential risk to human health associated with low levels of pharmaceuticals in the environment is a function of exposure and hazard. One way to assess this risk is to create a model to predict human exposure and evaluate the possibility of a health hazard. However, models are merely predictive and are only as good as the input data and assumptions used. Models can be constructed, using the principles discussed above, to estimate the possibility of human health risk associated with pharmaceuticals in the environment.

Screening models can be used to evaluate large numbers of compounds, employing conservative assumptions and readily available data, in order to identify those compounds with the greatest potential risk.

Detailed deterministic or probabilistic models can then be used to provide more definitive risk estimates for those compounds, and/or classes of compounds, identified in the screening analysis. Deterministic models are used to simulate site-specific conditions in a particular area, while probabilistic models are used to estimate the percentage of the population exposed to various levels of risk.

Hazard

There is no universally accepted methodology to measure the possible human health hazard associated with low levels of pharmaceuticals in the environment. One approach could be to use the therapeutic dose with a safety factor of 1000. Another approach is to use EPA's Reference Dose (RfD) methodology. The RfD is the amount of a chemical that a person, including sensitive subgroups, can be exposed to on a daily basis without causing adverse health effects over a lifetime. The RfD is derived from the no observed adverse effect level (NOAEL) by consistent application of generally order-of-magnitude uncertainty factors.

Exposure

Human exposure to environmental concentrations of pharmaceuticals is believed to be primarily through ingestion of drinking water and, for compounds that bioaccumulate, through ingestion of meat or fish.
The concentration of drug substances in drinking water depends on the following factors:

- The quantity of drug substance consumed by a given population;
- The extent to which the drug is metabolized in the body;
- Available dilution in public sewer systems and in receiving waters;
- Removal and partitioning in STPs and receiving waters; and,
- The degree of removal by drinking water treatment technologies.

After ingestion, human drugs undergo Phase I metabolism, which includes oxidation, reduction or hydrolysis; followed by Phase II metabolism, which involves conjugation (*e.g.*, addition of glucuronic acid, sulfate, acetic acid or amino acid). Depending upon the drug, these processes can yield various proportions of unchanged drug substance, active or inactive metabolites, or conjugates. There is evidence that conjugates can be converted back to the parent drug or metabolite through bacterial hydrolysis in a STP. The reduction in pharmacological activity following metabolism can range from zero to essentially 100 percent depending on the drug.

Understanding the Risk to the Environment

As with the risk to human health, the potential risk to the environment presented by the presence of pharmaceuticals is a function of hazard and exposure.

Potential for and Evaluation of Acute Hazards

Pharmaceutical companies sometimes conduct acute toxicity studies to support environmental assessments that are filed with NDA and MAA applications or to support internal programs. Acute toxicity to aquatic receptors is usually assessed by evaluation of several common species including typically a fish (usually fathead minnows, bluegills or rainbow trout), an invertebrate (usually a daphnid such as *Daphnia magna*) and an algal species. These acute toxicity studies last up to 96 hours. The endpoints measured may include growth and/or growth rate (algae), immobilization (*Daphnia*), and morbidity and/or mortality (fish) and are often expressed as a concentration that elicits an effect in a specified percentage of the test group. For substances which may be expected to partition into soils or sediments, studies in terrestrial species, such as earthworms, may be conducted.

Generally speaking, there is little potential for an acute environmental hazard to exist from the presence of pharmaceuticals that find their way into the environment through use by humans because the levels are so low. As for the potential for an acute environmental hazard to be presented from pharmaceutical manufacturing, there are regulatory and industry practices in place that minimize the risk. Environmental agencies have in place permitting programs that control acutely toxic manufacturing discharges.

Potential for and Evaluation of Chronic Environmental Hazards

There are several methods that are currently approved by regulatory agencies for the conduct of chronic aquatic toxicity studies. The most commonly used are the *Daphnia* 21-day toxicity study and the prolonged toxicity study in fish. Currently, such studies are conducted relatively infrequently for several reason: the relatively low introduction concentrations of drug substances into the environment, the tendency for many drugs to be metabolized to relatively more water-soluble and less active metabolites, and because drugs are primarily produced by batch vs. continuous manufacturing operations.

Conclusions

This paper has identified the many aspects of and the complexities around the presence of low levels of pharmaceuticals found in the environment. The pharmaceutical industry is committed to the protection and enhancement of human health, and is actively pursuing programs to place this issue into perspective, particularly given the extensive human health data generated by the industry to support the introduction of new pharmaceutical products.

[1] Ayscough, N. J., Farwell, J., Franklin, A.G., and Young, W. Review of Human Pharmaceuticals in the Environment, R & D Technical Report P390, Environmental Agency, United Kingdom (June 2000).

Presence of Pharmaceuticals in Wastewater and Ground Water in Hawaii and Studies of Their Fate in Soil Following Irrigation with Reclaimed Water.

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Abstract

Due to an increasing national emphasis on wastewater reclamation for purposes such as aquifer augmentation and agricultural irrigation over potable aquifers and as opposed to disposal, there is possibly considerable potential for transport of pharmaceuticals from treated wastewater through the environment and into many drinking water supplies. In Hawaii, nearly all of the drinking water supply is obtained from unconfined groundwater aquifers (present as Ghyben-Herzberg lenses) under each island. On Oahu, with a population of approximately one million, all of the drinking water supply is obtained from groundwater. Approximately 100 million gallons per day is withdrawn from the Pearl Harbor Aquifer, much of which in overlain with prime agricultural land. Oahu also currently has 35 golf courses in operation. Population expansion has placed pressure on water supplies such that there is a push (by the State Department of Health, DOH) to implement large scale recycling of treated wastewater for agricultural and golf course irrigation on Oahu (as well as the other major islands). Portions of the Pearl Harbor Aquifer and adjacent aquifers are contaminated with nematicides (EDB, DBCP) previously utilized by agriculture indicating their vulnerability despite deep unsaturated zones (200-600 ft) containing cohesive soil types (oxisols).

Efforts have only just recently begun to measure pharmaceuticals in 12 Oahu groundwaters, 10 wastewaters, and 5 reclaimed waters and to quantify relationships between wastewater treatment techniques and pharmaceutical removal efficiencies. We will be determining the fate and transport of spiked pharmaceuticals (and any naturally occurring pharmaceuticals in Oahu wastewater) during percolation through different soil types (oxisols and aridisols) in laboratory columns as well as in field test plots. The fate and transport of pharmaceuticals under field conditions in Central Oahu will be evaluated through collection of lysimeter percolate samples. A total of 9 ceramic-cup suction lysimeters located at two golf course test plots at depths of 12, 24, and 48 inches as well as a total of 6 pan lysimeters located in a pineapple field test plot at depths of 12, 18, and 24 inches are to be sampled.

We are using USGS National Water Quality Laboratory (NWQL) custom method LC9003 (HPLC/MS) with an recently aquired Agilent Corporation, 1100 Series HPLC-MSD. Method LC9003 is designed to determine 22 over-the-counter and prescription pharmaceuticals and their metabolites in filtered natural-water samples. The compounds are Acetaminophen, Amoxicillin, Caffeine, Cimetidine, Codeine, Cotinine, Digoxin, Digoxigenin, Diltiazem, Enalaprilat, Furosemide, Fluoxetine, Gemfibroxil, Ibuprofen, Lisinopril, Metformin, Paroxetine, (-)trans-4-(4-fluorophenyl)-3-(4-hydroxy-3-methoxyphenoxymethyl)piperidine, Ranitidine, Salbutamol, Sulfamethoxazole, Theophylline, Trimethoprim, Warfarin, and 1,7-dimethylxanthine. Sample preparation for method LC9003 includes solid phase extraction of 1-liter samples followed by methanol extraction and concentration.

The screening and fate and transport efforts are just getting underway for a recently approved grant award. Rapid progress is expected due to parallel efforts currently under support to examine the fate and transport of salts, nitrogen, phosphorus, bacteria and viruses through the same soils both in laboratory columns and field test plots. We anticipate having considerable data prior to the manuscript deadline of July 1, 2001 and for presentation at the conference in October.

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Occurrence of Antibiotics in Hospital Wastewater, Municipal Wastewater and Surface Waters

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Abstract

Antibiotics and other pharmaceuticals are widely used in both human medical care and for veterinary purposes. In recent years there has been a growing interest in the determination of these compounds in the aquatic environment, but there ist still insufficient data for an improved risk assessment of these substances. Especially the existence of antibiotics in the environment is discussed to contribute to the reported increase of antibiotic resistence concerning several human pathogen microorganisms. As part of the hospital complex of the University of Bonn, we used the data of the clinical pharmacy to compute the amounts of the most administered antibiotics within the hospital area. We developped an analytical method for the simultaneous determination of these compounds in the untreated wastewater of the hospital as well as in the municipal wastewaters of sewage treatment plants and in surface waters like water of the Rhine river at several points. Furthermore, we determined the concentrations in bank filtration and lake waters, used for the production of drinking water. In addition, several antibiotics used outside the hospital complex (public health area), which also were found in the wastewaters of the treatment plants, were added to the analytical program. 29 different compounds of different classes including macrolides, sulfonamides, penicillines, tetracyclines and chinolones were determined using a LC-MS/MS system (Agilent 1100 / Applied Biosystems, API 2000, ESI). Sample treatment consisted of filtration and SPE, using Baker SDB and Waters Oasis material at pH 4, with special elution conditions.
LC chromatography used HLB-phases, starting with 100% water eluent. Recovery rates ranged reproducible between 60 % and 110 %, and detection limits were 0,5 - 2 ng/l for most macrolides, 2- 10 ng/l for the most of the other compounds, up to 50 ng/l for vancomycin. The highest concentrations were found according to expectations in the untreated hospital wastewater effluents (Piperacillin up to 26 µg/l, followed by ciprofloxacin with 14 µg/l and sulfamethoxazol, trimethoprim and some macrolides up to 2-4 µg/l. In municipal wastewaters the influent concentrations (sum of all investigated compounds) ranged from 3 - 5 µg/l, the effluents showed concentrations of 0,5 - 1 µg(l, which indicates a reduction of ca. 80%. In river Rhine macrolides and sulfamethoxazol were found in concentrations between 0,005 - 0,1 µg/l (macrolides) and 0,03 - 0,3 µg/l (sulfamethoxazol). In bank filtration and lake waters sulfamethoxazole as main component besides traces of macrolide antibiotics were found with concentrations between 0,002- 0,015 µg/l. In drinking waters, stemming from surface waters or bank filtration waters, no antibiotics were detectable.

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Biographical Sketches

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Occurrence and Behavior of Iodinated X-ray Contrast Media in Surface and Ground water

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Introduction

Already 10 years ago it has been recognised that some municipal treatment plant effluents exhibit high concentrations of adsorbable organic halogenated (AOX) compounds. The differentiation of the group parameter AOX into AOCl, AOBr and AOI revealed that the AOI contributes a large part to the AOX (Oleksy-Frenzel et al., 1995 and 2000). Based on the consumption of halogenated pharmaceuticals Gartiser et al. (1996) showed that iodinated X-ray contrast media are causing elevated AOX concentrations of hospital waste water. Together with the fact that the weekly AOI concentration profile of a municipal treatment plant effluent, receiving hospital waste water, is characterized by low values over the weekend and high values during the week leads to the assumption that the iodinated contrast agents are responsible for the high AOI (Oleksy-Frenzel et al., 2000).

The worldwide consumption of the iodinated X-ray contrast media, which are all triiodinated benzene derivatives, is around 3500 t per year. For one medical examination about 100 g of X-ray contrast media (ca. 30 g AOI) are applied (Speck and Hübner-Steiner, 1999). Based on the required properties of the contrast agents they are very polar and persistent and are eliminated via urine or faeces unmetabolised after some hours of application. The very high water solubility and the persistency is a disadvantage from the environmental point of view. Municipal treatment plants are not able to remove the contrast agents (Kalsch, 1999; Ternes and Hirsch, 2000). Further more, in all parts of the influenced water cycle, as well as in raw drinking water, iodinated X-ray contrast media are detectable (Putschew et al., 2000; Ternes et al., 2000).
Up to now the release of the triiodinated compounds into the aquatic environment is thought to be harmless, because no toxic effects could be registered (Steger-Hartmann et al., 1999). But iodinated compounds are released in high amounts in the aquatic environment most of them are metabolized , but not mineralized and thus, fundamental knowledge about the behavior is urgently needed.

Triiodinated contrast media can be analyzed by the sum parameter adsorbable organo iodine (AOI) or by single compound analysis. To study the occurrence and behavior of triiodinated contrast media the AOI was monitored in a partly closed water cycle in Berlin. The concentration of selected contrast media was determined in the same water cycle.

Methods

Samples

Effluent samples of a municipal waste water treatment plant were provided as 24 h mixed samples by the Berlin Waterworks (Berliner Wasserbetriebe). All other samples are grab samples and were taken by the Free University Berlin, Department of Hydrologeology (Prof. A. Pekdeger) or by ourselves (Lake Tegel and Nordgraben). All samples were filtered (0.45 _m) and stored in dark at 4ºC if not analyzed immediately.

AOI

For the determination of the AOI samples were acidified to pH 2 with nitric acid and were than enriched on 80 mg activated carbon. By combustion the adsorbed organo halogens were reduced to the halogenides. For the quantification of the AOI the combustion gas was trapped in water and then analyzed by ion chromatography (DX-100; Dionex). For the separation of the halogenides a IonPac AS9-SC column (Dionex) was used. The ion-chromatography was equipped with a two-channel UV/VIS and a conductivity detector. Bromide was detected at 210 nm and iodide at 226 nm. In case of bromide and iodide UV detection is much more sensitive. Details about the AOX differentiation are given by Oleksy-Frenzel et al. (1995, 2000).

Single compound analysis

For quantification the iodinated X-ray contrast media were enriched by sequential solid-phase extraction. The extracts were concentrated and transferred into a 2.5 ml vial. Under a gentile nitrogen stream the solvent was evaporated. For analysis the samples were dissolved in 0.5 ml ultra pure water. In case of matrix poor samples 500 ml were extracted and the extraction volume of complex samples was 50 ml. A separation of the compounds is possible by high performance liquid chromatography using a reversed phase (particle size 3 _m), and water and methanol, both containing 0.05% trifluoracetic acid, as eluents. A tandem mass spectrometer was used as detector (Quattro LC, Micromass). Electro spray ionization was used in positive-ion mode and product ions, produced by collision induced dissociation were detected (for details see Putschew et al., 2001).

Soil columns

For degradation experiments four acryl glass columns (length of each column 1 m) were filled with inert quartz sand (size 0.71-1 mm). The four columns were connected in line by Teflon tubes. The columns were feed with surface water to which glucose and glutamine acid were added as carbon source. The addition of the easy available carbon source is necessary to receive and keep anaerobic conditions. The water needed about 8 day to pass the columns. Every two weeks the surface water was replaced by fresh surface water.

Results

The occurrence and behavior of triiodinated X-ray contrast media was studied by monitoring the amount of adsorbable organic iodine (AOI) in a influenced, partly closed water cycle in Berlin. Furthermore, selected X-ray contrast media were quantified in the same water cycle. Fig. 1 shows a map of the investigation site. The source of the AOI or triiodinated contrast media is a municipal sewage plant. The effluent of the sewage plant is introduced into the channel Nordgraben which is an inflow of Lake Tegel. A water work is located close to Lake Tegel with drinking water wells located 100 m at the bankside. The ground water pumped up here consists of up to 80 % of bank filtered water. To study the bank filtration process sampling point were drilled between the shore of Lake Tegel and a drinking water well (well 13; Fig. 1). The depth of sampling points 3311, 3310, 3309, 3308, 3307, 3306 and 3305 are 5–8 m. The other wells (3301, 3302, 3303 and 3304) are 20-23 m deep, like the drinking water well 13. The sampling points 3305 and 3304 are located behind the drinking water well, and provide back ground values.

AOI

Based on a monitoring program in 1995 and 1998/99 we know that the average AOI load of the municipal sewage plant effluent is around 50 _g/L I. The capacity of the municipal waste water treatment plant is around 200,000m^3/d thus, the AOI load which is released into the water cycle is around 3.7 t/a. Over the year the AOI load of the receiving channel Nordgraben varies between 10 and 55 _g/L I. The variation is due to the fact that just grab samples were analyzed. Further more, the samples were taken at different days whereby the AOI concentration of the sewage plant effluent depends on the weekday (see Introduction). When entering Lake Tegel the Nordgraben water is diluted, but even in Lake Tegel the AOI concentration can be classified as high. Over the year the average concentration in Lake Tegel is around 15 _g/L I. Even in the sampling wells of the transect and in the drinking water wells close to Lake Tegel AOI compounds can be detected. The transect as well as drinking water well 13 and Lake Tegel in front of the transect were sampled each month over one year and the AOI was determined. The data were statistically evaluated and are shown as box plots. Fig. 2 shows the AOI concentration determined in the shallow sampling points over one year and for comparison the AOI concentration of Lake Tegel. The annual mean value of the shallow sampling points are comparable with the annual mean value of Lake Tegel. In comparison with Lake Tegel the highest difference of the mean values is 4 _g/L I for sampling point 3311 and 6 _g/L I for sampling point 3305. Because of the complex hydrodynamic of the system and the intensive use of the drinking water wells located near the sampling points a detailed description about the behavior of the AOI is not possible in the shallow wells. It is known, e.g. that the bank filtered water can flow backwards is water is pumped out of the drinking water wells. But nevertheless it seems that under oxic conditions, as found in the shallow wells, the AOI is stable. A different behavior was found in the deep sampling wells. Fig. 3 showas the AOI concentration of the deep sampling points together with the amount of Lake Tegel and the drinking water well 13. The annual average of the AOI measured in sampling point 3301 is 7 _g/L I lower as in Lake Tegel. The AOI concentration of sampling point 3302 differ just by 2 _g/L I compared with 3301, and the

annual mean value of sampling point 3303 is the same as found for sampling point 3301. A further decrease is recognized in the drinking water well 13. In the drinking water well the initial AOI of Lake Tegel is reduced by 10 _g/L I. The variation of the AOI in well 13 is much higher as found for the other deep sampling point. The reason for the higher variation is probably caused by holes in the loamy clay layer. If water is pumped out of well 13 higher contaminated bank filtrated water flows through the holes in the region of well 13. The annual mean value of the back ground is 1 ug/L I, thus the AOI measured in the influenced water cycle represents the triiodinated X-ray contrast media. In the deep sampling points the conditions are anaerobic, thus during an anaerobic soil passage an AOI reduction can be expected. In the system studied here the initial AOI of the lake water is reduced by 70 % in the drinking water well and already by 50 % in well 3301. But it should be pointed out, that the soil passage is very slow here. It was not possible to determine the time the lake water needs to reach the drinking water well. But based on the AOI profile of Lake Tegel and well 3301 over one year, we were able to estimate the time which the lake water needs to reach point 3301. The distance between the lake shore and well 3301 is about 30 m and for this distance the lake water needs 4 to 5 month.

The observed anaerobic AOI elimination could be simulated in the laboratory using a soil column filled with quartz sand and feed with lake water. The AOI and the iodide concentration were monitored in the in- and outflow of the column. Fig. 4 shows the iodine balance of two experiments. After passage through the anaerobic column the initial AOI concentration is reduced by ca. 50-70 % and the iodide concentration is always higher in the effluent. Because most of the iodinated X-ray contrast media exhibit three iodine atoms and the observed AOI reduction is maximal 70 %, a partial deiodination seems to be responsible for the AOI reduction under anaerobic conditions.

Quantification of selected X-ray contrast media

The concentration of Iopromide, Diatrizoate and Iohexol, all triiodinated X-ray contrast media, were determined in different samples of the influenced water cycle as well as in sampling point 3311, 3301 and the drinking water well 13. Beside the X-ray contrast media some possible metabolites were available. Fig. 5 shows a chromatogram of a standard solution of all available triiodinated benzene derivatives and the structures. For the analysis of native samples the compounds were enriched by solid-phase extraction and were than analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS, for details see Putschew et al., 2001).

Table 1 summarizes the concentrations of the three X-ray contrast media in the different parts of the water cycle. None of the possible metabolites were detectable.

Table 1: Concentration of triiodinated contrast media.

	Iopromide _g/L	**Diatrizoate _g/L**	**Iohexol _g/L**
Effluent sewage treatment plant (24 h mixed sample)*	22 ± 2	14 ± 12	7 ± 1
Nordgraben*	9 ± 2	7 ± 1	2 ± 0.5
Lake Tegel**	3	2	0.5
3311**	0.62	0,76	n.d.
3301**	0.06	0.46	n.d.
Drinking water well 13**	0.04	0.15	n.d.

*same samples were cleaned-up and analyzed three times. **samples were taken at four different months. The concentrations are mean values over four month. n.d.: not detectable.

The concentration of the selected contrast media varies between 22 and 7 _g/L, depending on the compound, in the sewage plant effluent. The concentrations in the receiving channel Nordgraben are reduced, due to dilution and the fact that a grab sample was analyzed, whereas in case of the waste water treatment plant a 24 h composite sample was analyzed. Although the channel water is diluted when entering Lake Tegel, the concentrations of the three compounds are still high, with values between 3 and 0.5 _g/L. Iopromide and Diatrizoate are still detectable in the sampling points of the transect and in the drinking water well. Compared with other pharmaceuticals the concentration of the diagnostics is relatively high in the drinking water well with values up to 150 ng/L for Diatrizoate. Until the water reaches the soil passage the concentration of Iopromide is always higher than the concentration of Diatrizoate. The striking change

of the concentration shows that the behavior of the compounds is different during bank filtration. Up to know it is not clear what happens but transformation is probably the main reason, as indicated by the AOI values. Thus, the triiodinated X-ray contrast media are not as stable as assumed. A further hint, that a transformation of the compounds occurs,is based on the comparison of the AOI and the concentration of the single compounds. For some samples the concentration of the triiodinated compounds were calculated as AOI and were than compared with the AOI values determined for the samples. Table 2 shows the AOI amount of selected samples and the percentage of the AOI which can be explained by the quantified contrast media. It is obvious that the identified AOI amount decrease from the source to Lake Tegel, thus even in surface waters a transformation of the compounds occurs.

Table 2: AOI (_g/L I) and identified AOI (%)

Sample	**AOI (_g/l I)**	**Identified AOI (%)**
Effluent sewage treatment plant 24 h mixed sample	57	38
Receiving channel	33	29
Lake Tegel	13	20

Conclusion

Triiodinated X-ray contrast media are very polar compounds which are released into the aquatic environment in large quantities. Iodinated contrast media are detectable in all parts of the influenced water cycle as well as in drinking water. By bank filtration the amount of adsorbable organo iodine is reduced, if the soil passage is anaerobe. Based on laboratory experiments it seems that a partial deiodination is responsible for the AOI reduction. Further more, even in surface water a transformation of triiodinated X-ray contrast media is very probable, but even here the products are still unknown, but under investigation.
Because of the high quantities of triiodinated compounds which are released permanently into the aquatic environment, the unknown transformation products and their unknown toxicological behavior the permanent release can be classified as a risk.

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Biographical Sketches

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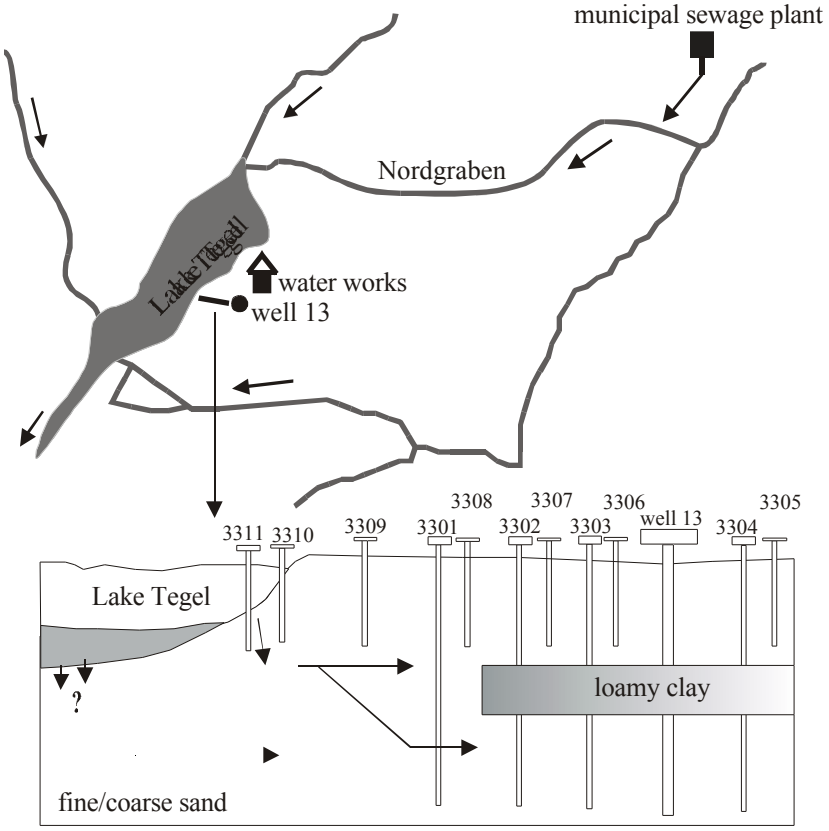

Fig. 1: Investigation site and transect scheme.

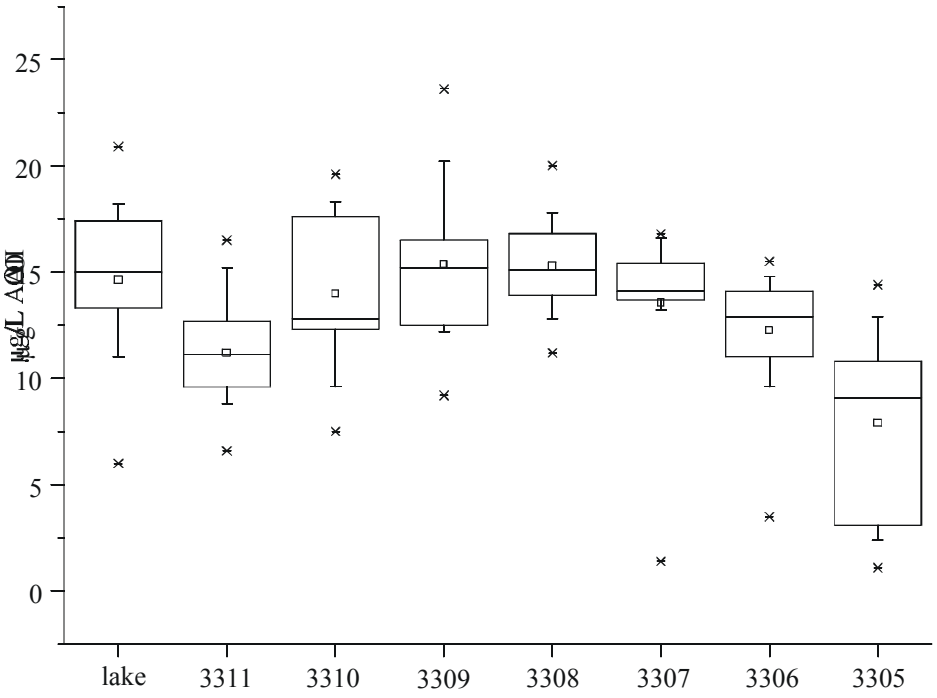

Fig. 2: AOI of the shallow sampling points.

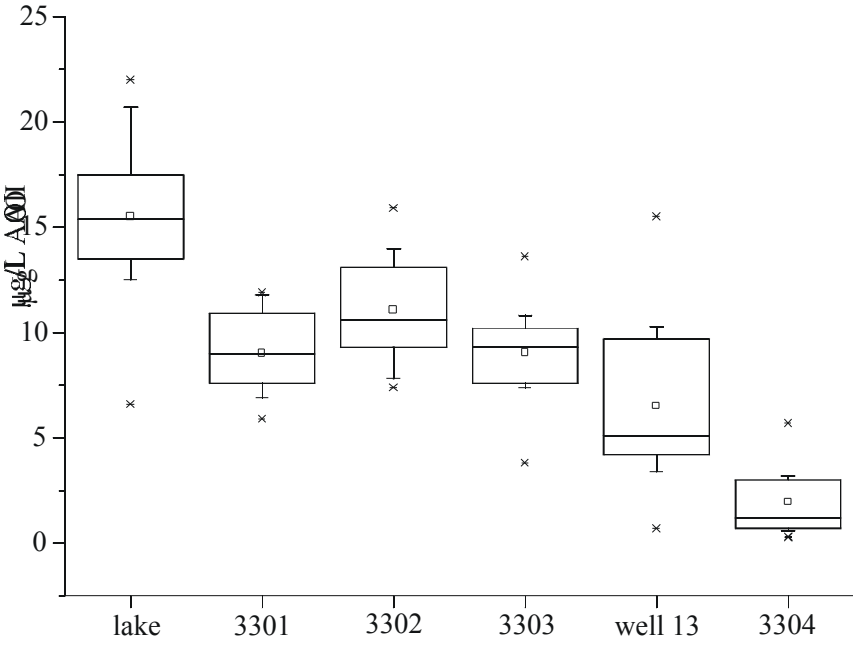

Fig. 3: AOI of the deep sampling points.

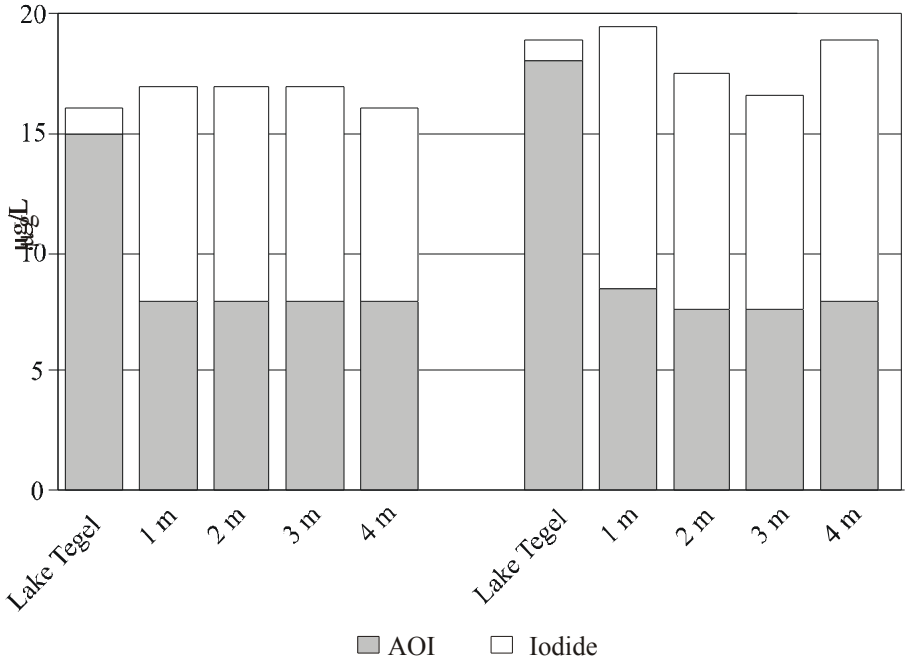

Fig. 4: Iodine balance of the anaerobic soil columns.

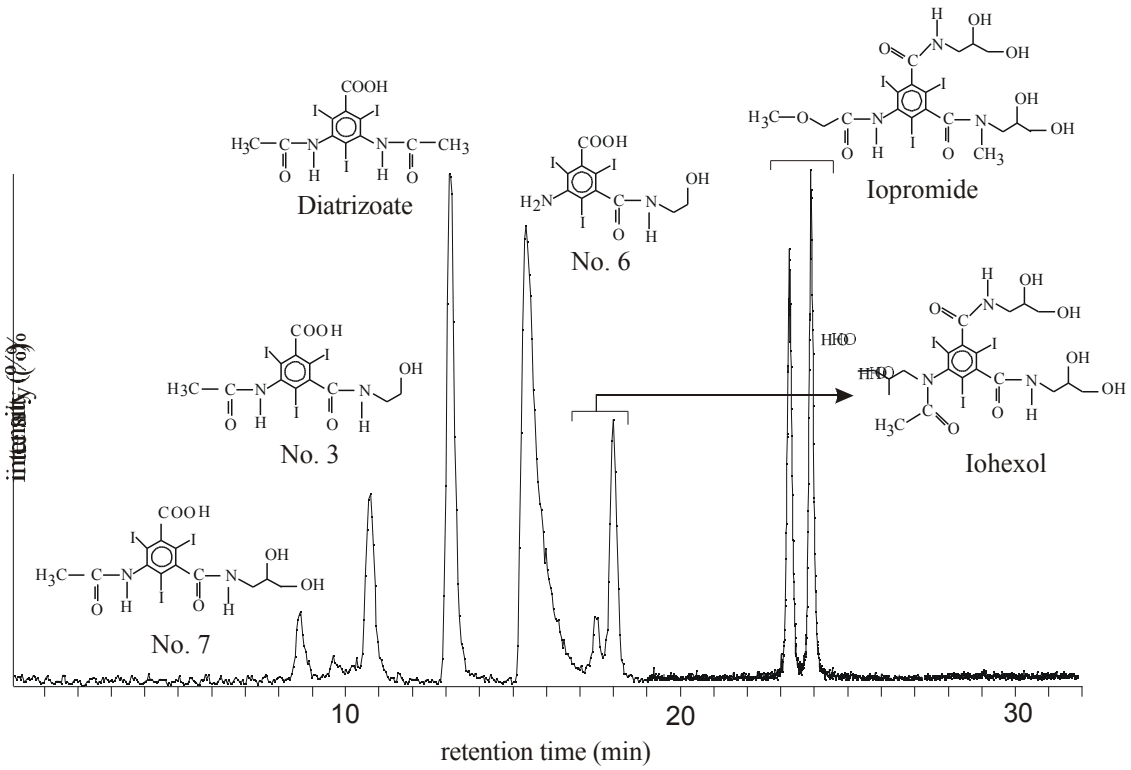

Fig. 5: Chromatogram (SRM fragments) of a standard solution and structures.

Comparison of Pharmaceuticals in Surface Water and Bed Sediment

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Abstract

Pharmaceutical compounds have emerged as important environmental contaminants. Nanogram-per-liter to low microgram-per-liter concentrations of pharmaceuticals previously have been reported in surface- and ground-water samples. Chemical properties vary among the diverse classes of pharmaceutical compounds. Although many pharmaceuticals are polar and highly water-soluble, compounds that are less polar should characteristically favor partitioning into the organic matter concentrated in bed sediment. In this study, solid-phase extraction (SPE) and accelerated-solvent extraction (ASE) procedures have been developed to isolate and concentrate pharmaceuticals from water and sediment, respectively. SPE was optimized by using a styrene divinylbenzene polymeric sorbent phase modified with vinylpyrrolidone. ASE was developed by using acetonitrile and water, which were compatible with the solvents used for high-performance liquid chromatography (HPLC), as the extracting solvents. Sample extracts were analyzed with electrospray ionization HPLC/MS (mass spectrometry) and HPLC/MS/MS, and results from analysis of the water and sediment samples were compared.

As expected, compounds known to have high log K_{ow} coefficients were detected in sediment either exclusively or in substantially greater amounts than in water samples. Compounds, such as diphenhydramine and thiabendazole with log K_{ow} coefficients of 3.27 and 2.47, respectively, were detected in bed-sediment samples at concentrations of 29 and 16 micrograms per kilogram. Concomitant surface-water samples show no detections for either of these compounds. The same surface-water samples did contain concentrations ranging from 75 to 130 nanograms per liter of other pharmaceutical compounds, such as acetaminophen, cotinine, and sulfamethoxazole, all having log K_{ow} coefficients less than one. However, the presence in sediment of pharmaceuticals with small log K_{ow} coefficients, such as caffeine and trimethoprim, suggests that other mechanisms might affect sorption of pharmaceuticals to sediment.

Presence of Pharmaceuticals in Treated Wastewater Effluent and Surface Water Supply Systems, Metropolitan Atlanta, Georgia, July–September 1999

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Abstract

Human and veterinary pharmaceutical compounds are a source of increasing environmental concern because the compounds are used in large quantities and the physical and chemical properties of the compounds make them likely to be transported into hydrologic systems. Effects on human health and aquatic ecosystems from the presence of small concentrations of pharmaceuticals in streams and rivers are generally unknown. Several pharmaceuticals have recently been detected in the US and Europe in surface waters that receive sewage effluent. Because Federal and state agencies do not routinely monitor these compounds, almost no data exist on the occurrence and distribution in US water supplies.

The Centers for Disease Control and Prevention (CDC) and the U.S. Geological Survey (USGS) initiated a study in 1999 to determine the occurrence of pharmaceuticals in treated effluent discharged from water pollution control plants (WPCPs), and in raw and finished drinking water at three drinking-water treatment plants in the Chattahoochee River watershed in Metropolitan Atlanta. Grab samples of water were collected at five effluent discharge points, and raw and finished water samples were collected at three drinking-water treatment plants downstream of the effluent discharges. Samples were collected once per month during low-flow conditions from July through September 1999. Two research analytical methods recently developed by the USGS Toxics Hydrology Program were used to quantify prescription and nonprescription pharmaceuticals, including antibiotics, at parts per billion (ppb) concentrations in filtered water samples.

Sixteen pharmaceuticals were detected in wastewater samples, 10 were detected in raw drinking-water samples, and three were detected in finished drinking-water samples. The prescription pharmaceuticals detected included diltiazem and dehydronifedipine (cardiac medications), metformin (anti-diabetic medication), and gemfibrozil (cholesterol-lowering agent). The nonprescription pharmaceuticals detected included caffeine (stimulant), 1,7-dimethyl xanthine (caffeine metabolite), cotinine (nicotine metabolite), cimetidine (gastrointestinal and ulcer medication), and acetominophen (analgesic). The antibiotics detected represent four groups including sulfonamides (trimethoprim, sulfamethazine, sulfamethoxazole, and sulfadimethoxine), macrolides (erythromycin-H_2O and roxithromycin), lincosamides (lincomycin), and fluoroquinolones (enrofloxacin). An additional seven prescription and nonprescription pharmaceuticals and fourteen antibiotics that were analyzed for were not detected.

One to three prescription pharmaceuticals were detected in 13 of 15 treated WPCP effluent samples. Concentrations ranged from low parts per trillion (ppt) to low ppb. Diltiazem was the only prescription pharmaceutical detected in raw drinking-water samples–detected at low ppt concentrations. No prescription pharmaceuticals were detected in finished drinking-water samples (minimum reporting levels (MRLs) ranged from 0.007–0.028 ppb). One to four nonprescription pharmaceuticals were detected in 11 of 15 treated WPCP effluent samples at low ppt to low ppb levels. Detection of nonprescription pharmaceuticals in the nine raw drinking-water samples ranged from no detections for cimetidine to nine detections for caffeine. The only three pharmaceuticals detected in finished drinking-water samples—caffeine, cotinine, and acetominophen—are widely used nonprescription pharmaceuticals. These compounds were detected in low ppb concentrations in two to eight of the nine finished drinking-water samples. One to five antibiotics were detected in 14 of 15 treated WPCP effluent samples at low ppt to low ppb levels, and in seven of 15 raw drinking-water samples at low ppb levels. No antibiotics were detected in finished drinking-water samples (MRLs ranged from 0.03-0.10 ppb).
Detection of antibiotics in raw drinking water is of particular concern because the presence of these chemicals in the environment may lead to the development of resistant bacterial strains, thus diminishing the therapeutic effectiveness of antibiotics. Detection of numerous prescription and nonprescription pharmaceutical compounds in treated WPCP effluent, and raw and finished drinking water; together with the absence of pharmaceutical

manufacturing facilities in the study area, suggests that human use of pharmaceuticals is one source of these compounds in water resources within the upper Chattahoochee River watershed.

Biographical Sketches

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Occurrence of Bacteriostatic Sulfonamide Drugs in Surface Waters and Their Behavior in Bank Filtration

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Introduction

Sulfonamide drugs with bacteriostatic effects are still used in human treatment (e.g. [1, 2]), though to a lesser extend than for treatment of livestock. In 1997, 60 tons of sulfmethoxazole, the most frequently used sulfonamide drug in Germany, were prescribed overall. For humans, daily intakes can reach up to 4 g/day [3]. Depending on the drug, up to 80% are not metabolised in the body and excreted renally [4].
Therefore, in an urban environment, sulfonamide drugs have an impact on the aquatic environment that is influenced by municipal waste water discharges. They should be detectable as micro pollutants in sewage treatment plants and in the receiving waters, if they are not removed by treatment techniques such as adsorption or biodegradation. In cases of partially closed water cycles, they even could reach drinking water supplies. We therefore developed an analytical screening method for fourteen sulfonamide drugs in environmental waters. A combination of solid phase extraction (SPE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) provided detection limits in the lower ng/L concentration range suitable for the investigation of the concentrations and remains of sulfonamide drugs a partially closed water cycle in Berlin [5].

Methods

Samples

All samples are grab samples and were taken by ourselves, except the transect samples and the samples of the drinking water wells which were provided by the Free University Berlin, Department of Hydrogeology (Prof. Dr. A. Pekdeger) in cooperation with the Berlin Water Works (BWB). All samples were filtered (0.45 _m) and stored in dark at 4ºC if not analyzed immediately.

Enrichment

For the enrichment a solid-phase extraction method using LiChrolute EN material (200 mg, Merck, Darmstadt, Germany) has been developed. After conditioning of the cartridges with 5 ml methanol, 5 ml methanol:water (1:1) and water (pH 2.5) the samples with a pH adjusted to 2.5, were percolated with a flow rate of 200 - 300 ml/h. The loaded cartridges were washed with 2 - 5 ml water (pH 2.5) and then eluted with 3 ml methanol:water (1:1) and 10 ml methanol. The eluates were combined and concentrated to approximately 1.5 ml using a rotating condensor Speedvac Plus SC110A (Savant, Germany). 40 _l of diluted trifluoroacetic acid and in some cases 300 _L methanol were added to maintain homogeneity. Internal standard sulphaphenazole was added before extraction and $^{13}C_6$-sulfamethazin just before analysis. Sulphaphenazol is used as a standard because it is not used in Germany and is thus not expected in the samples.

LC-MS/MS

For liquid chromatography a HP 1100 (Hewlett-Packard, Waldbronn, Germany) is used. The system is equipped with a vacuum solvent degassing unit, an automatic sample injector, and a column thermostat. The conditions for HPLC with MS detection are as follows: 0 to 50% B in 30 minutes and then, to clean the column, to 100 % B in 2 minutes. Reequilibration time at 0 % B was 7 minutes. Solvent A is acetonitrile-water (3:97) and solvent B acetonitrile-water (75:25), both containing 1 % formic acid (v:v). The column used is a 250×2 mm Supersphere RP18 encapped column, with a particle size of 4 _m (Knauer, Berlin, Germany) operated with a precolumn 20×2 mm containing the sorbent. The column oven is set to 45ºC and the flow rate is 0.3 ml/min.
For detection a tandem MS (Quattro LC, Micromass, UK) with a positive electro spray interface is used. The temperature of the interface is set to 300ºC and the source temperature to 100ºC , with a cone voltage at

40 V. With high sensitivity the sulphonamide drugs are detected in the selected-reaction-monitoring mode, thus product ions after collision induced dissoziation (CID) are recorded. Collision energies of 15 to 18 eV and dwell times between 0.35 and 0.5 s/sec are used.

Soil columns

For degradation experiments acryl glass columns (length of each column 1 m) were used. In case of anaerobic experiments four columns were connected in line by Teflon tubes and each column was filled with inert quartz sand (size 0.71-1 mm). Sampling is possible after each meter. The columns were feed with surface water to which glucose and glutamine acid were added as carbon source. The addition of the easy available carbon source is necessary to receive and keep anaerobic conditions. For experiments under aerobic conditions two columns, filled with filter sand of a water work were connected in line and were feed with a effluent of a waste water treatment plant. Here just the effluent can be sampled.

Results

Investigation site

The occurrence and behavior of sulfonamide drugs was studied in a influenced water cycle in Berlin. The investigated water cycle can be classified as partly closed. An effluent from a tertiary waste water treatment plant reaches, via the receiving water (Nordgraben), the recreational lake Tegeler See. A water work is located at the lake. The distance between the lake bank and the drinking water wells is about 100 m. The raw water pumped up here consists of up to 80 % of bank filtered surface water. Via monitoring/sampling wells in two different aquifer horizons, the quality of the bank filtered water was monitored concerning the load of sulfonamide drugs. A scheme of the transect is shown in Fig. 1.
The depth of the sampling points 3301 – 3304 varies between 20 to 23 m, for 3305 – 3309 between 5 and 8 m, and for 3310 and 3311 between 2 and 4 m. Well 3311 is located at the water side of the lake, the distance to the next well is 10 m (3310). The distance between all other wells is about 20m, except well 3304 an 3305 which are located ca. 60 m behind drinking water well 13, and provide background values. In the deep wells the concentration of oxygen is < 0.5 mg/L. The concentration in the shallow wells varies between 0 - 10 mg/L oxygen. Over half a year, from January to June 1999 the transect was sampled every month. Additionally, lake inflows were sampled randomly.

Field data

Three sulfonamide drugs (sulfmethoxazole, sulfadiazine and sulfamerazine) were detectable in the investigated water cycle. In the lake Tegeler See the average concentration of sulfmethoxazole is 231 ng/L, sulfadiazine 4 ng/L and sulfamerazine 5 ng/L (Table 1). The source of sulfonamide drugs detected in the lake is the effluent of the tertiary municipal waste water treatment plant. The concentration of sulfonamide drugs in the effluent varies between 5 and 210 ng/L in the morning and between 25 and 799 ng/l at mid-day (Table 1). In the receiving channel concentrations of up to 324 ng/L were detected. Lower concentrations of the drugs were found in all other lake inflows. The three sulfonamide drugs detected in the lake are also detectable in the sampling points of the transect and in the raw drinking water. The drugs show a different behavior during the soil passage, and thus will be discussed separately. The sampling period of 6 month does not allow to discuss seasonal variations, thus mean values of concentrations are used.

Table 1: Concentration of sulfonamide drugs in lake Tegeler See and the inflows. STD = sewage treatment plant, LOQ = limit of quantification, n.d. = not detectable.

	Sulfmethoxazole (ng/L)	Sulfadiazine (ng/L)	Sulfamerazine (ng/L)
Effluent STP (20.05.00, (8 a.m.)	210	11	5
Effluent STP (20.05.00, 1 p.m.)	799	33	25
Receiving channel (Nordgraben, 16.06.99)	607	24	23
Tegeler Fließ (14.07.99)	< LOQ	n.d.	n.d.
Oberhavel (22.06.99)	64	< LOQ	2
Tegeler See	231	4	5

Sulfmethoxazole: The concentration of sulfmethoxazole determined in the sampling points of the bank filtration transect varies between 75 and 2 ng/L (Fig. 2). The mean concentration of drinking water well 13 is 3 ng/L, and the background value (3304/3305) is 2 to 3 ng/L. The raw drinking water consist of up to 80 % out of the bank filtered lake water, thus the low concentration of sulfmethoxazole in the drinking water well indicates that the compound is removed up to 99 % by the soil passage. Conspicuously, the main reduction takes place during the first meters of bank filtration. Furthermore, the concentration in the shallow and deep sampling points are nearly equal, thus sulfmethoxazole is degradable under aerobic and anaerobic conditions.

Sulfadiazine: The concentration of sufadiazine varies between 4 and 1 ng/L in the sampling points of the transect (Fig. 3). The mean concentration in the drinking water well is 3 ng/L. No background value was measured in the shallow well 3305. The deep well provided a background of 1 ng/L of sulfadiazine. During the fist meters of bank filtration no reduction is observed, but furtheron a reduction is recognized in the shallow wells. In the first deep well, which distance to the lake shore is 40 m, the concentration is still comparable to the lake concentration. A lower concentration of sulfadiazine was measured in the two deep wells located 60 and 80 m from the water side. The reduced concentrations found in the deep wells 3302 and 3303 is probably caused by dilution. It is known that "shallow" bank filtered water can flow through holes in the loamy clay layer, if water is pumped out of the drinking water wells. The data show that sulfadiazine is degradable under aerobic conditions and stable in an anaerobic environment. Over the investigated time period sulfadiazine is just reduced by 25 % during bank filtration.

Sulfamerazine: The mean concentration of sulfamerazine varies between 16 and 2 ng/L in the wells of the transect between the lake shore and drinking water well 13 (Fig. 4). Difficult to explain is, that the concentration in the drinking water well is the highest concentration determined, and for this the background is not responsible. Sulfamerazine was not detectable in well 3304 and 3305, both located behind the drinking water well 13. In general the concentration in the deep wells is higher than in the shallow wells. Furthermore, compared to the first shallow well and the deep wells, as well as the drinking water well the lake concentration of sulfamerazine is lower. This phenomenon can only be explained by matrix effects, which reduce the ionization yield by electro spray ionization for MS/MS analysis, and thus the lake concentration determined is too low. The transect concentration profile of sulfamerazine is comparable with the profile of sulfadiazine. A degradation of sulfamerazine can be expected during an aerobic soil passage, but based on the data no reduction rate can be given.

Laboratory data

The degradation of sulfonamide drugs was studied in the laboratory using aerobic and anaerobic soil columns. For the anaerobic experiment the soil column was loaded over 14 days with 2 mg/L of each drug examined. The residence time was about 7 d/m. An anaerobic degradation was recognized for sulfmethoxazole. Already after one meter of anaerobic soil passage most of the compound was degraded. After 3 m of soil passage the compound was completely eliminated.

An aerobic degradation of sulfonamide drugs could not be observed, when over a short time high concentrations of drugs were loaded onto the soil column. But if the aerobic columns are spiked over long periods of time with low concentrations of sulfonamide drugs, then degradation occurs. Thus, the aerobic columns were spiked over 50 days with 30-100 _g/L of each drug. The residence time was 6 d/m. A aerobic degradation was registered for sulfmethoxazole, sulfadiazine and sulfamerazine, whereby the most easily degradable compound is sulfmethoxazole, with a degradation rate of around 95 % after 20 days. A complete reduction for sulfadiazine and sulfamerazine was achieved after ca. 40 days.

Conclusion

The three sulfonamide drugs sulfmethoxazole, sulfadiazine and sulfamerazine were detected in the water cycle under investigation. Concentrations of up to 800 ng /L of sulfmethoxazole, 30 ng/L sulfadiazine and 25 ng/L sulfamerazine were found in random samples of a waste water treatment plant effluent. Mean concentrations in a receiving lake were 230 ng/L sulfmethoxazole, 4 ng/L sulfadiazine and 5 ng/L sulfamerazine. In bank filtration, the further decrease in concentration depended strongly on the redox condition. Along the shallow monitoring wells and under aerobic conditions, a decrease of the three sulfonamide drugs along the filtration path was detected. Along the deep wells, under anaerobic conditions, only sulfmethoxazole was reduced. Therefore, the drop in concentration between lake and drinking water

wells was most pronounced for sulfmethoxazole. Mean levels below 10 ng/L were reached in the raw drinking water, while for sulfadiazine and sulfamerazine no significant reductions compared to lake concentrations were observed. However, random samples of tap water provided by the drinking water facility showed no sulfonamide drug residues.

Laboratory soil column experiments under aerobic and anaerobic conditions showed higher reduction rates but the same qualitative dependence of the degradation on the redox conditions. In the investigated system, the sulfonamide drugs are removed efficiently by the combination of bank filtration and drinking water treatment and therefore pose no health problems to drinking water.

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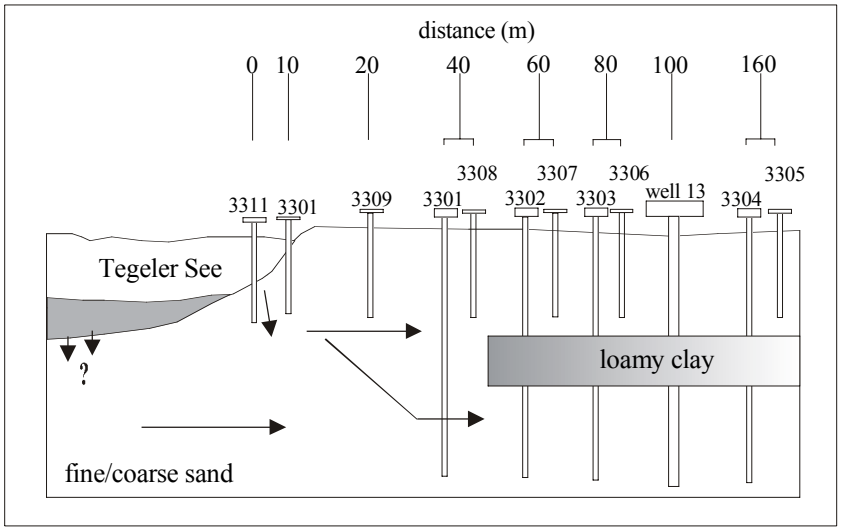

Fig. 1: Transect scheme.

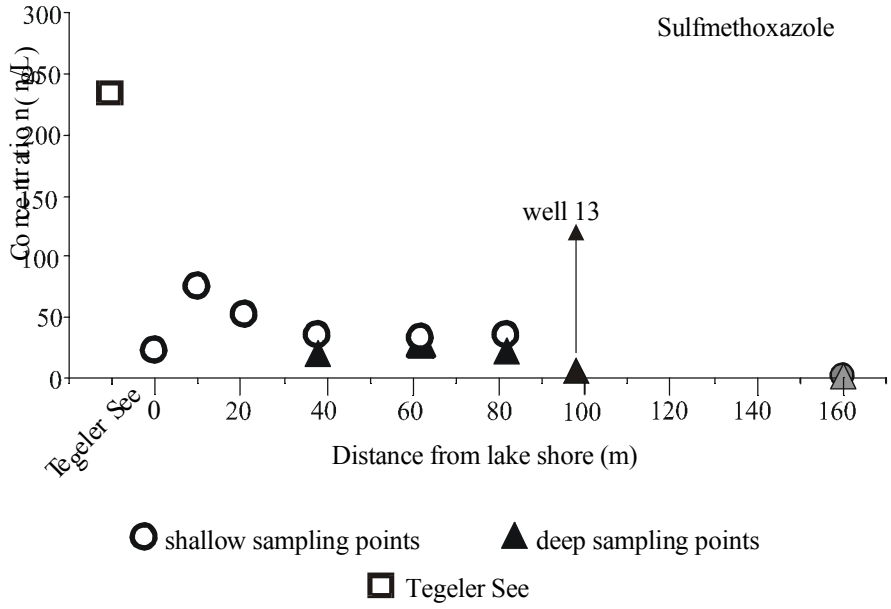

Fig. 2: Concentration of sulfmethoxazole (mean values).

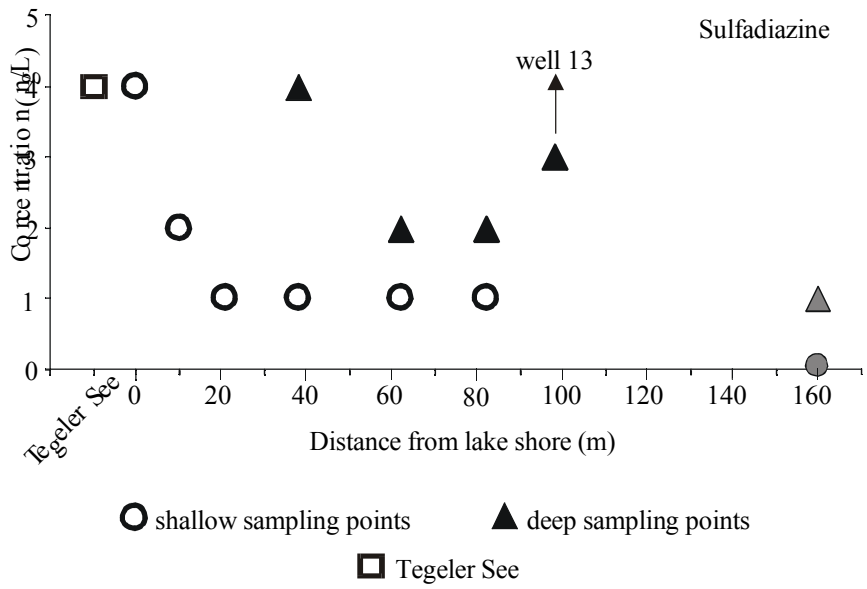

Fig. 3: Concentration of sulfadiazine (mean values).

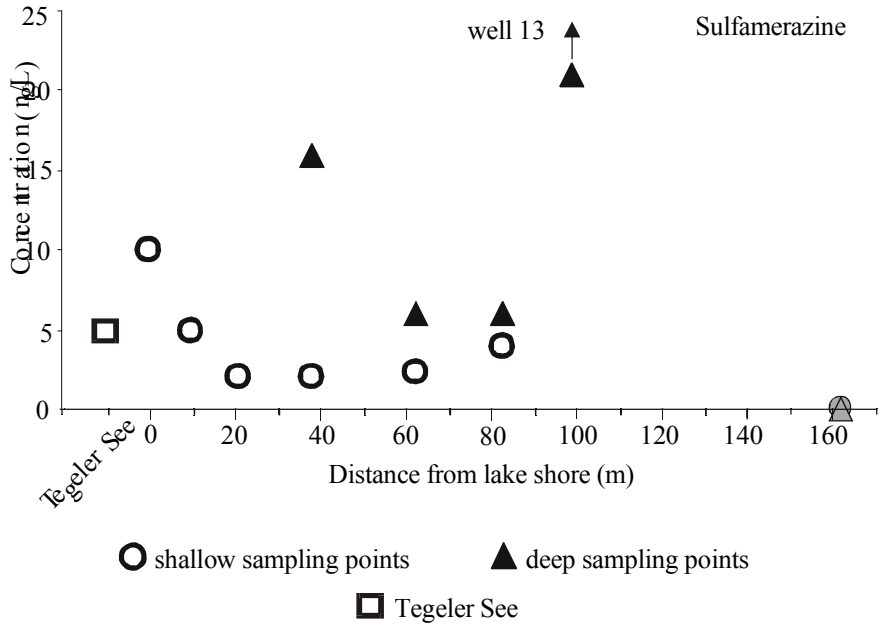

Fig. 4: Concentration of sulfamerazine (mean values).

Comparison of Solid Phase Microextraction, Solid Phase Extraction and Liquid Liquid Extraction coupled to GC/MS for Trace Analysis of Endocrine Disruptors in Wastewater

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Abstract

The studies performed dealed with investigations on the suitability of the solvent free Solid Phase Microextraction (SPME) for enrichment of Endocrine Disrupting compounds (ED`s), especially technical Nonylphenol, Bisphenol A and 17α-Ethinylestradiol, from wastewater. Method development included the selection of the optimum SPME-fibre and extraction time. Furthermore the optimum salt amount as well as the stirring speed were examined.

As comparative enrichment technique to SPME, Solid Phase Extraction (SPE) and Liquid Liquid Extraction (LLE) were investigated. Although SPE and LLE are time and substance-consuming procedures, this methods are linked to higher analyte accumulation factors. SPE was examined together with on-line derivatization coupled to Large Volume Injection-GC/MS. The LLE-extracts were derivatized off-line. Separation and detection were fulfilled by LVI-GC/MS.

For the three analytical procedures, SPME, SPE and LLE, recovery, precision data and detection limits as well as advantages and disadvantages will be shown and discussed.

SPE and SPME were used for the determination of ED´s in wastewater samples (influent and effluent). The results of both analytical procedures, SPE and SPME, will be presented and compared.

Biographical Sketch

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University of Kassel and

Dissertation: University of Leipzig (1993-1999), Germany; Autumn 1999 at the Centre for Environmental Rersearch Leipzig-Halle GmbH

Topic of Dissertation: Behaviour of Xenoestrogens, natural and synthetic Estrogens in Constructed Wetland Plants

On-Line Solid-Phase Extraction and Fluorescence Detection of Selected Endocrine Disrupting Chemicals in Water by High-Performance Liquid Chromatography

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Abstract

A high-performance liquid chromatographic method was developed to analyse selected endocrine disrupting chemicals in water by using automated on-line solid-phase extraction and with a fluorescence detector. The excitation and emission wavelengths of the fluorescence detector were 230 nm and 290 nm, respectively. The selected endocrine disrupting chemicals include hormone steroids such as estradiol (E2), estriol (E3), ethynylestradiol (EE2) and ethynylestradiol 3-methyl ether (MeEE2), and nonylphenols (NP), octylphenols (OP), POE(1-2) nonyl phenol (NPE) as well as bisphenol A (BP). Three types of on-line cartridges (C18, PLRP-s and PRP-1) were tested to pre-concentrate the endocrine disruptors in deionised water. It was found that the recoveries of these chemicals at 1 _g/L were close to 100% except for 4-octyl phenol and 4-n-nonyl phenol, which had recoveries of about 40% to 80%. The two polymer cartridges (PLRP-s and PRP-1) gave higher recoveries than the C18 cartridges. The addition of methanol at 5% to 10% in water significantly improved the recovery of 4-octyl phenol and 4-n-nonyl phenol. The addition of methanol also led to an improvement in the recovery with C18 cartridges. With the addition of methanol in water samples, these three types of cartridges gave similar recoveries for the chemicals. The detection limits of this method ranged from 50 ng/L to 100 ng/L.

A river water sample spiked with these chemicals was analysed using the above method and we found no interference with the peaks of the selected endocrine disrupting chemicals. Levels of endocrine disrupting chemicals ranging from 58 ng/L of estradiol to 50 _g/L of nonylphenol ethoxylates (NPE) were detected in four sewage effluents by this method. Interferences existed at lower retention time due to the very high level of organic substances in wastewater. This relatively simple method is useful for water analysis and screening, especially for laboratory study on environmental fate of endocrine disrupting chemicals in water.

Biographical Sketches

The authors are all working in the same program, Remediation of Contaminated Environments, in CSIRO Land and Water, Adelaide Laboratory. Research interests include environmental fate of organic pollutants such as steroids, pesticides, PAHs, and TCE, developing analytical methods and remediation technology.

Analysis of Selected Antibiotics from Water Associated With Fish Hatcheries

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Abstract

A method was developed for the solid-phase extraction of eight antibiotics from a 123-milliliter (mL) sample of water. The antibiotics consisted of three tetracyclines (tetracycline, oxytetracycline, chlortetracycline) and five sulfonamides (sulfamethazine, sulfamerazine, sulfathiazole, sulfachloropyridazine, and sulfadimethoxine). The antibiotics then were analyzed using liquid chromatography/mass spectrometry. Tetracyclines, as a class, exhibited losses of mass-to-charge (m/z) 17 and m/z 35, and a characteristic m/z 156 ion was identified for all five sulfonamides. A linear extraction curve was established from 0.1 to 5.0 micrograms per liter (µg/L) for the tetracyclines and from 0.05 to 5.0 µg/L for the sulfonamides. The extraction method was applied to water samples collected both upstream and downstream from fish hatcheries to determine the concentration of oxytetracycline dissolved from fish food. Raceways (a linear flow-through holding tank for fish) at the Milford Lake Fish Hatchery near Junction City, Kansas, contained only oxytetracycline at an approximate concentration range of 3 to 5 µg/L. Oxytetracycline was not found in the ground water used to fill the raceways. Results of a survey of water samples from many fish hatcheries across the United States, which is currently in progress, also will be presented.

Biographical Sketch

E.M. Thurman is a research scientist for the U.S. Geological Survey in Lawrence, Kansas. His work deals with pesticides, pesticide degradates and emerging contaminants in surface and ground water. Special interests include analytical techniques of liquid chromatography/mass spectrometry applied to emerging contaminants.

In-Situ Solid-Phase Extraction and Analysis of Ultra-Trace Synthetic Musks in Municipal Sewage Effluent Using Gas Chromatography-Mass Spectrometry, Full-scan Mode

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Abstract

Fragrance materials, such as synthetic musks in aqueous samples, are normally analyzed by GC/MS in the selected ion monitoring (SIM) mode to provide maximum sensitivity after liquid-liquid extraction of 1-L samples. A 1-L sample, however, usually provides too little analyte for full-scan data acquisition.We have developed an on-site extraction method for extracting synthetic musks from 60 L of wastewater effluent. Such a large sample volume permits high-quality, full-scan mass spectra to be obtained for various synthetic musk compounds. Quantification of these compounds was conveniently achieved from the full-scan data directly, without preparing SIM descriptors for each compound to acquire SIM data.

Keywords: Abselut Nexus™; Selected ion monitoring; Non-conditioning; SPE; Effluent; Synthetic musk

Notice: The U.S. Environmental Protection Agency (EPA), through its Office of Research and Development (ORD), funded this research and approved this abstract as a basis for an oral presentation. The actual presentation has not been peer reviewed by EPA. Mention of trade names or commercial products does not constitute endorsement or recommendation by EPA for use.

Biographical Sketch

Lantis Osemwengie is a Chemist in the Environmental Chemistry Branch at the U.S. Environmental Protection Agency's National Exposure Research Laboratory in Las Vegas, Nevada. From 1992 to 1999, he was an Environmental Chemist at the New York City Department of Environmental Protection, Special Project Laboratory. Currently a Ph.D. student in the Environmental Science Department (Chemistry Track), University of Nevada, Las Vegas.

Emerging Approaches for Assessing the Exposure of Children to Endocrine Disruptors.

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Abstract

Humans can be exposed to endocrine disrupting chemicals (EDC's) from environmental sources such as water, food, air, dust and soil. A major concern is that children may be exposed to higher amounts of pollutants than adults because of their different activity patterns, higher body-surface to volume ratios, and greater water and food consumption per kilogram body weight. A pilot study, titled the Children's Total Exposure to Persistent Pesticides and Other Persistent Organic Pollutants (CTEPP), examines the possible exposures of 256 preschool children between the ages of 18 months and 5 years to pollutants commonly found in their everyday environments. This three-year pilot study will quantify the aggregate exposures for these preschool children. Monitoring will be performed at their daycare centers and homes in twelve counties in North Carolina and Ohio during a 48-hour sampling period. Participants will be recruited from daycare centers and from the general population using a random digit dialing (RDD) method. Samples will be chemically analyzed for over 40 persistent pesticides and organic pollutants that children may contact in their daily surroundings. Samples collected over a 48-hour period include food, beverages, drinking water, urine, indoor and outdoor air, hand wipes, indoor floor dust, play area soil, dislodgeable pesticide residues, smooth floor wipes, and food preparation surface wipes. Samples will be chemically extracted and analyzed by GC/MS. These data will help to quantify the dermal, ingestion, and inhalation exposure pathways of these children to suspected EDC's such as herbicides (atrazine), insecticides (organophosphorus, carbamate, pyrethrin and organochlorine), phthalate esters (butylbenzyl, di-n-butyl), phenols, polychlorinated biphenyls, and polycyclic aromatic hydrocarbons. Total exposure concentrations will be determined for the preschool children through environmental sampling, time-activity diaries, food diaries, questionnaires and by the videotaping of up to 10% of the children in homes in Ohio. Potential doses will be estimated by analysis of the urine samples. Preliminary results will be discussed for water samples analyzed for atrazine from daycare centers and homes.

This work has been funded wholly or in part by the United States Environmental Protection Agency under contract #68-D-99-011 to Battelle. It has been subjected to Agency review and approved for publication.

Biographical Sketches

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Dr. Nancy K. Wilson is a research chemist with extensive experience in the human exposure field, including study design, sampling and analysis, and interpretation of results. She has a B.S. in chemistry from the University of Rochester and an M.S. and Ph.D. in physical chemistry from Carnegie-Mellon University. Battelle, Durham, North Carolina 27713, U. S. A., wilsonk@battelle.org, phone 919.544.3717 ext. 140, fax 919.544.0830

Occurrence and Screening of Treatment Alternatives for PPCPs and EDCs in Waters of Southeastern Louisiana

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Abstract

A method was developed for the quantitative determination of representative pharmaceutical and personal care products (PPCPs) and selected endocrine disrupting chemicals (EDCs) in water samples using GC/MS. The compounds include a metabolite of a lipid regulator (clofibric acid); three analgesics (naproxen, ibuprofen and acetaminophen); two steroids (estrone and 17β-estradiol); three fungicides and/or disinfectants (bisphenol-A, chlorophene and triclosan); an antidepressant (fluoxetine); and a human activity marker (caffeine). The method consists of solid phase extraction and derivatization using GC/MS for analysis. Based on methods developed to date, detection limits are estimated to range from 1 to 180 ng/L. The method can be applied to surface water and drinking water samples.

For this presentation, we will summarize our findings to date and present data from samples collected from the Mississippi River, Lake Pontchartrain, and tap water. In addition, we will present laboratory data from bench-scale experiments regarding effectiveness of drinking water treatment alternatives. Currently, we are examining the effectiveness of activated carbon adsorption and ozonation for treatment of PPCPs.

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Biographical Sketches

Glen Boyd is assistant professor with the Department of Civil and Environmental Engineering at Tulane University. He teaches courses in water and wastewater engineering design and groundwater contaminant fate and transport. His current research focuses on occurrence and treatment of PPCPs and EDCs, remediation strategies for DNAPL contaminants, and water quality in drinking water distribution systems.

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Deborah Grimm is Director of the Coordinated Instrumentation Facility at Tulane University where she oversees analytical instrumentation support of faculty and student research. She teaches an introductory course in organic and biochemistry for biomedical engineering students. In addition to PPCP research, she collaborates on environmental projects including monitoring of a local bayou.

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Occurrence, Distribution, and Fate of 4-Nonylphenol in Kansas Domestic Wastewater Treatment Plants

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Abstract

4-nonylphenol (4-NP), an endocrine disruptor, is one of the major metabolites of nonylphenol polyethoxylates (NPnEOs), the most commonly used non-ionic surfactants that are often discharged into wastewater treatment plants. Our investigation in three Northeastern Kansas wastewater treatment plants has shown that the concentrations of 4-NP in influent ranged from 3.39 to 169 :g/L, while effluent concentrations remained at lower levels. A significant portion of 4-NP was not degraded during the treatment processes but instead was mass transferred to the de-watered biosolids. The concentration of 4-NP in de-watered biosolids reached as high as 300 mg/kg on dry weight basis in one plant. Approximately 60% of the 4-NP in the biosolids was further degraded during biosolids composting process. An average dry weight basis concentration of 130 mg/kg for 4-NP was found in biosolids compost product that was eventually disposed of through land application. Given that about 7 million tons of biosolids are produced annually in wastewater treatment plants in the United States, several thousand tons of 4-NP could be potentially released to the soil environment through land application of biosolids. Biosolids land application is becoming the most common means of biosolids disposal as other disposal options become cost prohibitive or heavily regulated. The 4-NP released into the soil environment can potentially enter the water environment due to runoff and leaching. Future research on the transport and transformation of 4-NP in biosolids-amended soil and its impact on surface and ground water quality are needed.

Introduction

Alkylphenol polyethoxylates (APnEOs) represent an important class of non-ionic surfactants that have been widely used as detergents, emulsifiers, wetting agents, and dispersing agents in industrial cleaning products and industrial process aids (Thiele et al., 1997). Each year more than 200,000 tons of APnEOs, almost half of the global annual production, are produced in the United States (U.S. International Trade Commission, 1995). 4-nonylphenol (4-NP), an endocrine disruptor, is one of the major metabolites of APnEO surfactants. The majority of APnEOs is used in aqueous solutions and eventually is discharged through sewer systems into wastewater treatment plants (WWTPs).

Under anaerobic conditions APnEOs can go through step-wise de-ethoxylation, producing intermediate metabolites consist of APnEO compounds with lower number of ethoxylate groups and alkylphenol mono- and di-ethoxylates (AP1EO and AP2EO) (Maguire, 1999). Under aerobic conditions, both carboxylation and step-wise de-ethoxylation of ethoxy chain of APnEO parent compounds occur, producing alkylphenol mono- and di-ethoxy carboxylic acids (AP1EC and AP2EC) (Ahel, et al., 1994; Maguire, 1999; Di Corcia et al., 2000). Jonkers and coworkers (2001) recently observed concomitant oxidation of the nonyl chain with carboxylation and de-ethoxylation of the ethoxy chain during aerobic biodegradation of NPnEO parent compounds, leading to metabolites having both a carboxylated ethoxylate and an alkyl chain of varying lengths with a carboxyl functional group. It is believed that the intermediate anaerobic and aerobic metabolites of APnEO parent compounds can be further anaerobically degraded into alkylphenol such as 4-NP and 4-octylphenol (4-OP), however, complete degradation of those metabolites is believed to be difficult (Maguire, 1999).

Research by Ahel and coworkers (1994) has shown that at least 60-65% of APnEOs introduced to 11 Swiss WWTPs were discharged into the environment through effluent. They found that about 60% of the discharged compounds were NP1EO and NP2EO, while only a minor portion of 4-NP reached receiving waters directly via

effluents. The majority of 4-NP was found to be sludge-bound. Investigations mostly conducted in Europe have reported a wide range of concentrations of metabolites of APnEOs in various environmental matrixes, ranging from non-detectable level to several thousand Φg L^{-1} in WWTP effluents and surface waters and from several mg kg^{-1} up to several thousand mg kg^{-1} in biosolids (Bennie, 1999). Up to several Φg L^{-1} APnEOs metabolites were detected in groundwater and even in drinking water. Since APnEOs and their metabolites are not naturally occurring compounds, their wide spread presence in many environmental matrixes is believed to be due to domestic and industrial waste discharges (Bennie, 1999; Davi and Gnudi, 1999).

Compared to their parent compounds, degradation products of APnEOs are more toxic and estrogenic. There is an increase in the toxicity of APnEOs with decreasing ethoxy chain length (Yoshimura, 1986; Hall et al., 1989). Research has shown that APnEOs and their metabolites are acutely toxic to fish (LC_{50} = 17 - 3000 Φg L^{-1}), invertebrates (LC_{50} = 20 - 3000 Φg L^{-1}), and algae (LC_{50} = 27 - 2500 Φg L^{-1}) (Servos, 1999). This class of compounds can also bind to estrogen receptors (Routledge and Sumpter, 1996, Cooney 2000) resulting in the expression of several responses both in vitro and in vivo, including the induction of vitellogenin, a biomarker for estrogen exposure (Tyler et al., 1996). Solé and coworkers (2000) observed a positive correlation between the amount of NP detected in surface waters and vitellogenin induction in male carp. Gray and Metcalf's research (1997) showed that after being exposed to 50 Φg L^{-1} 4-NP for 3 months, 50% of the male Japanese Medaka (*Oryzias Latipes*) fish developed ova-testes. Elevated concentrations of NPnEOs and metabolites, especially 4-NP, in surface water are strongly believed to contribute to the observed widespread sexual disruption in wild populations of river fish throughout the United Kingdom, especially in the rivers that receive large amount of discharges from WWTPs (Jobling et al., 1998).

Although intensive investigations have been conducted in Canada and several European countries (Ahel et al., 1994; Paxéus, 1996; Bennie et al., 1998; Bennie, 1999; Di Corcia et al., 1994 and 2000), only few U. S. reports have documented the occurrence of 4-NP in influents, effluents, and digested sludge of U.S domestic and industrial WWTPs (Naylor et al., 1992; Chalaux et al., 1994; Field and Reed, 1996). The reported concentrations of 4-NP in inflent, efflent, and digested sludges of the investigated U.S. WWTPs range from non-detectable to 13 mg L^{-1}, 2 mg L^{-1}, and 400 mg kg^{-1}, respectively. None of the U.S research has reported comprehensive study on the occurrence, distribution, and fate of 4-NP during treatment processes in WWTPs.

The objectives of this research were to investigate the occurrence and distribution of 4-NP in 3 Northeast Kansas WWTPs that use different wastewater treatment methods and to understand its transformation during the treatment processes.

Methods and Materials

Selected wastewater treatment plants

The three wastewater treatment plants selected for this study are located along the Kansas River in Kansas. Plant 1 is a 0.75 MGD facility serving a population of 3,700 with no major industries. Plant 2 is a 5.5 MGD facility that serves a town of 50,000 with a few small industries, and Plant 3 is a 12 MGD facility serving a city of 150,000 with several medium scale industries. All three facilities are operated as activated sludge systems. Plant 3 was designed for carbon removal only; Plant 2 is operated for carbon removal and nitrification; and the operation of Plant 1 includes nitrification and denitrification (Figure 1). Effluents from those three plants are discharged into the Kansas River.

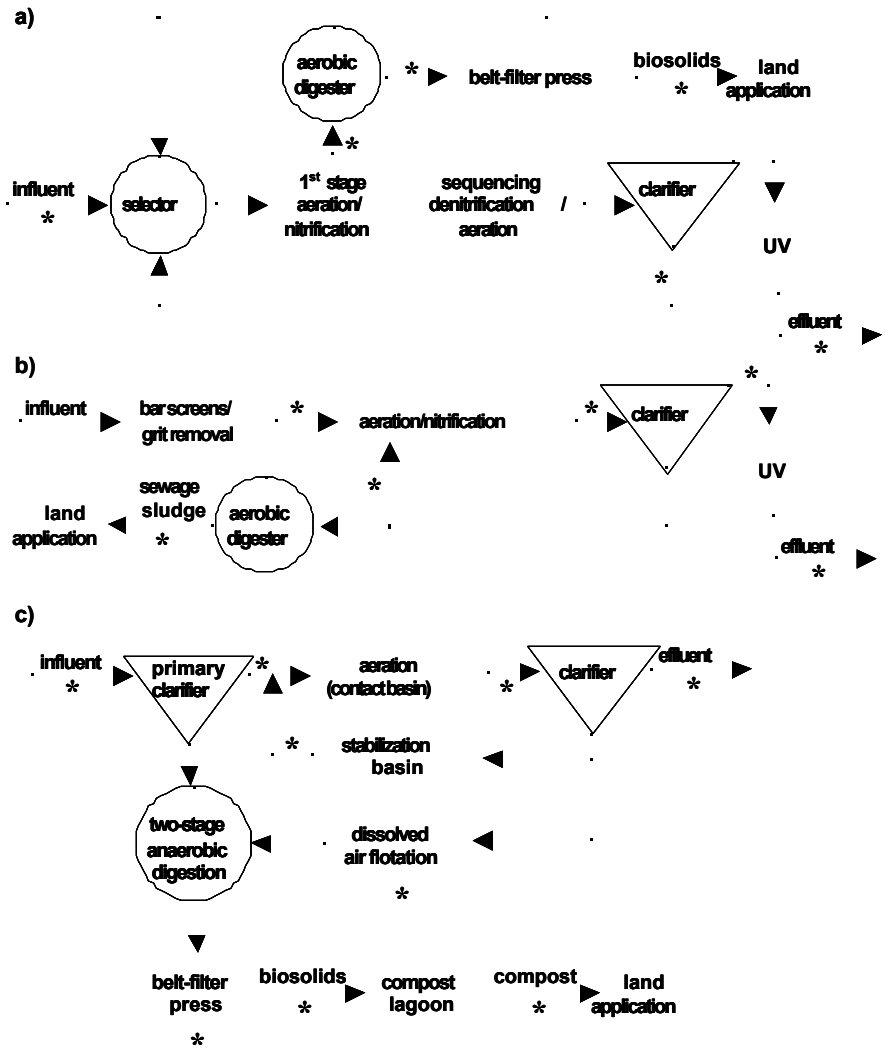

Figure 1. Process schematic of (a) Plant 1 (0.75 MGD), (b) Plant 2 (5.5 MGD), and (c) Plant 3 (12 MGD). Asterisk (*) indicates sampling locations.

Sample collection protocols

Grab samples of wastewater and/or mixed liquor were collected in the summer of 2000 at various points within each WWTP (Figure 1) in 1-L amber jars, immediately transported to the lab in coolers packed with ice, filtered with Whatman #41 paper, and stored at 4°C until extraction. For preservation, each filtered water sample was mixed with ~ 4 mL chloroform in order to retard microbial activities. Twenty-liter glass jars were used to collect grab samples of sludge from the aerators, return sludge lines and the digesters. The sludge samples were then transported immediately back to the lab where excess water was removed via centrifugation. The sludge sediment samples were then frozen at -10°C until analysis.

Sample extraction and cleanup

The solid phase extraction method previously reported by Lee (1999) was modified slightly for the determination of 4-NP in wastewater. Samples were extracted in duplicate with quality control samples and procedural blanks. Commercial C-18 columns (300 mg) were used in conjunction with a J & W Scientific vacuum manifold (Folsom, CA) for the wastewater extractions. Prior to acidifying the 200 mL wastewater samples with HCl (pH < 2), 100 Φg of 4-*t*-butylphenol (sublimed, Sigma Chemical, St. Louis, MO) were added as surrogate. The C-18 columns were pre-conditioned via gravity flow using sequentially 2 mL acetonitrile, 2

mL methanol, and 2 mL de-ionized water. Samples were pulled entirely through the columns under vacuum (_<10 mL/min). The columns were then dried under vacuum (-380 mm Hg) for 5 min. The 4-NP was eluted from the column with 2 mL methanol. A quantity of 125 Φg 2,4,6-tribromophenol (Sigma Chemical, St. Louis, MO), as internal standard, was then added to the eluant. Finally, the eluant was evaporated to dryness under N_2 (60°C), re-dissolved in 500 ΦL methanol, and stored at -10°C until HPLC analysis. Recovery for 4-NP determined from fortified water samples was 83 ± 12 %.

Sludge sediment samples were extracted using a soxhlet procedure similar to the one reported by Marcomini et al. (1990). Twenty grams (wet weight) of sludge were extracted for 8 hr with 130 mL hexane. The hexane extracts were then concentrated to ~ 5 mL under a gentle stream of N_2 (60°C) and cleaned up by passing through an aminosilica column pre-wetted with 5 mL hexane. The 4-NP was eluted from the column with 5 mL hexane-acetone (75:25), evaporated to dryness under a gentle stream of N_2 (60°C), re-dissolved in 500 ΦL methanol, and stored at -10°C until HPLC analysis.

Analysis of 4-NP by HPLC and GC-MS

4-NP was analyzed via reverse phase HPLC. Following reverse phase HPLC analysis, the presence of 4-NP was confirmed using GC-MSD. 4-NP purchased from Sigma Chemical (St. Louis, MO) was used as standard. A Hewlett-Packard 1050 HPLC containing a quantinary pump, diode array detector (DAD), and a fluorescence detector (FLD) was used for sample analysis. Injections (5 ΦL) passed through a 25 ΦL sample loop and the column was kept at 40°C. The DAD was operated under the following conditions: signal = 277 nm, bandwidth = 40 nm, and reference = 350 nm. Data was collected from the FLD at an excitation 8 = 230 nm, emission 8 = 301 nm, and pmtgain = 6 or 12. A 124 x 4 mm LiChropher 100-RP-18e (5Φm) column (Agilent Technologies, Santa Clarita, CA) was used for the reverse phase HPLC. A methanol: water mixture (8:2) was used as the mobile phase at a flow rate of 1.5 mL/min. The instrument detection limit for 4-NP was 0.09 ng/ΦL. The method quantification limit for 4-NP was 0.64 Φg L^{-1}.

A Hewlett-Packard 6890 Series GC-MSD was used to confirm the presence of 4-NP in the sludge and wastewater extracts. The GC-MSD utilized a 5972 model quadrupole mass selective detector and was operated in the electron ionization mode using helium as the carrier gas (12.9 psi; 1.1 mL/min). A 30 m x 0.25 mm x 0.25 Φm HP-5MS (Hewlett-Packard, Santa Clarita, CA) was used under the following conditions (De Voogt et al., 1997). The initial column temperature (100°C) was held for 0.5 min prior to a 10°C/min increase to 320°C which was maintained for 5 min. Injections (1 ΦL) were in the splitless mode with the following temperatures: injector 200°C and interface line 250°C. Both published spectra (Stephanou and Giger, 1982) and the 4-NP standard were used in the confirmation of 4-NP.

Results and Discussion

Figure 2 illustrates the concentrations of 4-NP in wastewater samples collected at various points within each wastewater treatment plant in the summer of 2000. Up to 169 :g L^{-1} of 4-NP was detected in the influent sample of Plant 3, a 12 MGD plant located in a medium-sized city that houses several medium-scale industries. Low levels of 4-NP also appeared at 5.94 :g L^{-1} and 3.39 :g L^{-1} in the influents of Plant 1 and Plant 2, respectively. Different from Plant 3, high levels of NPnEOs input to Plants 1 and 2 are not expected due to the less association of these plants with industries, a major source for NPnEOs. Nonionic nonylphenol polyethoxylate surfactants are known to enter wastewater collection systems as NPnEOs and 4-NP is not a naturally occurring organic compound. The detection of 4-NP in the influents of all three WWTPs suggests a rapid anaerobic transformation of its parent compounds, NPnEOs, in the sanitary sewers due to low dissolved oxygen levels in raw municipal wastewaters.

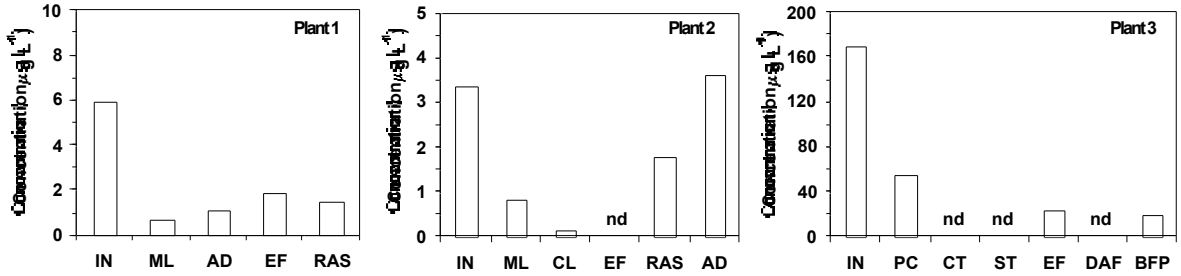

Figure 2. Concentrations of 4-NP in the wastewater samples collected at different treatment stages in three Kansas wastewater treatment plants. (nd, non-detectable). *Plant 1*: IN = influent, ML = mixed liquor from 1st stage aerator, AD = effluent of aerobic digester, EF = effluent, and RAS = return activated sludge from clarifier; *Plant 2*: IN = influent, ML = mixed liquor from aerator, CL = effluent from clarifier, EF = effluent, RAS = return activated sludge from clarifier, and AD = effluent of aerobic digester; *Plant 3*: IN = influent, PC = effluent from primary clarifier, CT = effluent from aeration contact basin, ST = effluent from stabilization tank, EF = effluent, DAF = mixed liquor in dissolved air flotation tank, and BFP = effluent from belt-filter press.

After the initial treatment stage of sedimentation to remove large particles and aeration/nitrification, the concentration of 4-NP in the wastewater decreased dramatically to 0.68 :g L^{-1}, 0.82 :g L^{-1}, and non-detectable (samples labeled CT and ST) in Plant 1, Plant 2, and Plant 3, respectively (Figure 2). At this stage, a combination of aeration and nitrification gave a lower 4-NP reduction (89% and 76% in Plants 1 and 2, respectively) compared to the 100% 4-NP reduction for Plant 3 with only aeration process. Nitrification might have promoted further transformation of parent compounds to 4-NP in Plant 1 and 2. However, production of 4-NP from NPnEOs is unlikely at this point in Plant 3 because of the aerobic condition. In Plant 3, the level of 4-NP decreased 68% to a concentration of 55 :g L^{-1} after sedimentation. Degradation and partition on to the biomass are speculated to be the possible reasons for the high reduction of 4-NP levels in the wastewater samples collected at the initial treatment stage. Due to its lipophilic nature, 4-NP present in the raw wastewater had great potential to partition on to the organic matter in the contact basin for aeration/nitrification. Partition on to the organic matter could potentially retard the biological degradation of 4-NP during the later treatment processes.

In Plant 1, after the 1st stage aeration/nitrification the sludge sediment was sent to aerobic digester for further treatment and the wastewater was directed to the denitrification/aeration tank. The appearance of 4-NP in wastewater of aerobic digester, return activated sludge, and effluent at levels of 1.15 :g L^{-1}, 1.5 :g L^{-1} and 1.9 :g L^{-1}, respectively, suggests further transformation of NPnEOs to 4-NP and/or possible release of organic-bound 4-NP in the denitrification/aeration tank and clarifier. In Plant 2, 0.11 :g L^{-1} and 1.53 :g L^{-1} of 4-NP were also detected in wastewater samples from clarifier and return activated sludge, respectively (Figure 2). The level of 4-NP in effluent of Plant 2 was non-detectable. The level of 4-NP in the wastewater from aerobic digester of Plant 2 was detected at 3.62 :g L^{-1}, a concentration level similar to that in the influent of this plant. Significant reduction of water input to the aerobic digesters in both plants might induce a concentration effect and, therefore, contribute to the elevated levels of 4-NP in the aerobic digester wastewater samples.

4-NP was found at 20 :g L^{-1} in the effluent of Plant 3 (Figure 2), a level much higher than that in the effluents of other two plants. However, compared with the 4-NP level in the influent of this plant, an 86% concentration reduction was achieved for the effluent. Activated sludge from the secondary clarifier at Plant 3 is first thickened using dissolved air flotation units and then pumped into the first stage of a two-stage anaerobic digestion process. Primary sludge from primary clarifier is pumped directly into the digesters. The two-stage anaerobic digestion process stabilizes the sludge and collects methane in the floating cover of the 2nd stage digesters. The digested sludge is then pumped to a series of belt-filter presses for dewatering. Figure 2 shows the 4-NP concentrations in the wastewater collected from the dissolved air flotation tank before the anaerobic digestion process and in the wastewater produced from the digested sludge that is thickened on the belt-filter presses after the anaerobic digestion. No 4-NP was detected in the water (DAF) before the anaerobic digestion. However, 20 :g L^{-1} 4-NP appeared in the aqueous phase (BFP) associated with the anaerobically digested sludge. Dissolved air flotation process might have promoted the partition of 4-NP on to the biomass in the

system and, therefore, decrease the amount of 4-NP in the wastewater. While during the anaerobic digestion process, some of NPnEOs might have been converted to 4-NP, causing an increase of 4-NP concentration in the wastewater produced.

Actived sludge in Plant 1 is also dewatered using a belt-filter press, but as seen in Figure 1, undergoes aerobic digestion instead of anaerobic digestion prior to dewatering. Figure 3 shows the dry-weight basis concentrations of 4-NP in the dewatered biosolids collected from Plants 1 and 3. Biosolids from Plant 1 were found to contain 1.3 mg kg^{-1} of 4-NP, while biosolids from Plant 3 contained as high as 300 mg kg^{-1} of 4-NP, more than 200 times higher than that found in the biosolids from Plant 1 and in the sewage sludge sample (1.24 mg kg^{-1}) from Plant 2. The biosolids produced in Plant 3 are transferred to compost lagoons for composting. More than half of the 4-NP in biosolids was removed during the composting process. The concentration of 4-NP in the composted biosolids was detected at 130 mg kg^{-1}.

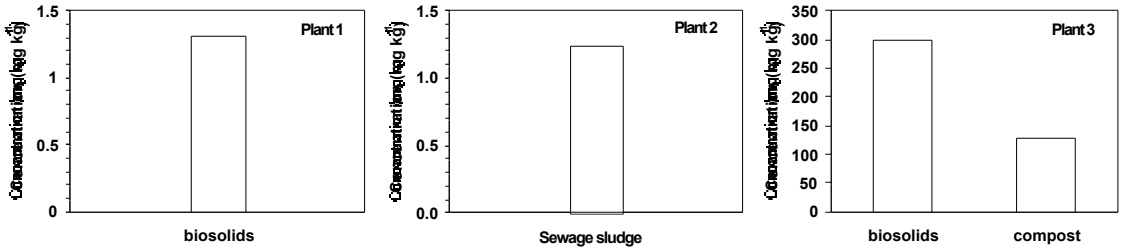

Figure 3. Concentrations of 4-NP in the dewatered biosolids, sewage sludge, and compost collected from the three plants. Detection limit for biosolids = 0.05 mg/kg.

Using the parameters listed in Table 1, mass distributions for 4-NP in the three plants were estimated for the day of sample collection (Figure 4). For Plant 1, the amount of 4-NP found in its biosolids was 54% of that entered into the plant with influent. The amount of 4-NP that was discharged out of Plant 1 with effluent was 31.9% of what was detected in the influent of the plant. Since there was a 13.7% discrepancy between the amount 4-NP that entered the plant and what was found in the effluent and biosolids, it is reasonable to assume that negligible amount of parent compounds NPnEOs were converted to 4-NP during the treatment processes in Plant 1. The discrepancy was likely due to degradation of 4-NP. Higher degradation efficiency (54.9%) was estimated for Plant 2 (Figure 4). About 45.1% of 4-NP that entered into this plant was partitioned on to the biomass in sewage sludge produced. No detectable level of 4-NP was found in the effluent of Plant 2. The total amount of 4-NP detected in the effluent and biosolids of Plant 3 is about 100% of what entered into the plant. Although certain amount of parent compounds were transformed into 4-NP at the final anaerobic digestion stage, a significant portion (88.8%) of 4-NP entered into Plant 3 was not degraded during the treatment processes but instead was mass transferred to the de-watered biosolids. The remaining 13.6% was discharged out of this plant through effluent.

Table 1. Total volume of raw wastewater and production of biosolids in the three plants.

Plant	Raw wastewater input (L day^{-1})	Biosolids/Sludge produced (kg day^{-1})
1	2.45×10^4	60
2	2.84×10^5	350
3	2.00×10^7	10,000

WWTPs (Plant 1 and Plant 2) that served communities with no medium or large-scale industries generally contained smaller quantities of 4-NP in their raw wastewater. It seems that the difference in the size of community that is served by the WWTP has less impact on the occurrence of 4-NP than the scale of the industries in the community. 4-NP was detected at a much higher level in the raw wastewater at Plant 3 that served a medium sized city with several medium-scale industries. Significant accumulation of 4-NP in biosolids/sewage sludge was observed for all three WWTPs investigated, no matter how much 4-NP was found in the raw wastewater of the WWTPs. Given that about 7 million tons of biosolids are produced annually in wastewater treatment plants in the United States, several thousand tons of 4-NP could be potentially released to

the soil environment through land application of biosolids. Biosolids land application is becoming the most common means of biosolids disposal as other disposal options become cost prohibitive or heavily regulated. The 4-NP released into the soil environment can potentially enter the water environment due to runoff and leaching. Future research on the transport and transformation of 4-NP in biosolids-amended soil and its impact on surface and ground water quality are needed.

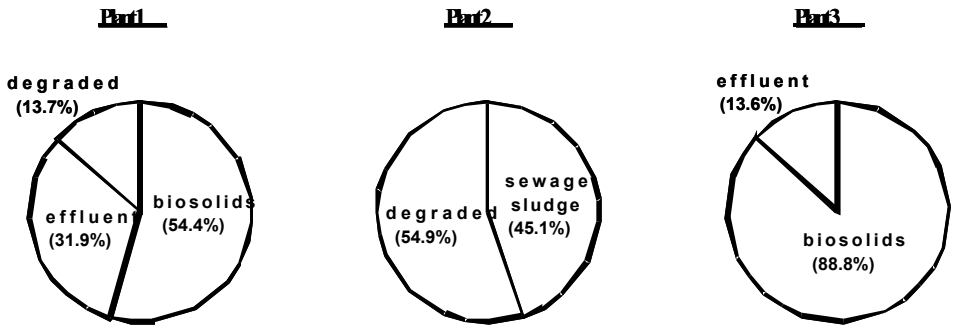

Figure 4. Daily mass distributions of 4-NP in the three plants.

Acknowledgements

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Presence of Wastewater Tracers and Endocrine Disrupting Chemicals in Treated Wastewater Effluent and in Municipal Drinking Water, Metropolitan Atlanta, 1999

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Abstract

Insecticides, detergents, processed foods and personal hygiene products contain a wide variety of organic chemicals. Municipal drinking water may contain these chemicals because these products are discarded in sewage systems and may survive treatment at water pollution control plants (WPCP) and at drinking water treatment plants (DWTP) that use surface water containing treated effluent from a WPCP. The occurrence and distribution in municipal drinking water of organic chemicals from commercial products are unknown because these chemicals are not routinely monitored in the U.S. water supplies.

The Centers for Disease Control and Prevention (CDC) and the U.S. Geological Survey (USGS) conducted a study to measure wastewater tracers (organic chemicals that survive WPCP treatment) and endocrine disrupting chemicals–in wastewater effluent and in drinking water. Treated effluent was collected at four municipal effluent discharge sites. Raw and finished water samples were collected at three drinking-water treatment plants downstream of the effluent discharges. Samples were collected once per month during low-flow conditions from July through September 1999 from WPCPs and DWTPs in the Chattahoochee River watershed. During these months, treated effluent contributed 1 to 35 percent of the stream flow at the DWTP intakes. Analytical methods developed at the USGS National Water Quality Laboratory used continuous liquid-liquid extraction with methylene extraction and selected ion monitor gas chromatography/mass spectrometry to quantify 31 wastewater tracers and 16 potential endocrine disruptors at parts per billion (ppb) concentrations in filtered water samples.

Of the 47 chemicals analyzed, 37 were detected in treated effluent samples, 15 were detected in raw drinking water (river water) samples, and 14 were detected in finished drinking water samples. All chemicals detected had concentrations in the low ppb range. Chemicals detected in treated effluent and in raw and finished water were plasticizers, products of combustion, disinfectants, flame retardants, surfactants, detergent metabolites, antioxidants, and wood preservatives. All treated effluent samples (N=12) contained tri(2-chloroethyl)phosphate, phthalic anhydride, triclosan, and tributyl phosphate. Eleven samples had 1,4 dichlorobenzene and 10 samples had cholesterol. All 9 raw drinking water samples contained caffeine. The next most commonly detected organic chemicals in raw drinking water samples were pyrene and fluoranthene (6 samples), followed by tri(2-chloroethyl)phosphate (5 samples). Finished drinking water samples contained tri(2-chloroethyl)phosphate (6 samples); phthalic anhydride (5 samples); caffeine, triclosan, fluoranthene, pyrene, 2,6-di-t-butylphenol and ethanol-2-butoxy-phosphate (3 samples), and tributyl phosphate in 2 samples. Seven additional organic compounds were detected in 1 of the 9 finished water samples. Potential endocrine disrupting chemicals were detected in 10 treated effluent samples, in 6 raw drinking water samples, and in 1 finished drinking water sample.

This study shows that some chemicals found in commercial household products survive WPCP treatment, ecologic conditions between a WPCP discharge and a DWTP intake, and DWTP treatment. These organic chemicals may go undetected in treated effluent and in municipal water supplies, either because water utilities do not test for these chemicals or the concentrations of these chemicals are below the detection limit of the analytical method. Additional surveys to measure these chemicals would characterize the scope and magnitude of these compounds in surface and municipal waters and would identify chemicals that may need further studies to assess their human and ecological effects at ppb concentrations.

Biographical Sketches

Dr. Alden K. Henderson , PhD, MPH. , is a toxicologist at the Centers for Disease Control and Prevention in Atlanta, GA. He received his Ph.D. in toxicology from the University of Arizona and his Masters of Public Health in epidemiology from the University of Hawaii. His projects deal with documenting exposures to environmental agents and determining the human health effects of these exposures.

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Processes Affecting the Fate of Nonylphenol During Wastewater Treatment

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Abstract

Hazardous organic compounds in wastewater pose a serious national and international problem. Wastewater treatment facilities routinely treat billions of gallons of domestic and industrial wastewater per day. Typical wastewater may contain hazardous organic contaminants including volatile organic compounds, polyaromatic hydrocarbons, oils, greases, and surfactants. In addition to the hazardous chemicals that enter a wastewater treatment plant, the intermediate and final breakdown products of these chemicals may pose environmental and human health risks. For example, alkylphenols, which are the breakdown product of commonly used nonionic surfactants, are highly toxic to aquatic life. Alkylphenols have also been implicated as endocrine disrupters. One specific chemical shown to be an estrogen mimic is nonylphenol.

Nonylphenol (NP) enters the wastewater stream in several ways. Nonylphenol in the influent to wastewater treatment plants could come either from industrial NP usage or as the leachate from particular plastics. However, a more significant source of NP is as the anaerobic breakdown product of nonylphenol ethoxylates (NPEOs). In the United States, NPEOs are widely used as nonionic surfactants in liquid detergents, paints, and cosmetics. Because of the high rate of NPEO usage in liquid detergents, many NPEOs eventually become part of the wastewater stream. While NPEOs are relatively harmless to the health of humans and to the environment, the daughter product, NP, is a stable, persistent, and extremely hazardous breakdown product. In order to form NP, NPEOs must be exposed to anaerobic conditions, which are the conditions purposely created in typical sludge digestion processes. Additionally, areas with anaerobic conditions are common throughout the wastewater treatment process.

Although the potential exists to form significant amounts of nonylphenol during wastewater treatment, the concentration of nonylphenol entering the environment from the effluent of wastewater treatment plants is relatively low. By surveying the levels of nonylphenol at different stages in the treatment process of a conventional activated sludge sewage treatment plant, we have shown that a significant amount of the nonylphenol present in the system is eliminated in the early stages of treatment. A large percentage nonylphenol loss in the treatment facility occurred between the influent and the primary treatment stages, between 40-45% based on data collected from the Essex Junction Wastewater Treatment Facility in Essex Junction, Vermont. Another large percentage (between 28-40%) total NP loss occurred during secondary treatment. Essex Junction has a typical activated sludge wastewater treatment plant with nitrification. Primary treatment consists of aerated grit chambers, aerated equalization basins, and primary settling tanks. Losses of NP in the process were attributed to sorption and volatilization. A high log Koc (4.4) was determined in our lab. Bench-scale laboratory results also showed significant loss of NP during aeration.

Implications of the volatilization of nonylphenol during traditional wastewater treatment include long-range transport and deposition of nonylphenol through the air. Potential for human exposure to nonylphenol through this pathway is high. Studies performed by other researchers have detected elevated levels of airborne nonylphenol in urban areas. Further work is being conducted to determine the significance of sorption and volatilization of NP in the wastewater treatment process.

Introduction

Wastewater containing hazardous organic compounds is a serious national and international problem. Daily, industrial and domestic wastewater treatment facilities treat billions of gallons of wastewater containing hazardous organic contaminants including volatile organic compounds, polyaromatic hydrocarbons, oils, greases, and surfactants. Pollution in the waste stream originates from domestic, industrial and agricultural sources. Typical wastewater contaminants include excess nutrients from agricultural runoff, degreasers and surfactants from industrial wastes, detergents from both domestic and industrial sources, greases and oils from storm runoff, and other organic contaminants from many sources. In addition to the hazardous chemicals that

enter a wastewater treatment plant, the intermediate and final breakdown products of these chemicals may pose environmental and human health risks. The primary purpose for wastewater treatment is to remove BOD, suspended solids, pathogens, and the organic and inorganic pollutants from the waste stream in order to allow safe discharge to receiving waters.

Wastewater treatment plants (WWTPs) are very effective at removing the majority of these compounds from the waste stream, but there are still some contaminants that are discharged to the environment. Both to preserve the quality of aquatic ecosystems and to safeguard human health, wastewater treatment mechanisms are needed that can safely remove specific, hazardous, synthetic organic compounds. Nonylphenolic compounds are one type of environmentally relevant pollutant. As well as being highly toxic to aquatic ecosystems, NP has been shown to be an estrogen mimic in fish and mammals.

Nonylphenolic compounds are made of a phenol ring with an attached side chain of nine carbons. Nonylphenol is typically a mixture of isomers with the various arrangements of the point of attachment of the carbon chain, as well as the degree of carbon chain branching. Most commercial produced NP is 4-NP, which is a mixture of isomers with the side chain located directly across from the OH chain. Additional compounds may be joined to the OH portion of the molecule. Nonylphenolic compounds include nonylphenol ethoxylates (NPEO) and its breakdown products such as short chain NPEO ($NP(EO)_{1-2}$) , nonylphenol (NP) and carboxylated nonylphenol ethoxylates (NPEC). Nonylphenolic compounds are an example of the more generic group of chemical referred to as alkylphenolic compounds.

The most common conventional wastewater treatment system for large communities is the activated sludge system. Activated sludge treatment typically includes preliminary treatment processes, primary treatment, and secondary treatment. Depending on the waste characteristics, tertiary treatment may also be implemented. The effluent is disinfected before discharge to the receiving water source.

Preliminary treatment is used to remove the gross solids from the waste stream. This typically involves some combination of screens and grit removal chambers. The grit chamber is often aerated and kept mixed at a rate adequate to keep the smaller solids suspended, but let the heavy grit settle. Aeration in this stage is not done as a treatment process, but may lead to inadvertent air stripping of pollutants.

Primary treatment involves solids settling in the primary clarifier. Quiescent conditions allow solids to settle and other materials to float. Secondary treatment is the process of biological treatment and subsequent settling of the microorganisms from the wastewater. The microorganisms grown in this stage consume the organic matter measured as biochemical oxygen demand (BOD) in the wastewater. Environmental conditions are kept in the range that is conducive to their growth. Aeration is required by the microorganisms for metabolism of the BOD. A portion of the sludge settled in the secondary clarifier is recycled into the aeration tank. This ensures that there is an adequate microbial population to remove the BOD from the wastewater.

Tertiary treatment involves processes to remove nutrients, colors, and odors from wastewater. Advanced treatment methods may also need to be implemented to remove high levels of other pollutants from the wastewater that cannot be effectively removed with secondary treatment.

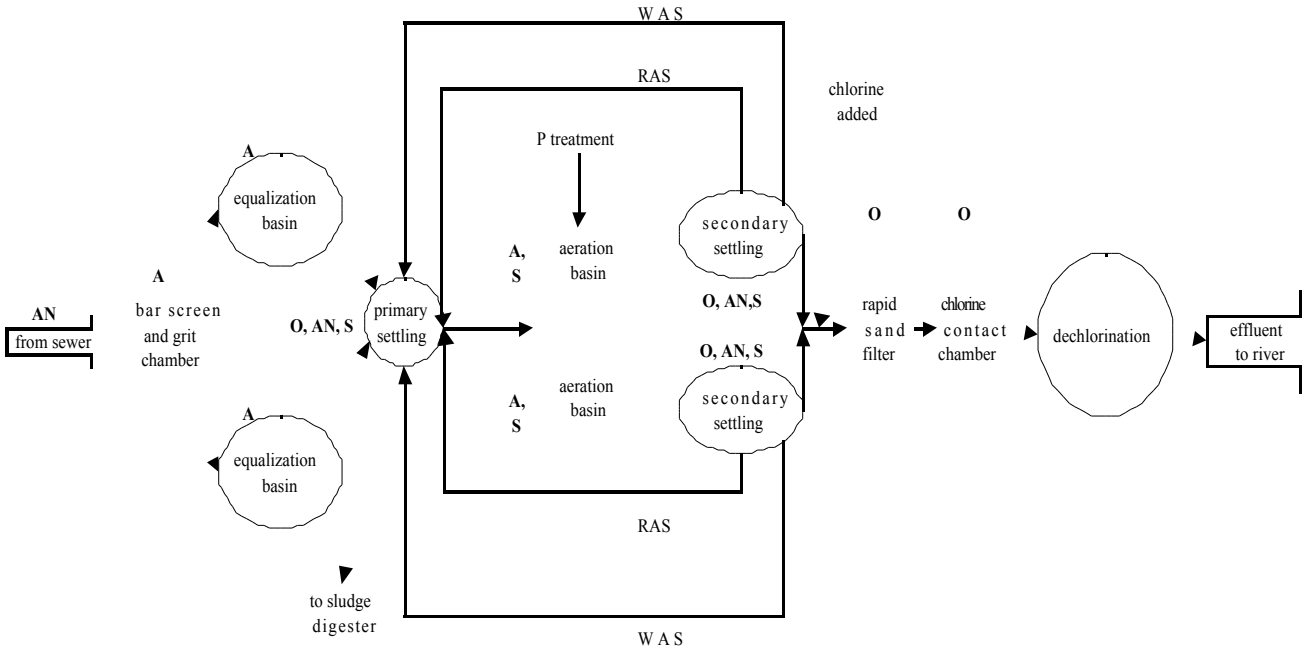
In addition to the treatment of the wastewater itself, the byproduct sludge that is settled in the primary and secondary clarifiers needs disposal. Typically the volume of excess sludge is reduced through sludge digestion processes. Sludge digestion can be designed to take place under either aerobic or anaerobic conditions. Sludge digestion reduces the volume of sludge as well as eliminates the majority of pathogens in the sludge. Sludge may also be dewatered to reduce the final volume for disposal. Depending on the properties of the sludge and the regulatory considerations, it may be used as fertilizer, incinerated, or landfilled.

The Essex Junction Wastewater Treatment Facility (EJWWTF), located in Essex Junction Vermont, is an activated sludge facility that also performs nitrification. Wastewater from domestic and local business sources enter the plant at an average flow rate of 1.8 million gallons per day (MGD). The schematic shown in Figure 1 is marked to indicate potential zones of aerobic and anaerobic conditions that may be important for determining contaminant fate. Particularly of concern for NP fate and transport during wastewater treatment are areas that are aerated, are open to the atmosphere, have high concentrations of solids, or have the potential to become

anaerobic. For example, when tanks are aerated, the risk of loss of semi-volatile NP through volatilization is increased and in open tanks, surface volatilization can occur. In treatment processes that have high concentrations of solids, there is an increased chance of NP loss to adsorption. Under anaerobic conditions, NP can be formed as a breakdown product of NPEO.

The wastewater passes through screens and a grit chamber in the headworks and then flows into two flow equalization chambers. Equalization chambers dampen variations in flow and loading providing better treatment. Additionally, the chambers are aerated and mixed to prevent solids deposition and formation of odors. The wastewater is mixed with wasted activated sludge (WAS) from secondary clarifiers and flows through primary clarifiers where solids settle. The effluent from primary is mixed with return activated sludge (RAS) and flows to the aeration tanks. After aeration, the wastewater flows into the secondary clarifiers where the microorganisms and other solids settle and are recycled to the aeration tanks and wasted to the primary clarifiers. After the secondary clarifiers, effluent is disinfected and passes through a sand filter to remove fine and suspended solids. It is then dechlorinated, and discharged as into the Winooski River. The settled sludge from the primary clarifier is decomposed in an anaerobic sludge digester.

Figure 1. Schematic of the Essex Junction Wastewater Treatment Facility
Legend: **A** = aerated, **AN** = possible anaerobic conditions, **O** = open tank, **S** = high solids



The overall objective of this research was to investigate nonylphenol fate and transport in the wastewater treatment process. Specific objectives of this paper were to:

1) review and compare existing data on nonylphenolic compounds in wastewater treatment plants;
2) survey the levels of NP in the EJWWTF and determine the possible NP elimination methods in a typical activated sludge wastewater treatment plant ; and
3) perform laboratory scale studies to test proposed mechanisms that influence the environmental fate of NP.

Background

Nonylphenol becomes part of the wastewater stream in several ways. Nonylphenol could be present in the influent to wastewater treatment plants, either from industrial usage or as the leachate from certain plastics. A more significant source of nonylphenol is as the anaerobic breakdown product of NPEOs. Nonylphenol

ethoxylates are nonionic surfactants widely used throughout the U.S. in cosmetics, paints and especially detergents. Because of its detergent use, most NPEOs eventually become part of the wastewater stream. The NPEOs are relatively innocuous, but the daughter product, NP, is a stable, persistent, and extremely hazardous breakdown product.

Under anaerobic conditions, NPEOs can be transformed into dangerous and persistent NPs as final products. NPEOs undergo a series of breakdown transformations both within a sewage treatment plant and when discharged to the environment. Breakdown steps involve shortening of the ethoxylate chain. In general, the shorter the ethoxylate chain becomes, the more hydrophobic, persistent, and toxic the substance becomes. Interestingly, NPEOs will only degrade to NP under anaerobic conditions, which are the conditions purposely created in typical sludge digestion processes. Additionally, local zones with anaerobic conditions are common throughout the wastewater treatment train. On the macro-scale, corners of aerated tanks as well as entire settling basins and clarifiers can become anaerobic. On the micro-scale, local anaerobic zones may be found within the floc particles in the aeration basin. Anaerobic zones may also occur in sewage lines, storm drains, and pipes as the wastewater is in transit to the treatment facility.

NP is both hydrophobic and moderately volatile. Volatility is typically expressed as the Henry's law constant. The Henry's law constant is the ratio of the concentration of a compound in air to the concentration of the compound in water at equilibrium conditions. Henry's law constants for NP are given in Table 1. The general definition, as given by Lyman et al. (1990), of a volatile compound is one with a Henry's law constant greater than 3×10^{-7} (dimensionless). Literature values vary from 0.6×10^{-3} to 1.7×10^{-3}.

One factor that can significantly affect the Henry's law constant in a particular situation is the salinity of the solution. As outlined by Snoeyink and Jenkins (1980), when salt concentration increases, the solubility of molecular species decrease and affect the Henry's law constant. The salting out coefficient, Ks, is generally in the range of 0.01 to 0.15. The ionic strength, μ, and Ks are used to calculate γ and modify the Henry's law constant. For wastewater, conductivity (as measured in our lab with a conductivity meter) is ~ 1000 μmho. Then $\mu = 1.6 \times 10^{-5}$ * conductivity (in μmho) = 0.016. Using the range of Ks values, and the relationship $\log \gamma = Ks * \mu$, $\gamma = 1.000$ to 1.006. This shows that γ is ~ 1, suggesting that the ionic strength of wastewater will not affect the Henry's law constant of NP in wastewater.

Table 1. Henry's law constants for 4-NP (mixes of isomers) converted to typical units.

Pa-m^3/mol	atm-m^3/mol	Dimensionless	y/x (atm/mole fraction)	Reference
3.04 to 4.05	3×10^{-5} to 4×10^{-5}	1.25×10^{-3} to 1.67×10^{-3}	1.67 to 2.22	Dachs et al., 1999
3.65	3.6×10^{-5}	1.5×10^{-3}	2.00	Van Ry et al., 2000
1.5075	1.55×10^{-5}	6.46×10^{-4}	0.86	Shiu et al., 1985

Lee et al.(1998) state that volatilization from the surface of primary and secondary systems in a wastewater treatment plant are important fate mechanisms for compounds with moderate to high volatilities, they are defined as H= 0.001 to 0.1 (dimensionless). NP falls within this range. Volatilization should be considered as one of the pathways that determine the fate and transport of NP both within wastewater treatment plants and the environment at large.

Elevated levels of airborne nonylphenol have been detected in urban areas (Dachs et al., 1999, Van Ry et al., 2000). This, in addition to the reported Henry's law constant values for NP, points to volatilization as an important pathway in the fate of NP. Based on their studies, Van Ry et al. (2000) estimate the half-life for NP in the Lower Hudson River due to volatilization to be 9 days.

Studies with other hydrophobic, synthetic organics have found volatilization to be a significant loss mechanism as well. Jeremiason et al. (1994) determined that the decrease in PCBs in the water of Lake Superior was due to loss of PCBs by volatilization. In this study historic levels of PCBs in the aqueous phase in the lake from 1978-1992 were compiled and analyzed. Sediment core samples were used to evaluate losses by adsorption. A mass balance calculation revealed that volatilization was the dominant loss process. It was expected that, since PCBs are hydrophobic, sedimentation would account for most of the losses over time. In fact, estimates from the data

predicted that losses of PCBs due to net volatilization were an order of magnitude higher than losses attributable to net sedimentation. For example, in 1986, the PCB lost through volatilization was estimated at ~1900 kg, whereas the PCB lost through sedimentation was estimated at ~110 kg. However, the solids concentration in Lake Superior is very low compared to the concentrations found in wastewater treatment and sorption processes may dominate over volatilization.

The hydrophobicity of NP influences its environmental fate and transport and is measured using various partition coefficients. NP octanol-water partition coefficient (K_{ow}) and log K_{ow} values are listed in Table 2. Log K_{oc} values have been calculated using Kow values and various estimation equations. Sekela et al. (1999) report a log K_{oc} for 4-NP in sewage treatment effluent of 4.7 mL/g. Ekelund et al. (1993) list the log Koc for NP as approximately 4. Romano (1991) reports findings from sediment adsorption tests on 4-NP with values of Log K ranging from 3.3 to 3.7. These values indicate that NP will sorb to sediment in the environment, bioaccumulate in animal fat, and attach to solids during wastewater treatment.

Adsorption to solids is a major factor in the fate of NP in the environment. Because it is hydrophobic, NP preferentially sorbs to solids rather than remaining in the aqueous phase (Marcomini et al., 1990, Eljertsson et al., 1999, Bennie et al., 1997, Bennettt and Metcalfe, 1998).

Table 2. Log K_{ow} values for mixtures of 4-NP isomers

Property	Value	NP isomer	Reference
Log K_{ow}	> 4.0	4-NP, unspecified chain	Thiele et al., 1997
Log K_{ow}	3.80 to >4.75	4-NP, branched chain	Romano, 1991
Log K_{ow}	4.48	4-NP, unspecified chain	Shiu et al., 1994
Log K_{ow}	4.48	4-NP, branched chain	Ahel and Giger, 1993a

Solubility is another factor that influences the fate of NP in the environment. Reported values of NP solubility in water are given in Table 3. Because it is a phenol, the solubility of NP will vary depending on temperature and pH (i.e. it will be more soluble at higher pH).

Table 3. Solubility of NP in distilled water

Solubility (mg/L)	NP isomer	Reference
6	4-NP, branched chain	Muller and Schlatter, 1998
@ pH 5 = 4.6 @ pH 7 = 6.24 @ pH 9 = 11.9	4-NP, branched chain	Hellyer, 1991
5.43	4-NP, branched chain	Ahel and Giger, 1993b

Biodegradation of NP is also of interest in determining its fate in wastewater treatment. Verschueren (1983) found that 45% of NP could be biodegraded by adapted sewage sludge in 135 hours. Ekelund et al. (1992) examined the biodegradation of NP in seawater and sediment. They found that under aerobic conditions, after a 58-day trial, microorganisms had mineralized 50% of the NP added to a seawater/sediment sample. It is important to note that the Ekelund et al. (1992) study did not account for possible NP loss through volatilization during the experiment. Conversely, Ejlertsson et al. (1999) studied anaerobic degradation of short-chained NPEOs in sludge. They found that NP was formed as a persistent end product that would not be degraded further. Similarly, Razo-Flores et al. (1996) found that NP on anaerobically digested sludge was not biodegradable under methanogenic conditions. Romano (1991) reports that under anaerobic conditions, NP at 61 mg/L shows no degradation in 60 days. Typical hydraulic detention times in aeration basins in wastewater treatment are 8-14 hours, suggesting that NP may not be significantly biodegraded in the wastewater treatment process.

Photodegradation may play an important role in the breakdown of nonylphenolic compounds in the environment. Ahel et al. (1994a) examined degradation of NP by sunlight in natural river water. They estimated a half-life for aqueous NP at the surface would be 10-15 hours with light equivalent to midday, summer sun on a clear day. Half-life estimates were 1.5 times slower in the water 20-25 cm below the water surface. It was also found that the rate of photolysis for NP increases in the presence of organic matter. In the

wastewater treatment process light penetration is severely limited in many of the processes due to high solids concentrations and therefore photodegradation may not be an issue.

Nonylphenol has been measured in the atmosphere and in surface waters and associated sediment. Reported concentrations of NP vary, but observed levels indicate low levels of global contamination. Especially troubling are findings of NP in the air that indicates NP could travel long distances and increase the risk of human and environmental exposure. Observed levels of NP from the literature are reported in Table 4. Additionally, as shown in Table 5, analyses of the levels of nonylphenolic compounds at various stages in wastewater treatment plants indicate that NPEO and its breakdown products are not effectively removed during traditional treatment processes. None of the reported studies include air emissions from the treatment facilities, and so actual degradation rates of NP may be overestimated.

Table 4. Environmental Levels of NP found by various researchers.

Matrix	**Location**	**NP Concentration**	**Reference**
Solid (sewage)	Cape Cod, MA	> 1000 μg/L in all samples	Rudel et al. (1998)
Solid (sediment)	St. Lawrence River Valley	0.17 to 0.72 μg/g in 9 samples	Bennie et al. (1997)
Solid (sediment)	Upper Great Lakes	up to 37 μg/g	Bennett and Metcalfe (1998)
Solid (sediment)	Venice Lagoon, Italy	0.15-0.37 μg/L	Marcomini et al. (1990)
Liquid (surface water)	St. Lawrence River Valley	quantifiable in 24% of the water samples at levels up to 0.92 μg/L	Bennie et al. (1997)
Liquid (surface water)	Glatt River, Switzerland	above 1 μg/L in 84% of samples, maximum level of 45 μg/L	Ahel et al.(1994b, 1996)
Liquid (surface water)	England and Wales	up to 12 μg/L	Blackburn and Waldock (1995)
Liquid (surface water)	Lake Mead, NV	non-detect to 1140 ng/L	Snyder et al. (1999)
Liquid (surface water)	Trenton Channel, MI	0.269-1.190 μg/L	Snyder et al. (1999)
Liquid (surface water)	Tampa Bay, FL	Non-detect	Potter et al. (1999)
Air	Hudson River Valley	2.2 to 70 ng/m^3	Dachs et al. (1999)
Air	Hudson River Valley	coastal: nd – 56.3 ng/m^3 suburban: 0.13 – 0.81 ng/m^3	Van Ry et al. (2000)

Modeling

The EPA developed the WATER9 model to determine air emissions from wastewater treatment plants as well as emissions from waste transport and storage facilities for volatile organic contaminants. Additionally, Lee et al. (1998) designed a steady state model to predict the fate of hydrophobic and volatile contaminants in activated sludge systems. Both models are steady-state models that use the concept of mass balance to determine the fate of the target compound. Each of these models relies on user input of physical properties of the wastewater treatment system as well as physical and chemical properties of the pollutant of concern.

The WATER9 model has a graphical interface that allows the user to schematically represent the treatment plant on the computer screen. Physical characteristics of each unit in the treatment train are associated with the screen images. Process information as well as properties of the waste stream are required. If requisite parameters for the pollutant are missing, the program will attempt to estimate them.

The WATER9 model considers primary removal mechanisms for volatile organics compounds (VOCs) to be air emissions and biodegradation and the model focuses on these areas and does not include adsorption, photodegradation, hydrolysis, oxidation/reduction, hydroxyl radical reactions, runoff and mitigation. The model was developed for use with VOCs, and therefore may not be appropriate for with semi- or non- volatile compounds.

Table 5. Nonylphenolic compounds in wastewater treatment plants (all liquid concentrations converted to µg NP /L; all solid concentrations converted to g NP/Kg solids)

Type	Sampling Points	Concentration of Nonylphenolic compounds	Reference
Activated Sludge	Primary Effluent	1440 µg/L total nonylphenolic compounds: 43.2 µg/L NP, 172.8 µg/L $NP(EO)_{1-2}$, 43.2 µg/L NPEC, 1180.8 µg/L NPEO	Giger and Ahel (1991)
	Secondary Effluent	400 µg/L total nonylphenolic compounds: 16 µg/L NP, 88 µg/L $NP(EO)_{1-2}$, 184 µg/L NPEC, 112 µg/L NPEO	
Activated Sludge	Influent	21 µg/L NP, 30 µg/L $NP(EO)_1$, 16 µ g/L $NP(EO)_2$	Brunner et al. (1998)
	Stabilized sludge	0.12–2.2 g/Kg NP, 0.06–0.68 g/Kg $NP(EO)_1$, 0.02-0.28 g/Kg $NP(EO)_2$	
	Primary Effluent	15 µg/L NP, 30 µg/L $NP(EO)_1$, 17 µ g/L $NP(EO)_2$	
	Activated sludge	150 µg/L NP, 530 µg/L $NP(EO)_1$, 110 µ g/L $NP(EO)_2$	
	Secondary Effluent	2.7 µg/L NP, 16 µg/L $NP(EO)_1$, 15 µ g/L $NP(EO)_2$	
	Raw sludge	2850 µg/L NP, 3900 µg/L $NP(EO)_1$, 3000 µ g/L $NP(EO)_2$	
	Digested sludge	78000 µg/L NP, 6700 µg/L $NP(EO)_1$, 1820 µ g/L $NP(EO)_2$	
Activated Sludge	Influent	29-145 µg/L NPEO	DiCorcia et al. (2000)
	Effluent	1.7-6.6 µg/L NPEO, 0.6-15 µg/L NPEC, 0.05-24 µg/L $C(AP)_{3-8}EC$	
Activated Sludge	Influent	1.81-22.69 µg/L NP, 2.2-71.3 µg/L $NP(EC)_1$, 1.4-15.4 µg/L $NP(EC)_2$, 123.4-414.9 µg/L NPEO	Lee and Peart (1998)
	Primary Effluent	1.59-10.92 µg/L NP, 3.3-11.1 µg/L $NP(EC)_1$, 4.3-16.4 µg/L $NP(EC)_2$, 43.1-166.9 µg/L NPEO	
	Final Effluent	0.56-2.12 µg/L NP, 9.1-43.8 µg/L $NP(EC)_1$, 14.3-41.1 µg/L $NP(EC)_2$, 3.3-32.4 µg/L NPEO	
Various	Primary Effluent	2.8-30 µg/L NP	Lee and Peart (1995)
	Post-chlorinated Effluent	0.8-15.0 µg/L NP	
	Sludge	0.137-0.470 g/Kg NP	
Various	Effluents	142.5-272.4 µg/L NPEC	Field and Reed (1996)
Various	Effluent	0.171 µg/L to 37 µg/L NP, non-detect to 332 µg/L NPEO	Snyder et al. (1999)
Sludge digestion	Digested sludge (anaerobic)	0.45-2.53 g/Kg NP	Giger et al. (1984)
Sludge digestion	Digested sludge (anaerobic)	0.638 g/Kg and 0.326 g/Kg NP	Sweetman (1994)

Abbreviations used: NP = nonylphenol; NPEO = nonylphenol ethoxylate with unspecified length EO chain; $NP(EO)_X$ = nonylphenol ethoxylate with EO chain of length X; NPEC = nonylphenol ethoxylate with a carboxylated EO chain; $NP(EC)_X$ = nonylphenol ethoxylate with a carboxylated EO chain of length X; $C(AP)_XEC$ = alkylphenol ethoxylate with carboxylated EO and alkyl- chains, alkyl- chain is of length X.

Materials and Methods

The 4-NP used to make standards and NP-water solutions was obtained from Arcos Organics. OPTIMA and HPLC grade methanol were obtained from Fisher Scientific. All glassware was triple washed with methanol, double washed with acetone and baked at in a 105°C oven overnight prior to use.

Wastewater and activated sludge samples from EJWWTF were collected in acid-washed glass BOD bottles with ground glass stoppers and stored at 4°C for a maximum of 4 hours before processing. Liquid samples were filtered though a 47mm Gelman Laboratory glass fiber filter, Type A/E to remove any solids. Alternately, some liquid samples were centrifuged at 1500rpm for 10 minutes to remove solids. No significant differences were observed for the two methods of solid separation. Acidification to below pH 2 was also tested as a method to arrest biological activity of the liquid samples. Acidification produced inconsistent, and typically higher results for NP concentration during GC analysis and was determined to be a poor way to preserve samples. As another method to stop biological activity in the samples, refrigeration or freezing was used. Frozen liquid samples seemed to provide questionable analytical results. However, freezing the RAS biosolids was used as a method to inactivate the solids for the partitioning test. In cases where freezing was used, both the liquid and the sludge samples were frozen at approximately 0°C. Prior to analysis, covered samples were thawed at room temperature under a ventilation hood. Samples were protected from ultraviolet light at all times.

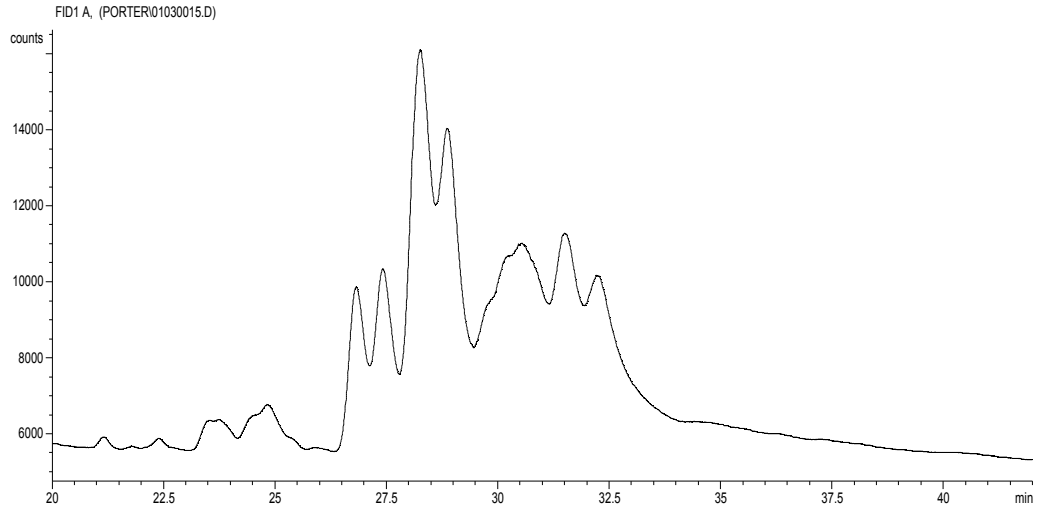
NP saturated water solution was made by coating glass beads with pure NP. The glass beads were packed into a glass column with machined stainless steel plates and one opening on each end. Distilled water was pumped up through the bottom on the column and collected in a glass jar at the outlet on the top. This method consistently provided NP-water solutions with concentrations between 4.36 and 5.44 mg/L

Partitioning to inactivated biosolids was determined by addition of an NP-water solution to 50 or 100 mL activated sludge samples that had been prepared as indicated above. Partitioning tests were done at room temperature, which was 20 $\pm$ 1°C over the course of the experiments. The NP-water solution was added to the sludge samples and stored in glass bottles with ground glass stoppers. Bottles were filled to a total volume of 300mL. Care was taken to minimize headspace in the bottles. Bottles were shaken on a reciprocating shaker for 24 hours. Samples were removed and let to settle for 28 hours. Liquid was decanted from each bottle and analyzed by GC after solid phase extraction (SPE) for clean up and concentration. Solids were extracted with a shaking extraction method followed by GC analysis.

Quantitative analysis of samples was performed on HP 5890 Series II Gas Chromatograph using the flame ionization detector (FID), and a J&W Scientific Phase DB5 column with 1.5 micron film thickness, 30 m x 0.53 mm I.D. Temperature program was initial temperature of 100°C for 10 min., ramp at 10°C/min. to 140°C and hold for 40 min. Each peak in the chromatogram represents a different isomer of NP and is shown in a typical chromatogram (Figure 2). Peaks could not be completely separated even with low temperature isothermal runs of extended duration. In order to quantify NP, a baseline was drawn from 23 minutes to 37.5 minutes and the resulting combined area under the peaks was calculated. Accurate calibration curves were obtained by this method with a calibration limit of 2.5 mg/L. By combining this analytical method with solid phase extraction (SPE) for sample clean-up and concentration, detection limits of 0. 01 mg/L were obtained.

Verification of detected NP levels by an outside laboratory was unsuccessful. A laboratory in the U.S. with existing methods for NP detection could not be found. One Canadian firm, Maxxam Analytics, Inc. (Ontario), was contracted to test replicate wastewater samples for NP and NPEO. Unfortunately, their detection limit for NPEO was 5mg/L, which is higher than what would be expected in an environmental sample. For NP analysis, they did not request any sample preservation and returned levels of non-detect in all samples processed (detection limit 0.001mg/L). It is difficult to get accurate outside confirmation for NP without specific knowledge of how the samples were handled and processed. Without preservation, NP loss to volatilization, photodegradation, or aerobic biodegradation are likely.

Figure 2. Typical NP chromatogram from GC analysis.



The NP SPE method used was adapted from Lee (1999). SPE cartridges filled with the bonded-phase sorbent Octadecylsilane (C_{18}) were obtained from Waters Corporation. The Sep-Pak Vac 6cc cartridges with 1g of sorbent were modified in our laboratory to connect to glass reservoirs with copper tubing. To use the C_{18} cartridges, a 20-position vacuum manifold (Waters Corporation) drew liquids through the sorbent. The cartridges were pre-conditioned prior to sample loading with 5mL of acetonitrile followed by 5mL of methanol. Next, 10mL of distilled water was flushed through the cartridges as a final preparative step. Aqueous samples were loaded at a rate of 6-9mL/min. Finally, the NP was eluted with 5mL of methanol and collected for GC analysis.

Shaking extractions for solid matrices were adapted from Schwab et al. (1999). A 10 mL portion of sludge solids was shaken for 1 hour on a reciprocating shaker with 10mL of acetone in a 20 mL crimp cap glass bottle. Bottles were removed from the shaker and centrifuged at 1500 rpm for 10 minutes. Supernate was decanted and stored in a crimp capped glass bottle. A new aliquot of 10mL acetone was added to the solids in the bottles and the process was repeated. Three cycles of shaking were done and the supernate for each of the cycles was combined to make one sample. The combined sample was analyzed by GC as previously described.

Results and Discussion

Figure 3 and Table 7 show data for two different sampling days. A large portion of NP removal from the wastewater stream takes place by the time the wastewater leaves primary treatment and occurs where microbial biodegradation is not likely. This reinforces the importance of physical processes, especially volatilization, as removal mechanisms. There is continued loss of NP between the primary and secondary treatment outlets. Losses in this segment of the treatment train may be due to sorption to the solids, volatilization in the aeration basin, and biodegradation. Biodegradation is not thought to be a significant removal mechanism within the wastewater treatment plant as reported biodegradation half-life for NP in adapted sewage is 150 hours (Verschueren, 1983). The literature confirms that sorption, as shown by the K and K_{oc} values, as well as by the detected levels of NP on RAS and digested sludge solids, is likely a significant removal method. In the EJWWTF, the level of NP on RAS from 6/21/01 were 2.42 g/Kg.

Samples taken on a given date were taken at approximately the same time. There is inherent variability in the influent to the wastewater treatment plant, and samples of influent on a particular day do not correspond directly to the effluent that is collected at that time. For this study we assumed steady state conditions for the plant for both flow rates and concentration of NP in the influent. Obviously if the influent load of NP and NPEO varies considerably, then the correlation between the influent and effluent will not be good. Other variability in the data may be attributable to changes in operating and environmental conditions such as water temperature, pH, flow rates, and solids concentrations. However, a similar trend was noted for NP concentration in the two

different days that sampling occurred. Approximately 72-89% of the influent NP had been lost during the entire process.

Figure 3. Levels of NP at various stages of treatment in the Essex Junction Wastewater Treatment Facility.

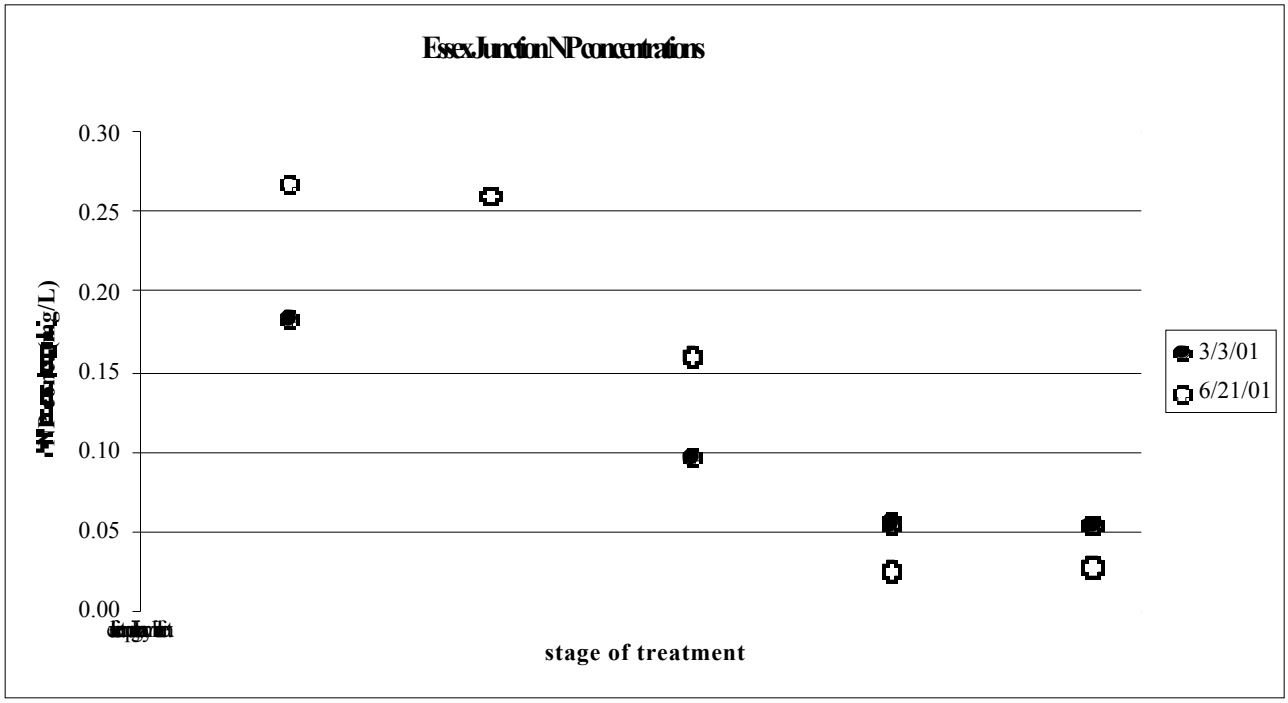


Table 7. Levels of NP in samples from the Essex Junction Wastewater Treatment Facility (nc = not collected)

Sample	NP concentration 10/6/00	NP concentration 3/3/01	NP concentration 6/21/01
influent	0.10 mg/L	0.18 mg/L	0.27 mg/L
grit chamber	nc	nc	0.26 mg/L
after primary	nc	0.10 mg/L	0.16 mg/L
after secondary	nc	0.05 mg/L	0.03 mg/L
effluent	nc	0.05 mg/L	0.03 mg/L

The laboratory test to measure the adsorptive capacity of return activated sludge solids for NP was used to determine the partition coefficient for NP to RAS. As detailed in Table 8, log K ranged from 3.96 to 4.21 and log K_{oc} ranged from 4.36 to 4.61, where K_{oc} refers to organic carbon. The log K value was slightly higher than the range of 3.3 to 3.7 that Romano (1991) reported for NP on sediment. The log K_{oc} value was similar to the value of 4.7 found by Sekela et al. (1999) for NP in sewage effluent and slightly higher than the value of approximately 4 given by Ekelund et al. (1993).

Table 8. Partition coefficients for nonylphenol to return activated sludge solids. Values given are in the following format: mean (standard deviation).

samples	K (mL/g)	Log K (mL/g)	Koc (mL/g)	Log Koc (mL/g)
all	16166.90 (12852.0)	4.21	40437.40 (32146.1)	4.61
w/o outliers	10180.40 (2227.34)	4.01	25463.74 (5571.13)	4.41
for 100mL RAS	9135.97 (2101.61)	3.96	22851.35 (5256.66)	4.36
for 50mL RAS (w/o outliers)	11921.12 (1121.50)	4.08	29817.72 (2805.15)	4.48

At equilibrium conditions, K was determined as follows (as explained in Lyman et al., 1990):

x/m = μg NP adsorbed/g of solids

C = μg NP/mL of solution

and $x/m = KC^{1/n}$ (assume that n = 1)

K_{oc} was calculated with the formula $K_{oc} = (K / \%oc) * 100$. The units for all reported K and K_{oc} values are mL/g.

Percentage of organic carbon was determined using the total suspended solids (TSS) and the volatile suspended solids (VSS) in conjunction with some logical assumptions. Since TSS and VSS were both measured for each sample, it is known that VSS= 75.30% of the TSS. It is assumed that all of the VSS is organic matter made of cells and that the composition of the cells is given by $C_5H_7O_2N$. Using this formula for the VSS, organic carbon is 53.1% of the VSS on a weight/weight basis. This means that the organic carbon is 39.98% of the TSS (since 75.30% * 53.1% = 39.98%). Actual values for organic carbon for the sludge were not determined at this time.

Other laboratory tests also provided insight into the probable fate of NP during wastewater treatment. Bench-scale sequencing batch reactors were used to study the fate of NP under different conditions. The conditions examined here included NP in distilled water, NP in wastewater and NP in a mixture of wastewater and activated sludge. In all cases the experimental set-up used aerated flasks. After approximately one hour of aeration, the units were allowed to settle for one hour and then sampled. After samples were removed for analysis, the systems were dosed with more nonylphenol and either distilled water or wastewater. Levels of NP in the sample solution were determined for each case during each cycle. An example of the results is shown in Figure 4.

Figures 4 shows that the highest levels of NP were found remaining in the wastewater case, followed by the distilled water case, and the wastewater and activated sludge case. In the distilled water case approximately 85% of the NP added was not recovered from solution. In the distilled water case, adsorption to solids and microbial biodegradation were not feasible loss mechanisms. Adsorption to glassware and photodegradation were investigated and eliminated as loss mechanisms, therefore we propose that in the majority of the NP loss was due to volatilization during aeration. This theory is supported by several lab scale studies we did on NP in distilled water to determine loss mechanisms, shown in Table 9. Flasks received constant aeration (which would be similar to the experimental conditions), sporadic aeration (three short bursts of aeration over the course of the evaluation), or no aeration. Under conditions of constant aeration, there was an 81% loss of the NP. Volatilization of NP by aeration seems to be the factor that accounts for the NP loss in the controls of the bench scale studies.

In the reactors where solids were present, it is unclear how much loss was due to volatilization or sorption. However, solids concentrations were about 1500 mg/L in the wastewater and activated sludge case and about 150 mg/L in the wastewater alone case.

There is a dynamic relationship between the NP adsorbed to the solids in the system and the NP that can be volatilized. During the aeration cycle, NP would both partition to the solids and into the air. Because NP is both hydrophobic and semi-volatile, there is a competition between different fate mechanisms as the NP levels try to come to equilibrium. The possibility of the formation of additional NP from NPEO under the anaerobic settling conditions compounds the problem of trying to do a mass balance on the system. The specifics of this complicated interaction are still being investigated.

It seems likely that in the cases of high solids concentration, sorption is playing a significant role. Based on an average log K value of 4.07 for partitioning of NP to RAS, 93% of the initial NP added to the WAS flasks would be expected to adsorb to the solids. This could account for the low values of NP in the WAS flaks.

Using the EPA WATER9 model and adjusting the NP chemical and physical properties to match those outlined in the background section of this paper, the model predicted approximately 3% of an initial NP concentration of 0.2 mg/L would be lost to volatilization. As described previously, the model is intended for volatile compounds, and significant errors can occur with analysis of semi-volatile chemicals. The model also did not take into account sorption to solids. The EPA database included with their WATER9 computer model, as well as the EPA Federal Register (1998), give the Henry's law constant for NP as 400.00 (y/x). They also note that

values ≥ 0.1 (y/x) are of concern for air emissions during wastewater treatment. This very high Henry's law constant value for NP is listed in conjunction with a solubility value of 5000mg/L, which is 3 orders of magnitude higher than most other reported solubilities for NP (see Table 3). Because of these inconsistencies, we assumed the correct value for Henry's law constant for 4-NP to be in the range of 0.86-2.22 (y/x). These values are still high enough to indicate potential air emissions problems for wastewater treatment streams containing NP. When the properties of NP, as given in the WATER9 database, were not adjusted, ~75% of the NP would be predicted to volatilize. This higher number is congruent with our other laboratory studies.

Figure 4. NP levels in batch reactors with initial NP loading of 2.38 mg/L.

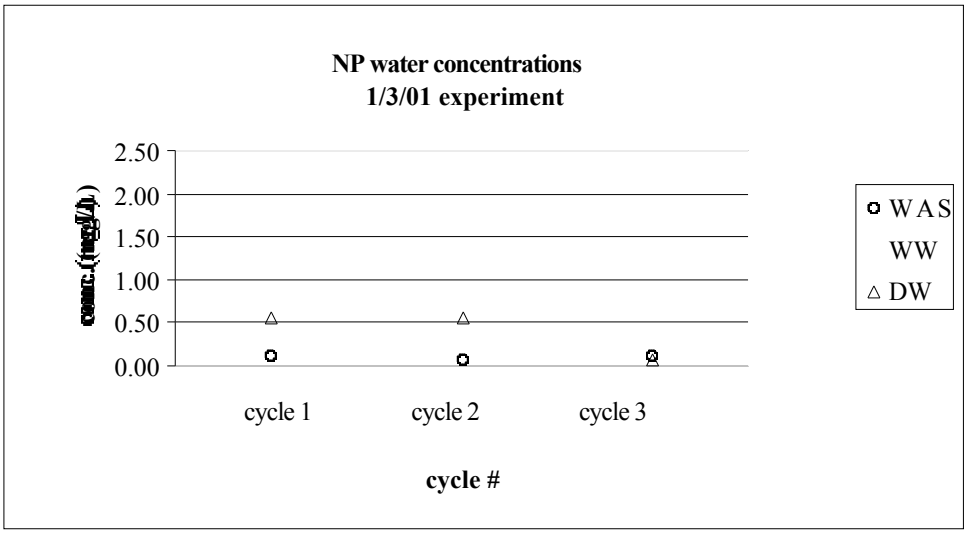


Table 9. Levels of NP in distilled water aerated for 0 min., 120 min. (constantly), or 3 min (one minute per hour). Initial concentration in each case was 4.96 mg/L.

Time of aeration	Concentration of NP in solution	% Loss
no air	mean = 4.83 mg/L (st. dev. = 0.05)	2.6
3 min. air	mean = 2.91 mg/L (st. dev. = 0.10)	41.3
120 min. air	mean = 0.96 mg/L (st. dev. = 0.27)	80.6

Summary and Conclusions:

Nonyphenolic compounds are not adequately degraded during traditional wastewater treatment. Likely mechanisms that affect the environmental fate of NP are volatilization and sorption. Based on field values for NP concentrations at a typical activated sludge plant, as well as results from laboratory scale experiments using real wastewater and RAS, it was found that the most significant mechanisms for NP elimination during wastewater treatment are volatilization and adsorption. Both volatilization into the atmosphere and adsorption to sludge solids have implications regarding human health and increased risk of environmental exposure. NP sorbed to solids may provide a long-term source of NP to the environment while airborne NP has the potential to travel great distances and distribute pollutants worldwide. Additionally NP in the air represents a dangerous exposure pathway for humans. To adequately treat nonylphenolic compounds in the wastewater stream, alternate methods of wastewater treatment need to be explored.

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Monitoring of Pesticide Residues in Surface and Underground Water by Capillary Gas Chromatogaphy using C_{18} Column

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Abstract

Screening of more than 50 samples of under ground (hand Pump) and surface water (Giri river) samples at regular monthly interval for organochlorines and organophosphorous pesticide residue pollutants in Himachal Pradesh were carried out to know the extent of pesticide use pattern in agriculture. All the compounds kept under investigation can be reliably analyzed by using chemelut C_{18} column below the maximum tolerances for drinking water fixed at 100 ng/l (ppt). All the under ground water samples were found polluted with HCH residues in the range of 0.0018-1.848 ppb while surface water to the tune of 91.6% in the range of 0.038-1.265 ppb. DDT residues were found in two samples of surface and four samples of underground water in the range of 0.036-0.084 and 0.028-0.096 ppb, respectively. Malathion residues (0.041-0.820 ppb) were also detected in surface water (January to June) as well as underground water (January to August) samples. Positive results of the screening for malathion residues were re-confirmed by GC-MS in a selected ion monitoring (SIM) mode.

Biographical Sketch

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Fate of the Endogenous Hormones 17ß-Estradiol and Testosterone in Composted Poultry Manure

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Abstract

Relatively little attention has been paid to the fate and environmental impact of the potent hormones, 17ß-estradiol (E) and testosterone (T), eliminated in animal and human waste. Current socio-economic forces have increased the animal numbers per farm operation, but have limited the distance manure or litter can be transported. Hence, considerable amounts of manure are stockpiled, lagooned, or composted before being applied to farmland. Land application of manure has a considerable potential to affect the environment adversely. Transport into surface waters can readily occur after heavy rains. Exposure of livestock, poultry, and wildlife to these compounds during key stages of development may play a role in subsequent developmental and/or reproductive problems.

Composting is known to effectively remediate soils contaminated with toxic organic compounds. The present study was undertaken to quantitatively assess whether 17ß-estradiol and testosterone could be degraded during composting.

Chicken layer manure was obtained from a commercial producer and was mixed as follows: manure (3.3 parts), old hay (2 parts), straw (2 parts), partially decomposed leaves (4 parts), and starter compost (2 parts) to achieve a C:N ratio of 30:1. The mixture was adjusted to ~60% moisture, and composted in windrows (23 x 2 x 1.75 m). Two windrows were constructed with the above mixture, and two more windrows, which additionally contained 1 part Christiana clay were also on-site. All windrows were turned weekly using a compost turner to brings the outer portions of the piles into the self-heated core zone. Composting involved the peak heating and curing phases. Temperature and oxygen in the composting mass were monitored to ensure that thermophilic temperatures were maintained. Three subsamples were removed weekly during the first two weeks and then biweekly until the end of the experiment. Samples were homogenized in a blender with dry ice. An aliquot was removed, extracted with ultra-pure water, and centrifuged. Commercial enzyme immunoassay kits were used to quantitate the levels of 17ß-estradiol and testosterone in the aqueous extracts.

The results demonstrated that aerobic microorganisms could slowly degrade 17ß-estradiol in chicken manure during the active composting process. Standard composting resulted in a slow decline in estradiol levels from 95 ppb to 42 ppb during the first 83 days. Testosterone levels also declined during standard composting, from an initial concentration of 196 ppb to 26 ppb at 83 days. Testosterone degradation by the microorganisms present in standard compost was estimated to be 3 times higher than for estradiol. Neither hormone was degraded to background concentrations by day 83. Degradation of E and T in clay-amended compost did not differ significantly from standard compost. These data suggest that composting may be an environmentally friendly technology suitable for reducing, but not eliminating, the concentrations of these potent endocrine disrupting hormones at concentrated animal operation facilities.

Introduction

Animal wastes contain significant quantities of the endogenous sex hormones 17ß-estradiol (E) and testosterone (T). These wastes are a potentially significant source of sex hormones in the environment because they are directly applied to land. In fact, livestock manure has been shown to be a source of environmental contamination for these hormones (Callantine, 1961; Knight, 1980). Laying chickens excrete high levels of these hormones, 50 and 250 ng/g dry manure/day, respectively (Shemesh and Shore, 1994; Shore, 1988; Shore, 1995). In contrast, the endogenous concentration of E in cattle urine averages 13 ng/L (Erb, 1977). However,

the levels can be even higher, because E is frequently administered as a growth hormone to increase muscle mass and decrease fat deposition (Popp, 1997). Several studies have demonstrated that sex hormones appear in soil, surface water, and groundwater as a result of manure application (Peterson, 2000). Exposure to exogenous endocrine-disrupting chemicals, e.g. estradiol and testosterone, in the environment has been associated with widespread physiological and reproductive disorders in a variety of wildlife (Colburn, 1993) and in humans (i.e., reduced sperm counts, Sharpe & Skakkebaek, 1993; and increased breast cancer, David & Bradlow, 1995). 17_-Estradiol is classified as a carcinogen by the US Dept. of Health and Human Services (NIEHS, 1994) based on its link to breast cancer (Dickson, 1986). Dramatically altered sex ratios were observed in salmon bred in waters that contained 250-5000 ng estradiol/L (Nakamura, 1984). Fish deaths have also been noted when sufficiently high concentrations of E were measured (Kramer, 1998).

Little is known about the transport and fate (degradation) of these hormones in field settings or in hydrological systems. Preliminary studies by individuals involved in this study have shown that estradiol and testosterone are strongly sorbed to soils. The lack of literature on the fate of these hormones is interesting given the fact that they are vastly more potent in interacting with the estrogen and androgen receptors than most man-made environmental contaminants. In vitro studies have reported that estradiol has a 10^4-10^6 fold greater affinity for the estrogen receptor than alkylphenol ethoxylates (Jobling and Sumpter, 1993).

Composting is a self-heating, aerobic process that accelerates the degradation of organic materials by the successive action of a diverse group of microorganisms, mesophiles, thermophiles, bacteria, including actinomycetes and fungi (Finstein and Morris, 1975). Composting is a beneficial waste management option that stabilizes organic by-products, such as manure, reduces its weight, destroys pathogens and weed seeds, and produces a low odor soil conditioner that also has some fertilizer value. Active composting generates considerable heat (>60˚ C) during the 3-4 week peak heating phase of the process, during and after which time the organic matter is converted into gaseous CO_2 and water vapor. It can also provide an inexpensive and straightforward solution to managing hazardous waste or remediation of contaminated soil. For instance, a greater than 99% degradation was achieved when 2,4,6-trinitrotoluene contaminated soil was composted for 160 days (Williams, 1993). Depending on the level of degradation achieved, costs of disposal of remediated soil resulting from composting are far lower than from incineration or landfilling.

The purpose of the present study was to evaluate the fate of the potent endogenous hormones, 17_-estradiol and testosterone, present in high concentrations in layer chicken manure, during a typical composting operation. Results will be used to project the efficacy of composting in decreasing the environmental load of these hormones originating from farms, particularly concentrated animal feeding operations.

Materials and Methods

Composting

Chicken layer manure was obtained from a commercial layer operation in Pennsylvania, and hauled to the Composting Research facility at the Beltsville Agricultural Research Center, Beltsville, MD, where composting was performed. The composting facility handles about 19,000 cu. yd. generated at the 7000 acre farm and research center. The operational surface of the composting facility consists of an outdoor 77,000 ft^2 pad which is 8 inches thick and comprised of coal ash from area power plants, cement kiln dust, quicklime, and the existing Christiana clay subsoil. The composting center is surrounded by an 8 acre orchardgrass field. The site is located in the Anacostia River watershed, which ultimately is received by the Chesapeake Bay. The manure was analyzed for moisture content and carbon:nitrogen ratios (C:N). Water was regularly added to maintain moisture content of approximately 60% by weight. The initial C:N ratio was adjusted to 30 by addition of hay, straw, leaves, and starter compost. The standard compost composition was old hay (2 parts by weight), 3-week-old peak heat starter compost made with no poultry, dairy and swine manure (2 parts), poultry litter (3.3 parts), autumn leaves (4 parts), and straw (2 parts). In the clay-amended compost, an additional 1 part of crushed Christiana clay was added. The amended manure was placed into double windrows which were 160 feet long, 5 feet wide, and 40 inches high. After the first week of composting, during which the windrows were left undisturbed but monitored for temperature, windrows were turned once a week to aerate, and to facilitate mixing and exposure of all parts of the mass to the high temperature core regions of the windrows. After initial

thermophilic conditions (>60° C) were achieved, temperatures in the pile core and ends were monitored weekly for 12 weeks until temperatures reached ambient again.

Sampling

Three subsamples of the litter were obtained from the windrow, and mixed to provide a sample for time=0 measurements. Three subsamples of thermophilic sites within the compost windrows were obtained at the following intervals: 6, 13, 27, 45, 55, 69, and 83 days and placed into plastic bags. The subsamples were all mixed to assure non-preferential sampling. The samples were frozen at -20° C, and transported on ice to the Biosciences Research Laboratory, Fargo, ND. They were immediately frozen at -30° C and stored until analyzed.

Sample preparation

The frozen compost samples were removed from storage, placed into a Waring® blender with dry ice. The contents were blended until homogeneity was achieved. The contents were placed into a plastic bag, and dry ice was allowed to evaporate in a –4˚ C freezer. An aliquot was removed, weighed and dried until a constant weight was obtained (dry weight). This dry weight was used for all calculations and concentration data presented in the Tables or Figures.

Hormone Assays

A 200 mg aliquot of the homogenized sample was placed into a 125 ml Ehrlenmeyer flask with 50 ml of double distilled water. The mixture was shaken at 25˚ C for 2h on a reciprocating water bath. The sample was centrifuged at 1124 x g, and the aqueous layer was decanted and used for all assays. Aqueous extraction was sufficient to remove all extractable hormones at the measured levels, since acetone or methanol extracts of litter or compost did not result in higher levels of extractability of E or T (data not shown).

Estradiol and testosterone enzyme immunoassay (EIA) kits were purchased from Cayman Chemical (Ann Arbor, MI), and were used according to the manufacturer's instructions. EIA analyses were run in triplicate with blanks and standards, and both non-specific and maximum binding were determined. The analyses were repeated twice with two separate kit lots. The estradiol-EIA kit cross-reactivities were 17% for estradiol glucuronide, 4% for estrone, and less than 1% for all other common steroids. Cross reactivities for the testosterone kit were 21% for 5_-dihydro testosterone, 12.4% for 11-keto testosterone, 10% for 5_-dihydroxy testosterone, 3.6% for androstenedione, 1.2% for 11-hydroxy testosterone, and less than 1% for all other steroids. Quantitation was performed using a Wallac Model 1420 96-well plate reader and measuring the amount of 5-thio-2-nitrobenzoic acid released in an enzymatic reaction at 405 nm. Blank EIA analyses for straw, hay, leaves, starter compost, and Christiana clay were not performed.

Results

At the initiation of the standard composting study (day 0), after the poultry litter had been diluted with hay, starter compost, leaves, and straw, the mean E concentration was 95 ppb. A very slight decrease in E was observed over the course of the 83 day study (Table 1). Concentrations of E at day 45 were greater than twice that measured at the beginning of the study. Contamination with estradiol-like activity was suspected and this sampling point was excluded from all calculations (see Discussion). The decrease in E was modeled with a first-order with time expression:

$$dC/dt = kC \qquad (1)$$

where C is the concentration of E (ng/g compost) and t is time (days). The rate expression was integrated and written in linear form (Eq. 2).

$$\ln (C/C_o) = kt \qquad (2)$$

where C is the EIA-measured concentration of estradiol at the sampling times, and C_o is the initial concentration of E. The rate constant *k* for the degradation of E was estimated after creating a plot of the data according to Eq. 2, then determining the slope of the best fit through the data points (Figure 1). During the thermophilic stage of the study, i.e., the first 83 days, the estimated degradation of E was –0.0086/day. The concentration of E did not reach 0 ppb by the conclusion of study period.

Table 1. Average concentrations of 17_-estradiol present in composted poultry manure. Aliqouts of compost were extracted with distilled water and analyzed for estradiol with an estradiol-specific enzyme immunoassay kit (Cayman, Ann Arbor, MI). Reported values are in ng estradiol/g compost (ppb) on a dry weight basis (n=9).

Sampling time (days)	Standard compost (ppb)	Compost with clay (ppb)
0	95 ± 21	116 ± 64
6	72 ± 22	70 ± 15
13	40 ± 20	34 ± 15
27	92 ± 60	51 ± 11
45	218 ± 91	213 ± 70
55	66 ± 19	52 ± 17
69	56 ± 14	80 ± 12
83	42 ± 31	36 ± 17

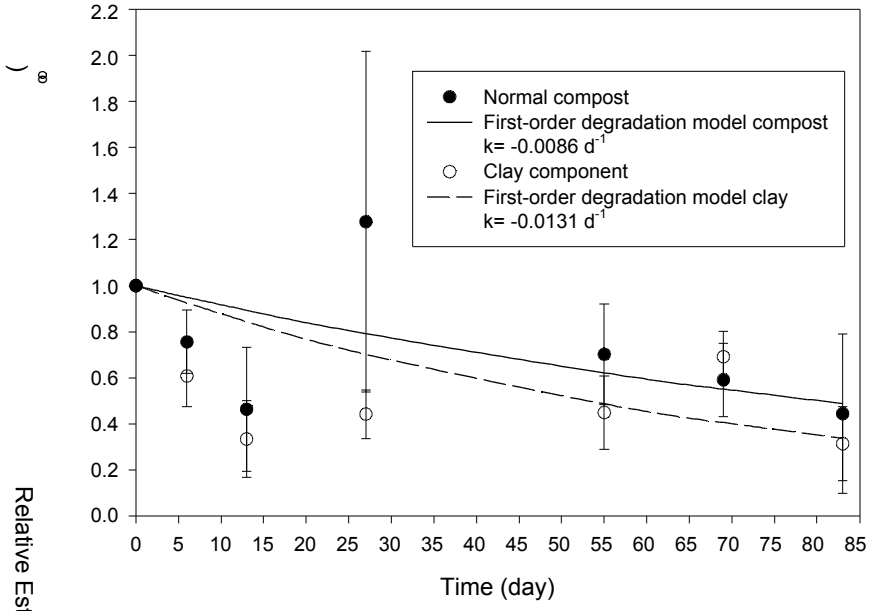

Figure 1. Average relative concentration of 17_-estradiol through time in a poultry manure composting operation with first-order degradation model fit. Error bars are one standard deviation.

At the initiation of the standard composting study, after the poultry litter had been diluted with hay, starter compost, leaves and straw, the average concentration of testosterone in the composting mass was 196 ppb (Table 2). The concentration dropped quickly by day 6, at which time it was 81 ppb, and continued to decrease throughout the study period of 83 days. The concentration of T in the compost never reached 0 ppb during active composting. As with estradiol, the decrease of testosterone was modeled with a first order with time model (Figure 2). During the 83 days of the study, the rate constant *k* for the degradation of T was -0.0291/day.

Table 2. Average concentrations of testosterone present in composted poultry manure. Aliqouts of compost were extracted with distilled water and analyzed for testosterone using a testosterone-specific enzyme immunoassay kit (Cayman, Ann Arbor, MI). Reported values are in ng testosterone/g compost (ppb) on a dry weight basis (n=9).

Sampling time (days)	Standard compost (ppb)	Compost with clay (ppb)
0	196 ± 63	198 ± 61
6	81 ± 13	82 ± 16
13	79 ± 32	104 ± 24
27	49 ± 19	40 ± 5
45	48 ± 5	57 ± 18
55	31 ± 4	35 ± 6
69	26 ± 4	32 ± 6
83	26 ± 2	32 ± 4

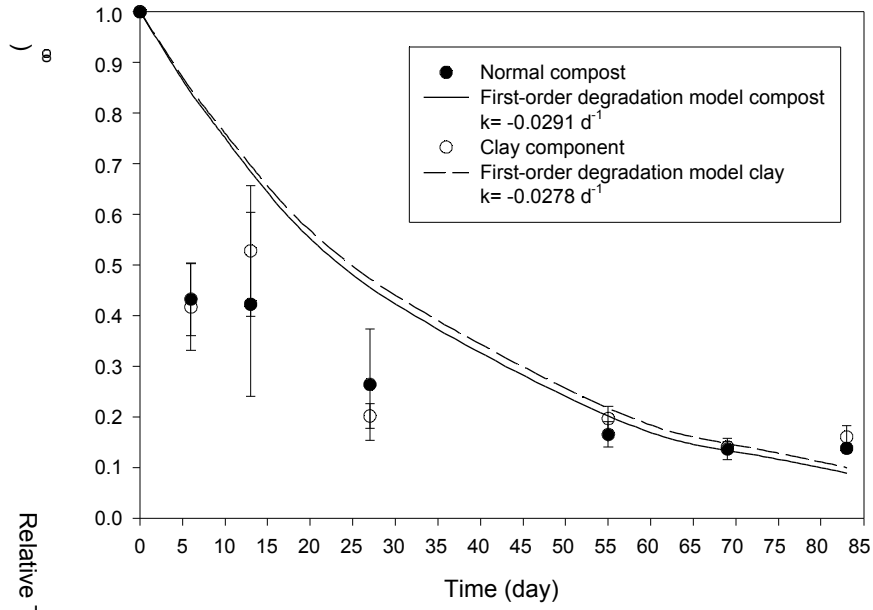

Figure 2. Average relative concentration of testosterone through time in a poultry manure composting operation with first-order degradation model fit. Error bars are one standard deviation.

Christiana clay is commonly added to compost used on strawberry fields to provide a more desirable topsoil texture. The addition of one part Christiana clay to the compost mixture did not substantially affect the degradation rates for E or T during composting. The rate constant *k* for the degradation of E with time was –0.0131/day with clay, and -0.0086/day in the standard compost that contained no clay (Figure 1). The rate constant *k* for the degradation of testosterone was –0.0278/day for compost containing clay, and –0.0291/day for standard compost (Figure 2).

The average temperature of the standard compost pile indicated that thermophilic conditions (>60˚ C) were achieved by day 3, and remained there until at least day 21 (Figure 3). Mesophilic conditions (35-55˚ C) characterized the standard compost windrows during the time between day 41 and 83. By 8 weeks (56 days), the volume of material had been reduced in half, and therefore, windrows were combined. Identical conditions were observed with the Christiana clay-amended windrows (data not shown), and therefore, they were managed in the same manner.

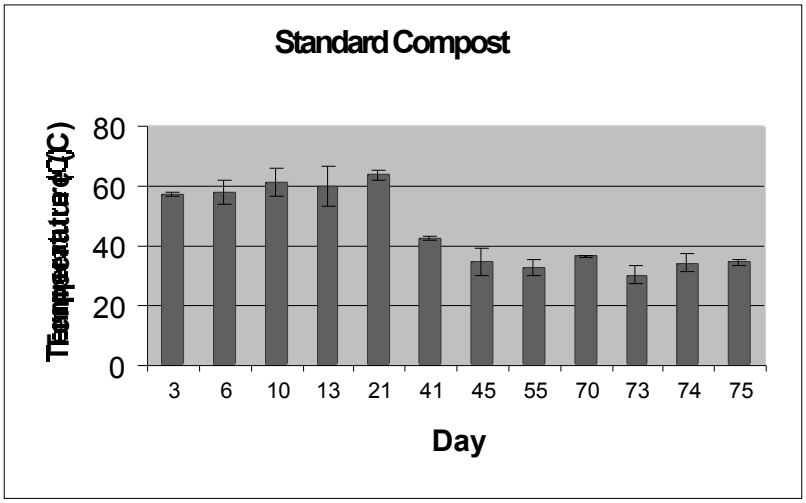

Figure 3. Average temperatures measured in the standard compost pile during the first 75 days of composting. Means represent three temperature measurements taken from two pile core regions and one pile end region.

A commercial soil amendment processed from agricultural waste was also analyzed for residual E and T. The material was available in both swine- and cattle manure-derived formulations. The cattle manure-derived product contained E and T at 18 and 4.5 ng/g, respectively (Table 3). The concentrations of the two hormones in the swine manure-derived product were 144 and 52.5 ng/g, respectively.

Table 3. Average concentration of 17_-estradiol and testosterone in a commercial soil fertilizer/amendment derived from either cattle manure or swine manure. An aliquot of the commercial product was extracted with distilled water and analyzed for the hormones with an enzyme immunoassay kit specific for the hormone. Results are expressed in ng hormone/g compost (ppb) on a dry weight basis.

Source of commercial product	Estradiol (ppb)	Testosterone (ppb)
Cattle	18	4.5
Swine	144	52.5

Discussion

The results of this study demonstrated that the microorganisms present in standard compost produced from poultry manure degraded the hormones estradiol and testosterone. Standard compost concentrations for E and T that started at and average of 95 and 196 ng/g dry weight (ppb), respectively, and were reduced to 42 and 26 ppb at day 83. This represents approximately a 56% and 87% reduction, respectively, in the levels of these potent hormones during active composting. Aerobic microbial degradation of E and T in poultry manure and starter compost, however, was unable to completely degrade the hormones during the 83 days of thermophilic and mesophilic composting. The impact of these results is that hormone levels can be reduced during composting, but apparently not eliminated.

Estradiol was clearly metabolized by the compost microorganisms at a much slower rate than testosterone. The rate of degradation of E in poultry litter compost by 83 days was about one-third that observed with T. The difference was probably due to resistance in metabolizing the aromatic A-ring of E. The relative rate of degradation of T has been previously compared to E. The removal and mineralization of E and T from the aqueous phase of a municipal wastewater treatment plant (WTP) was measured, and it was reported that T was degraded approximately 2x faster than E (Layton, 2000). Adaptation of the bacteria to the media was concluded to be an important factor in mineralization of E and T. Municipal plant microbial populations mineralized E by 84% over 72h, while bacteria from industrial biosolids only degraded 4% of the E under identical conditions. Testosterone was not as affected by bacterial adaptation, in that only slight differences were noted for degradation of T when comparing the two systems, i.e. 65% vs. 55%, respectively. Differences in the degradation of these hormones can also arise from different microbe populations present in differing treatment processes. A Brazilian WTP reported >99% degradation of E in its wastestream, while only 64% of E was removed in a German WTP (Ternes, 1999). Microbe populations may also differ in poultry compost obtained from different regions of the country due to antibiotic administration or other management practices. Additional compost degradation studies should be performed to assess this potential difference.

The measured hormone concentrations showed a considerable degree of variability, particularly in E, which were reflected in relatively high standard deviations in the raw immunoassay kit (Tables 1 and 2), and low correlation coefficients for the degradation rate constants (Figures 1 and 2). Compost extracts may have contained compounds that cross-reacted in the immunoassay. According to the manufacturer, only estrone was reported to be cross-reactive with the estradiol kits. Many factors can influence the composting process itself, and may also have contributed to the high standard deviations observed in the results. Fully aerated conditions must be maintained continuously to avoid the onset of anaerobic conditions, which will lead to degradation of organic materials by a different, slower pathway (NRAES, 1992). Moisture content must also be maintained between 40-65% to maintain thermophilic degradation. However, water in each windrow was being constantly driven off by the high temperature, self-heating conditions of composting. In addition, carbon:nitrogen ratios of 25:1 to 40:1 must be maintained to provide an adequate feed source for the microorganisms. Porosity and particle size of the composting material can greatly affect the degradation of organic materials, by limiting air movement and providing too small a surface area for decomposition.

At day 45, the measured E levels were much higher than at the initiation of the study. A repeat of the analyses demonstrated that the measured values were accurate. The testosterone concentrations at day 45 were not affected in the same manner (Table 2). Two possible explanations exist for the elevated measurements at day 45. The first may relate to sampling. It was possible that the subsamples removed on day 45 were from a region or "pocket" of the windrow where T degradation was unhindered, but where E degradation was limited. Another explanation may be that the sample was somehow contaminated with estradiol or estradiol-like activity. This explanation was preferred, because all samples obtained obtained from different windrows and shipped on day 45 from the composting site displayed extremely high E concentrations, despite being at different stages of the composting process (data not shown). Due to the suspected contamination, the day 45 measurements were removed when deriving degradation rate constants for E and T, and a repeat of the study is being planned.

The apparent resistance of E and T to undergo complete biological degradation via composting was in agreement with EIA analyses performed on extracts obtained from a commercial soil amendment product made by processing animal waste. The commercial product was produced from cattle or swine manure by a proprietary process that involved biological treatment in three stages, de-watering, and pelletization to produce a material used as a fertilizer or soil amendment. Aqueous extracts of the swine manure-derived product contained 144 and 53 ppb, E and T, respectively (Table 3). These concentrations were lower for the cattle manure-derived product. These data suggest that the introduction of these hormones into the environment occurs from multiple sources, and that geographical surveys should be performed to evaluate the levels of these hormones in various settings.

Very little data has been produced discussing the fate of endogenous hormones following field application of manure, or their fate in a hydrological system. A previous report indicated that soil bacteria are incapable of degrading estradiol (Zondek, 1943). The present study indicates that the microbes present in a common composting operation could slowly metabolize E, while T was more rapidly degraded. Estradiol is mobile in a

field setting, and was measured in runoff from a simulated rainfall event (Nichols, 1997). Groundwater levels of E have been clearly shown to have their source in animal waste, because concentration trends for fecal coliforms and E in a mantled karst system were the same (Peterson, 2000). Much of the E runoff potential into surface waters was reduced when fields were surrounded with grass filter strips (Nichols, 1998).

The degradation products of estradiol and testosterone were not determined in the present study. Despite the observed degradation of E and T via composting, a critical issue becomes whether the lack of full degradation, and the possible formation of nonextractable metabolites is a satisfactory endpoint for these hormones. In some previous studies, it was demonstrated that metabolism of organic substances to hydroxylated metabolites was followed by covalent coupling to humic and fulvic acids in soil (Richnow, 1994; Calderbank, 1989). This led to long-term immobilization, but not the complete destruction of these compounds. Residual E and T in compost applied to fields are water soluble at the ppb level, and therefore, would be mobile during rainfall events. Therefore, the possibility exists that even after composting, these lowered levels of E and T may enter the environment with potentially detrimental effects to both plants and animals. Estradiol at concentrations of 0.0054-0.54 ppb in soil increased alfalfa growth, while 54.4-5,440 ppb decreased alfalfa growth (Shore, 1982). Brown trout gonad development and feeding were halted at 50-300 ppb E in water (Ashby, 1957). Estradiol levels of 10-200 ppb in water resulted in Masu salmon death, while 0.25-5 ppb resulted in 84-100% feminization (Nakamura, 1984). E in poultry litter fed to heifers caused premature udder development (Shore, 1988).

To summarize, this study demonstrated that the endogenous hormones, 17_-estradiol and testosterone, excreted in poultry manure, were degraded to differing degrees by a typical composting operation. Testosterone was degraded approximately 3X as rapidly as estradiol by composting for 83 days. Neither hormone was degraded completely by the end of an 83 day thermophilic and mesophilic period of composting. Addition of Christiana clay, a topsoil texturing agent, to compost mixture did not affect the rate of degradation of the hormones. Composting may provide an effective means of reducing, but not eliminating, the introduction of these potent hormones into the environment from poultry manure.

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Biographical Sketch

Heldur Hakk has been with the USDA/ARS/Biosciences Research Laboratory for 22 years, and is a Research Chemist in the Animal Metabolism-Agricultural Chemicals Research Unit. His areas of research include: mammalian metabolism of dioxins, PCBs, and flame retardants, protein transport of chemicals in mammals, effect of exposure to xenobiotics on circulating hormones, applications of NMR to assess impact of foreign compounds to central metabolic pathways, and the fate of native hormones in the environment.

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Sorption, Mobility and Fate of 17ß-Estradiol and Testosterone in Loam Soil and Sand

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Abstract

In this study, transport and fate experiments of [^{14}C] testosterone (T) or [^{14}C] 17ß-estradiol (E) were conducted by individually applying T and E to columns packed with loam soil (Glyndon silt loam) or sand. The T and E were applied at concentrations that are found in poultry litter that is applied in the field.[1] The columns were eluted with 0.01M $CaCl_2$. No radioactivity from either T or E was found in the effluent of the loam soil column, even after 10 pore volumes were displaced. Combustion analysis of the soil sections showed that 80% and 96% of the ^{14}C from T and E, respectively, remained in the top five centimeters of soil with most of the ^{14}C from T and E sorbed in the top centimeter (41% and 49%, respectively). A nonequilibrium sorption convective-dispersivetransport model was applied to the concentration profiles of the soil columns provided estimates of K_d values of 182 and 1661 cm^3g^{-1} for T and E, respectively. Toluene extracts of the top three cm of soil from the E column, there appeared to contain at least two metabolites of E by thin-layer chromatography (TLC). The toluene, ethyl acetate and methanol extracts from the first 3 centimeters of the T soil sections each showed a predominate metabolite (11.1%-32.3% of the ^{14}C) in of the soil column, parent T (4.6-17.8%) and a minor metabolite (1% of the ^{14}C in the second centimeter). Further, T and E remained strongly sorbed to the soil, because at least 50% of the ^{14}C from both T and E remained and was non-extractable from the soil after sequential extraction with toluene, ethyl acetate and methanol. There does not appear to be significant mobility of T or E in the loam soil because there was no detectable ^{14}C in column effluent, or significant ^{14}C in the lower soil levels of either of the columns. Most of the ^{14}C from the T and E (87% and 85%, respectively) was eluted from the sand columns after 1.08 and 1.06 pore volumes were eluted, respectively. From TLC data, this ^{14}C appeared to be intact T eluting from the T column; however, a metabolite of E appeared to elute from the E sand column. In each case, the sand column was divided top to bottom into four sections and little ^{14}C remained in the sand. Batch studies using the Glyndon silt-loam were conducted at 48, 96, and 178 h incubation times and Freudlich isotherms were fit to the observed data. For both T and E, the sorption isotherms were similar for all three incubation times and indicated that equilibration was reached by 48 h. Sorption isotherms for T and E were both linear, which suggests sorption was a first-order process. The sorption coefficients (K_d) for the T (348 cm^3 g^{-1}) and E (4388 cm^3 g^{-1}) were high and indicated that these chemicals readily sorbed to the soil. The column K_d values were smaller in the column studies than for the batch experiments but within the same order of magnitude. Both column and batch experiment K_d values for E were an order of magnitude larger than previous published values from river sediment, which have lower organic matter content.

Introduction

Within the last 50 years concern has increased about the prevalence of endocrine-disrupting chemicals in the environment. In the environment endocrine-disrupting chemicals may interfere with the endocrine system of humans, and small amounts can contaminate large amounts of water because they often function at the part per billion level.[2] The presence of xenoestrogens in the environment has become a concern in numerous countries, as expressed by international organizations (e.g. World Health Organization, United Nations), and in Congressional Bills. Recently, a bill (H.R. 1712) was submitted to the 106th Congress that proposed to amend the Federal Water Pollution Act to authorize an estrogenic substance-screening program. Past research has centered greater attention on endocrine-disrupting chemicals emitted into rivers and estuaries by sewage treatment plants,[3-7] but less attention has been given to animal wastes as a direct source of these chemicals.[1,8-10] Animal wastes can potentially be a more significant problem because they are applied untreated directly to the land surface.

It is estimated that the Eastern Shore of Maryland produces 200,000 metric tons yr^{-1} of manure from broiler chickens alone, containing 30 ng g^{-1} (30 ppb) of the E steroid. This amounts to about 6 kg/yr of E being applied to fields when the manure is used as a fertilizer. In 1997 the U. S. poultry industry produced over ten billion kg of broiler litter, 90%

of which was applied to pastures and hayfields. Little is known about how these steroids are degraded. Work conducted over 50 years ago observed no metabolism of E and T by soil or fecal bacteria.[11,12] On the contrary, Layton et al.[6] demonstrated that biosolids in wastewater treatment systems can mineralize E and T.

A study on fields where chicken manure had been applied at levels of 28 ng g^{-1} and 34 ng g^{-1} of manure showed significant levels (upper limit of 5 ng L^{-1} of E or T) in a nearby, free flowing stream.[13] It was reported that E reaches the streams by surface runoff while T reaches streams by both groundwater and surface water routes. In another study of steroid run-off, E and T from broiler litter containing 65 ng g^{-1} dry weight of E and 133 ng g^{-1} dry weight of T was applied to 0.8-ha pasture or hayfield plots. Background levels of E and T in surface runoff from these plots were determined to be 40-50 ng L^{-1} and 14-19 ng L^{-1}, respectively. When 7200 kg of broiler litter was applied to these plots, E and T levels increased from background to 150-2300 ng L^{-1} and 63-1000 ng L^{-1}, respectively, in runoff occurring one day after application. In runoff measured 12 days after application, E and T dropped to 46-100 ng L^{-1} and 42-120 ng L^{-1}, respectively, and by 200 days, levels of E and T had dropped to background.[1] Tall fescue grass filter strips (6.1 m wide), used to filter run-off from a poultry litter application, reduced the transport of E in run-off by 79%. When an 18.3 meter filter strip was used, the transport of E and T in runoff was reduced by 98%.[14]

There has been limited research reported on the fate and transport of endocrine disrupting chemicals in soils; however, some research has been done on the sorption of estrogen chemicals to river sediments.[15,16] Estrogenic chemicals are hydrophobic organic compounds that have a high octanol-water partitioning coefficient (K_{ow}), which makes them likely to sorb to soil particles from the dissolved phase. Jurgens et al.[15] used models based on E K_{ow} and some experimental data to show that these chemicals will partition to river sediments. Furthermore, Lai et al.[16] showed there was kinetic partitioning of various steroid estrogens and that these estrogen compounds competed with each other for sorption locations. They also concluded that sorption depends on the total amount of organic matter and to some extent particle size. The main goal of this study was to identify the fate and transport of T and E present in animal wastes applied in the field to loam soil and sand through the use of laboratory batch and disturbed soil column experiments.

Materials and Methods

Sorption studies on T and E were conducted using a batch equilibration technique with a Galesburg loam (coarse silty, mixed, superactive, frigid, aeric calciaquoll) obtained from Galesburg, ND, USA. Soil to water (0.0l M $CaCl_2$) ratio used was 1.6g to 8ml, respectively. [^{14}C]- Spiked T or E was added to separate vials so that the solution concentrations were 0.725, 0.0725, and 0.00725_g/ml or 0.15. 0.015, and 0.0015 _g/ml, respectively. Each of the concentrations was run in triplicate. The slurries were agitated by rotation of the vial top to bottom (360°/5 sec). At 48, 96 and 168h, the bottles were centrifuged at 1,700 rpm, triplicate 100_l samples removed, and radioactivity analyzed by liquid scintillation counting (LSC).

Two column miscible-displacement experiments were conducted using the Glyndon silt loam which was dried at 85° C for 24h, and 1126g was evenly packed in a glass column (8.4cm x 15.2cm; 842ml) with a stainless steel bottom end cap. Sandwiched between the soil and the end cap were a 40 mesh stainless steel screen and six layers of cheesecloth. The soil was slowly wetted with 0.01M $CaCl_2$ solution from the bottom over a 24h period. This was done to maintain soil structure and reduce the amount of entrapped air. The pore volume, the space inside the packed soil column occupied by liquid was calculated to be 417ml for both columns. After saturation, steady-state pore water velocities were achieved with T (3.7mm min^{-1}) and E (3.4mm min^{-1}) column experiments with 0.01M $CaCl_2$, from the top of the column. Once the steady-state flow velocity was achieved, a pulse of [^{14}C] T (0.91_Ci, 1.2 mg) or [^{14}C] E (0.65 _Ci, 0.2 mg) was applied to the surface of separate soil columns in volumes of 40 ml, and eluted with at least 4.4 L of 0.01M $CaCl_2$ (approximately 12 and 10.6 pore volumes). The steady-state pore water velocities did not change during the elution of either column experiment. The column effluent was fractionated and each fraction (ca. 20ml) analyzed for ^{14}C. The soil columns were extruded from the columns and cut into one cm sections. During the extrusion process the soil columns were found to be compressed approximately one centimeter. The sections were dried and assayed for ^{14}C by combustion analysis on a Packard Model 307 Oxidizer. The top three sections from each T and E soil column were individually extracted sequentially with toluene, ethyl acetate, and methanol in an Accelerated Solvent Extractor (Model 200; ASE; Dionex, Sunnyvale, CA, USA). Thin-layer chromatography (TLC) analysis on these fractions was conducted using silica gel plates (250_m; Whatman Lab. Div., Clinton, NJ, USA) with the following solvent system: tetrahydrofuran:ethyl acetate:hexane (12.5:12.5:25).

As a comparison, standard, washed laboratory sand (Catalog No. 3382-5, J. T. Baker Chem. Co., Phillipsburg, NJ, USA) was packed in the same size column as the soil, and the pore volume was calculated to be 303ml, assuming

saturation. Again, the sand was saturated with a solution of 268mL of 0.01M $CaCl_2$ from the bottom over a 24h period, to maintain structure and reduce the amount of entrapped air. A steady-state flow velocity of 4.8mm min^{-1} was then established with 0.01M $CaCl_2$ from the top of the column. After steady-state flow velocity was achieved, a pulse of [^{14}C] T (0.86_Ci, 1.2 mg) or [^{14}C] E (0.96 _Ci, 0.2mg) was applied to the surface of separate sand columns in a volume of 40ml and eluted with 3.7 L and 3.1L, respectively, of 0.01M $CaCl_2$ (approximately 12.2 and 10.2 pore volumes), and the steady-state flow velocity did not change. The column effluent was fractionated and each fraction (ca. 20ml) analyzed for ^{14}C. The sand column was then eluted with methanol, and extruded in four sections and analyzed for ^{14}C by LSC and TLC analysis conducted as described above.

Model Description

A Freundlich sorption isotherm was used to describe the equilibrium batch experiments:

$$s = K_d C^n \qquad [1]$$

where s is the concentration of T or E sorbed to the soil (mg g^{-1}), K_d is the adsorption coefficient (cm^{-3} g), C is the dissolved concentration (mg L^{-1}), and n is an empirical constant controlling the shape of isotherm function (n=1 is linear). A least-squares approximation method was used to obtain the best-fit of Eq. [2] to the observed data and the coefficient of determination (r^2) was calculated to measure the goodness of the fit.[17] The parameter K_d and n were optimized to achieve the best-fit description of the observed data.

The computer model HYDRUS-1D[18] was used to describe the transport of T and E through the soil. No chemical was present in the effluent of the columns; however, there was significant redistribution of both T and E through the column profile. HYDRUS-1D was used inversely, fitting the model solution to the observed data, to estimate the fate and transport parameters. The following one-site chemical nonequilibrium convective-dispersive model was used to describe the concentration profiles:

$$\frac{f\ C}{f\ t} = D\frac{f^2\ C}{f\ x^2} - v\frac{f\ C}{f\ x} - \frac{\alpha\rho_b}{\theta}\left[K_d C - s\right] \qquad [2]$$

$$\frac{f\ C}{f\ t} = \alpha\left[K_d\ C - s\right] \qquad [3]$$

where C is the solute concentration (mg L^{-1}), t is time (min), D is the dispersion coefficient (cm^2 min^{-1}), x is depth in the column (cm), v is the pore water velocity (cm min^{-1}), α is the fist-order mass transfer coefficient (min^{-1}), ρ_b is the bulk density (g cm^{-3}), θ is the volumetric water content (cm^3 cm^{-3}), K_d is the adsorption coefficient (cm^{-3} g), and s is the solid phase concentration sorbed to soil (mg g^{-1}). Equation [1] describes the convective-dispersive transport, and Eq. [3] describes the first-order exchange of solute between the dissolved and sorbed phases. This model is solved numerically by HYDRUS-1D and is applied inversely using a least-squares optimization routine. Transport parameters are iteratively changed and optimized until the best-fit model solution to the observed data is achieved. The r^2 of the model versus the observed data is reported by HYDRUS-1D to assess the goodness of fit, where the fit of the to the data improves as r^2 values approach unity.

Results and Discussion

The batch (Fig. 1) experiments indicated that both T and E readily partitioned out of the dissolved phase into the sorbed phase. The K_d value for the T and E was 348 and 4388 cm^3 g^{-1}, respectively. The n values for the T and E were 0.94 and 0.98, respectively, which indicates that the sorption process was first-order because the isotherm was approximately linear (i.e., $n \approx 1$). The K_d value for E was two orders of magnitude greater than what Lai et al. [16] reported, who investigated sorption of estrogen compounds to river sediment. The higher K_d values from this study may have resulted from using a mollic soil epipedon, which has high organic matter (_0.6% organic carbon content). Lai et al.[16] noted in their study that sorption depends on the total amount of organic matter.

There was no detection of radioactivity in the effluent of either the T and E miscible-displacementcolumn experiments, which indicated that no radiolabel chemical broke through after 10 pore volumes. However, when the columns were extruded, it was found that both T and E were significantly redistributed through the soil column profile (Fig. 2). After the combustion analysis for ^{14}C, it was found that 99.6% and 84.2% of the applied T and E were retained in the soil column. The ^{14}C from E in the first three centimeters of the soil column was extracted using the ASE extractor, only the toluene extract of each section contained ^{14}C (16.6%-26.1%), while 83.4%-73.9% of the ^{14}C from E remained

sorbed to the extracted soil. This further indicated that E was very strongly sorbed to the loam soil. In contrast, ^{14}C from T in the first three centimeters of the soil column was extracted in all three solvents [toluene (0-16.0%), ethyl acetate (20-34%) and methanol (9.1-23.8%)], and generally less ^{14}C remained nonextractable than with E (26.9-70.9%, mean = 42.5%).

In contrast to the to the silt-loam column experiments, it was found that T and E were readily transported through the sand column. Within the first two pore volumes of elution, 87% and 85% of T and E came out in the effluent. This indicated that these chemicals do not readily sorb to the mineral sand size fraction. This result may also indicate that the smaller particle sizes or the higher organic matter content of the silt-loam greatly influence the sorption and transport of T and E.

The ^{14}C from E toluene extract was analyzed by TLC, and two E metabolites were observed (R*f*s= 0.43 and 0.56, authentic E, R*f*= 0). The toluene, ethyl acetate and methanol extracts from the T soil sections each showed a predominate metabolite at R*f*= 0.58, representing 32.3%, 22.3% and 11.1% of the radioactivity in the first, second and third centimeters of soil from the top of the column, respectively. Parent T was detected in these sections also [R*f*=0.64 (4.6-17.8% of the ^{14}C in the sections)]. A minor metabolite of T (1% of the ^{14}C in the second centimeter section) was detected at R*f*=0.93. The ^{14}C eluting from the T and E sand column was analyzed by TLC and ^{14}C from the T column appeared to be intact T (R*f*=0.44; authentic T, R*f*=0.46); however, a metabolite of E appeared to elute from the E column (R*f*=0.46, authentic E R*f*= 0). The identification of T and E metabolites has not yet been determined.

Figure 1 also shows the fit of the transport model (Eq. [2] and [3]) to the T and E total resident concentrations. The inverse application of this model provided best fit K_d values for E of 1661 $cm^3\ g^{-1}$ and 182 $cm^3\ g^{-1}$ for T. In general the K_d values for both T and E were lower for the column experiments than for the batch experiments on the same soil. This may be caused by a mass transfer limitation in the column experiments such as decreased lateral diffusion from convective transport. Nonetheless, the column K_d values fell within the same order of magnitude as the batch K_d values, and both values were higher than those previously reported by Lai et at.[16] Again the higher K_d values may have resulted from using higher organic matter content soil. The K_d value for T was lower than the E value, which was consistent with what Shore et al.[8] suggested. Shore et al.[8] suggested that the phenolic group of E causes it to bind more firmly to soil, which makes it less mobile than T.

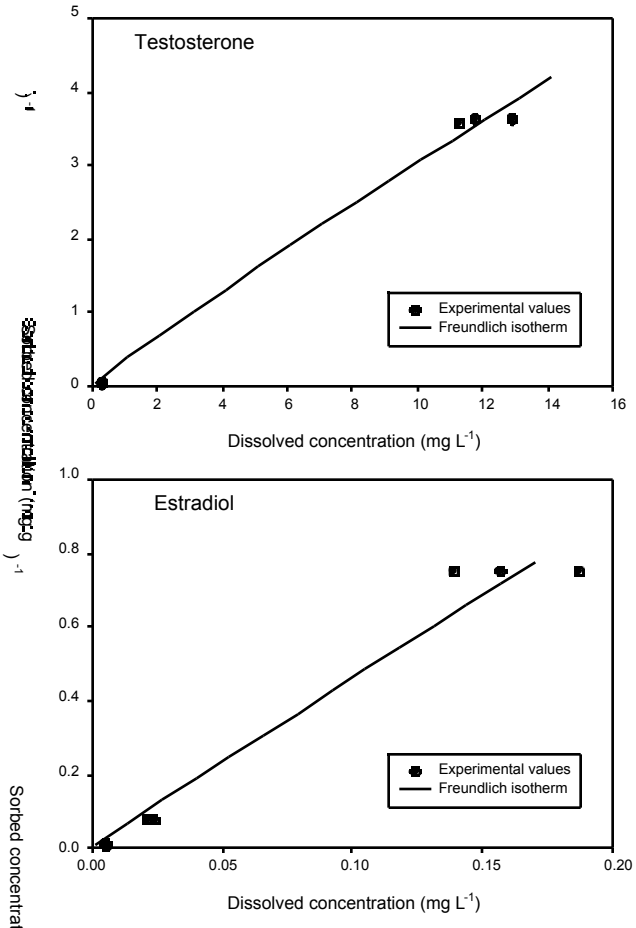

Fig. 1 Batch equilibrium sorption studies of testosterone and estradiol on Glyndon silt-loam. The measured data was fit with a Freundlich isotherm.

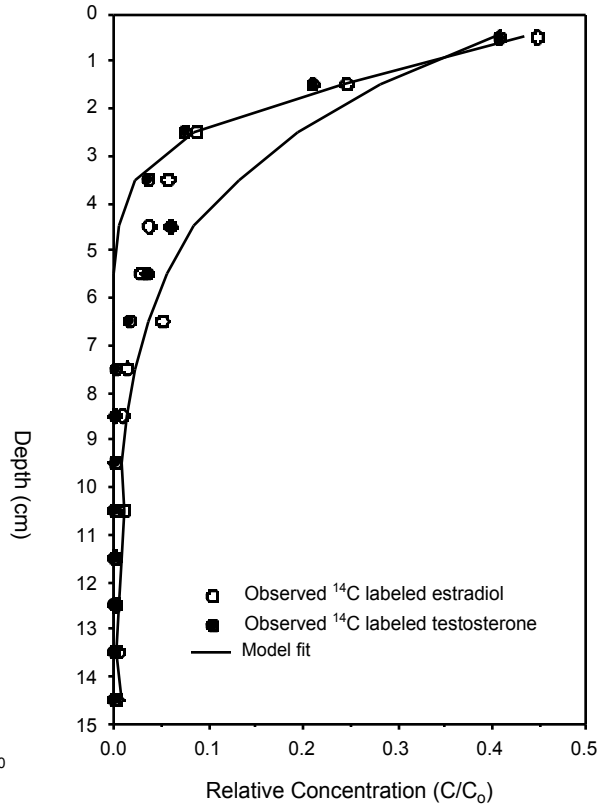

Fig. 2 Distribution of testosterone and estradiol in repacked Glyndon silt-loam columns with nonequilibrium sorption conventive-dispersive model fit.

Although it was found that T and E were strongly held to soil, they appear in groundwater.[8,10,14] Numerous studies have clearly shown that water and chemicals can move through soil along preferred pathways,[19-22] macropores, cracks, root channels, worm holes, funnel flow, and instability of wetting front. These preferential flow pathways can result in rapid movement of chemicals to the water table and may be a leading cause of water contamination by agricultural pollutants. When screening models ignore preferential flow pathways they do not accurately predict observed spatial patterns of groundwater contamination (USEPA, 1992). Preferential flow is especially important for those chemicals that are toxic in parts per billion or trillion concentration,[2] such as T and E. Consequently, we hypothesize that T and E can be leached to significant depths and eventually to groundwater in structured soils where preferential flow can occur. Significant leaching of highly sorbed chemicals, such as phosphorous, can occur, bypassing much of the soil matrix and entering the groundwater. Gachter et al.[23] reported that soil macropores and artificial drainage systems are the most important pathways for the vertical and lateral transport of soluble reactive phosphorus (SRP) from the soil into surface waters. To our knowledge, no study to date has addressed transport of steroid compounds in soil especially by preferential flow.

Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others than may also be suitable.

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Biographical Sketch

Dr. Gerald Larsen has been with the USDA for 30 years and is the Research Leader of the Animal Metabolism-Agricultural Chemicals Research Unit. His research has focused on the elucidation of metabolic pathways, building, transport and disposition of chemicals in animals with regard to food safety. Along with his colleagues, he has shown that a source of dioxins in U.S. beef result from animal's exposure to technical pentachlorophenol (i.e. treated wood). Most recently, his research has involved the metabolism and disposition of brominated flame-retardants and fate of estrogenic chemicals in the environment.

Sorption, Mobility, and Fate of Tetrabromobisphenol A (TBBPA) in Loam Soil and Sand

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Abstract

In this study [^{14}C] TBBPA was applied to the soil and sand columns at the upper limit found in contaminated Swedish sediment (500ppb). No radioactivity was eluted from a loam soil even after 11 pore volumes were displaced. It was found that 71% of all applied ^{14}C from TBBPA was retained in the soil column after combustion analysis and that 16.2 % of the ^{14}C remained in the top centimeter of soil. Six to seven percent of the ^{14}C was recovered in each of the next four 1cm sections below top. Generally, radioactivity in the remaining sections of the column appeared to taper off from four to two percent, except for a slight raise in activity (five percent) three to five centimeters from the bottom. The concentration profile of ^{14}C labeled TBBPA was inversely modeled with an analytical solution to an equilibrium sorption convective-dispersive model. The inverse analysis provided an estimate of the adsorption coefficient (K_d) of 207 cm^3 g^{-1}, which indicates high sorption. Only the toluene extracts of selected sections contained ^{14}C and was characterized as TBBPA by TLC. Although it appeared that TBBPA was strongly sorbed to the soil, because there was no radioactivity in the column effluent, there was significant redistribution of the TBBPA to a depth of 15 cm. A small amount of ^{14}C (4.5 %) was found in the effluent of the sand column in the fractions from between 0.86 to 1.4 pore volumes (260 to 430 ml). After 14 pore volumes had been displaced the sand column was eluted with methanol, which removed 73 percent of the ^{14}C as TBBPA. This indicates that the TBBPA may also be adsorbed to the mineral fraction of the soil as well as the organic. Little ^{14}C was detected in sand after it had been eluted with methanol (2.5% total). Batch studies at 48h showed that 97.9, 92.6, and 93.6% of the 0.025, 0.25, 2.5_g/ml [^{14} C] TBBPA, respectively, were bound to the soil. Sorption of [^{14}C] TBBPA to the soil at the later time points (i.e. 96h and 178h) were similar. Batch experiments were fit with Freundlich isotherms and it was found that the isotherms were linear, indicating first-order sorption. The independently estimated K_d value from the batch experiment was higher than the column experiment but within the same order of magnitude, 657 cm^3 g^{-1}. These results show that TBBPA is extensively sorbed to loam soil and sand. Environmental levels of TBBPA were shown to have no significant biological activity in the Frog Embryo Tetrogensis Assay: *Xenopus* bioassay.

Introduction

Tetrabromobisphenol A (TBBPA) is the most abundant brominated flame retardant (BFR) currently in use. Over 150,000 tons are produced annually.[1] Tetrabromobisphenol A can serve as a reactive or additive flame retardant. About 90% of TBBPA use is for the production of reactive flame-retarded polymeric epoxy and polycarbonate resins, where they are generally used in printed circuit boards.[2] The monomeric nature of TBBPA is lost on polymerization. The remainder of TBBPA is used as an additive flame retardant in acrylonitrile-butadiene-styrene (ABS) resins and in high-impact polystyrene, which are used in automotive parts, appliances, packaging, and consumer disposable products. TBBPA has an octanol-water partition coefficient (K_{ow}) of 4.5-5.3, and is a weak acid with two phenolic hydroxyls (pK_{a1} = 7.5 arid pK_{a2} = 8.5).

It was originally thought that TBBPA was poorly absorbed in rats because 95% of a single oral dose was excreted in the feces as unchanged parent at 72h.[2] However, Hakk *etal.*[3] have shown through the use of bile-duct cannulated rats that TBBPA is readily absorbed by rats. Once absorbed the TBBPA was shown to be metabolized to the glucuronic acid and sulfate ester conjugates in the liver and extensively excreted into the gastrointestinal (GI) tract via the bile. Subsequent deconjugation in the GI tract resulted in excretion of 92% of the dose in the feces as unchanged TBBPA.[3]

Little is known about the biological activity of TBBPA. However, TBBPAs' nonbrominated parent - bisphenol A (BPA) is a ubiquitous compound employed in the production of epoxy resins used to line food and beverage cans and as a building block for polycarbonate plastics. BPA has been demonstrated to exhibit estrogenic activity though it is not definite whether it poses an environmental concern.[4] Little is known about the biological activity of TBBPA which is employed as a flame retardant.

A commonly employed bioassay for the study of hormonally active agents is the Frog Embryo Teratogenesis Assay: *Xenopus* (FETAX). The FETAX bioassay examines the effects of aqueous agents on *Xenopus* embryo development during the first 96 hours of development. The endpoints examined including mortality, malformation rate, and growth inhibition / acceleration as indicated by a change in embryo length and the presence of features indicative of earlier / later stages.[5-8]

Research has indicated that sodium and potassium content influence *Xenopus* embryo development and that the mineral content of the media may influence the susceptibility of frogs to biologically active compounds.[9,10] This has raised questions with regard to the conditions under which bioassays should be performed to screen environmental samples for the presence of hormonally active agents. As a result, the ability of BPA and TBBPA to effect *Xenopus* development was examined using the standard mineral media and a media (S5511) in which the sodium and potassium concentrations are barely sufficient to prevent developmental retardation.

One objective of this study was to determine the fate and transport of TBBPA in soil at levels that are found in the environment. To do this, soil sorption and transport experiments were done using ^{14}C labeled TBBPA. Concentrations of TBBPA found in the upper limit in contaminated Swedish sediment (500ppb)[11] were used in miscible-displacement column experiments and batch experiments to identify fate and transport parameters in the soil and to identify the soil interactions. Another objective was to determine if environmental levels of TBBPA were biologically/hormonally active in the Frog Embryo Tetrogensis Assay: *Xenopus* bioassay.

Materials and Methods

A Glyndon silt loam (coarse silty, mixed, superactive, frigid, aeric calciaquoll) obtained from Galesburg, ND, USA was dried at 85° C for 24h, and 1126g was evenly packed in a glass column (diameter = 8.4cm, length =15.2cm) with a stainless steel end cap at the bottom of the column. Sandwiched between the soil and the end cap were a 40 mesh stainless steel screen and six layers of cheesecloth. The packed soil column was then slowly wetted from the bottom with 0.01M $CaCl_2$ solution over a 24h period. This was done to maintain soil structure and reduce the amount of entrapped air. The volume of liquid filled pores (pore volume) inside the column was calculated to be 417 mL. After the column was saturated, a steady-state flow velocity of 4.1 mm min^{-1} was established with 0.01 M $CaCl_2$ from the top of the column. Once steady-state flow velocity was achieved, a pulse of [^{14}C] TBBPA (0.39 _Ci, 563 _g) was applied to the soil surface in a volume of 135 mL, and eluted with 4.6 L of 0.01 M $CaCl_2$ at the same steady-state flow velocity. The column effluent was fraction collected in 20 ml volumes, and each fraction was analyzed for ^{14}C. The soil column was extruded from the column and cut into 1cm sections. The sections were dried and assayed for ^{14}C by combustion analysis on a Packard Model 307 Oxidizer. Selected sections [14 (top), 12, 10, 5, 3, and l cm from column bottom; 10 g] were individually extracted sequentially with toluene, ethyl acetate, and methanol in an Accelerated Solvent Extractor (Model 200 ASE; Dionex, Sunnyvale, CA, USA). Thin-layer chromatography (TLC) analysis on these fractions was conducted using silica gel plates (250_m; Whatman Lab. Div., Clinton, NJ, USA) with a methylene chloride:hexane (1: 1)solvent system.

Bis-[^{14}C phenol-A was prepared from [UL-^{14}C]phenol (2.0 mCi, 25 mCi mmol^{-1}) and acetone.[12] [^{14}C]2,2-bis(4-hydroxy-3,5-dibromophenyl)propane(TBBPA) was synthesized by brominating bis-[^{14}C]phenol-A with 4.2 equiv. bromine in 1 : 1 methanol : water. The resulting specific activity of TBBPA was 48.4 mCi mmol-' as determined by gas chromatography and radioactivity measurements. The purity of TBBPA (> 98 %) was determined by TLC (see above) and HPLC (Cl8; H_20:CH_3CN gradient). Radiolabelled TBBPA was diluted with unlabelled material until the desired specific activity was achieved (3,950 dpm ug^{-1}).

An equilibrium convective-dispersive transport model was inversely fit to the TBBPA concentration distributions with depth. This was done to estimate the solute transport parameters of TBBPA in soil. The analytical solution

of the following equilibrium sorption, convective-dispersiveequation was fit to experimental data using the model CXTFIT ver 2.1:[13]

$$(1 - \frac{\rho_b K_d}{\theta}) \frac{f C_r}{f t} = D \frac{f^2 C_r}{f x^2} - v \frac{f C_r}{f x} \qquad [1]$$

where ρ_b is the bulk density (g cm^{-3}), K_d is the adsorption coefficient (cm^{-3} g), θ is the volumetric water content (cm^3 cm^{-3}), C_r is the resident concentration (mg L^{-1}), t is time (min), v is the pore water velocity (cm min^{-1}), D is the dispersion coefficient (cm^2 min^{-1}), and x is depth in the column (cm). The model, CXTFIT, uses a least-squares approximation and iteratively changes select transport parameters so that the best-fit model solution to the observed data is achieved. Coefficients of determination (r^2) values are calculated by CXTFIT as a means to describe the best fit, where an r^2 value closest to unity is best.

As a comparison, standard, washed laboratory sand (Catalog No. 3382-5, J. T. Baker Chem. Co., Phillipsburg, NJ, USA) was packed in the same size column as the soil, and the pore volume was calculated to be 303 ml. The sand was saturated with a solution of 0.01 M $CaCl_2$ from the bottom over a 24 h period, to reduce the amount of entrapped air. A steady-state flow velocity of 4.8 mm min^{-1} was then established with 0.01 M $CaCl_2$ from the top of the column. After steady-state flow velocity was achieved a pulse of [^{14}C] TBBPA (0.34 _Ci, 490 _g) was applied to the sand surface in a volume of 135 ml and eluted with 4.4 L of 0.01M $CaCl_2$ (approximately 14 pore volumes) at the same steady-state flow velocity. The column effluent was fractionated and each fraction (ca. 20 ml) was analyzed for ^{14}C. The sand column was then eluted with methanol, and extruded in four sections and analyzed for ^{14}C by liquid scintillation chromatography (LSC)

TBBPA soil sorption was done using a batch equilibration technique. The soil used was the Galesburg loam described above. Soil was placed in 10 ml glass bottles with water at a ratio of 1.6 g to 8 ml, respectively. Then ^{14}C spiked TBBPA was added so that the solution concentrations were 2.5, 0.25, and 0.025 _g ml^{-1} in 0.01 M $CaCl_2$ in separate vials. Each of the concentrations was run in triplicate. The slurries were agitated by rotation of the vial top to bottom (360°/5 sec). At 48, 96 and 168 h, the bottles were centrifuged at 1,700 rpm, triplicate 100 _l samples removed, and radioactivity analyzed by LCS.

Equilibrium batch experimental data was described using Freundlich isotherms:

$$s = K_d C^n \qquad [2]$$

where s is the concentration of TBBPA adsorbed to the soil (mg g^{-1}), C is the TBBPA in the dissolved phase (gm L^{-1}), and n is an empirical constant controlling the shape of isotherm. A least-squares approximation method was used to obtain a best fit of Eq. [2] to the observed data. The parameter K_d and n were optimized to achieve the best-fit description of the observed data.

The FETAX bioassay was performed according to ASTM guidelines[8] as previously modified.[9] In addition, the embryos were reared using both the commonly employed FETAX mineral media (10.7mM NaCl / 1.14 mM $NaHCO_3$ / 0.403 mM KCl / 0.135 mM $CaCl_2$ / 0.349 mM $CaSO_4 \cdot 2H_2O$ / 0.623 mM $MgSO_4$) and a mineral media (S5511) whose composition is barely sufficient to prevent developmental retardation and is similar to pond water encountered in Minnesota, USA. The composition of S5511 is 0.22 mM NaCl / 0.13 mM KCl / 0.41 mM $MgSO_4$ / 0.25 mM $CaCO_3$. No differences in the results obtained were observed upon using plastic petri plates in place of glass.

The stock solutions of BPA and TBBPA were prepared in ethanol, which resulted in an ethanol concentration in the FETAX growth media of 0.015%. In control experiments an ethanol concentration of 0.015% in the FETAX growth media had no effect on *Xenopus* embryo development in the FETAX bioassay. The sensitivity of the embryos in the FETAX bioassay to teratogens and agents capable of retarding embryo development was confirmed using 6-aminonicotinamide(6AN) and water from a site previously shown to induce developmental delay (ROI2). Embryos raised in 6AN displayed the characteristic effects of AN exposure, namely 5.5 mg 6AN / L induced a mortality rate of 0% and a malformation rate of 12%, while 2.5 g 6AN /L induced a mortality rate of 92% with all surviving embryos malformed. When the embryos were reared in water from ROI2 a mortality of 4% was observed and all remaining embryos were developmentally delayed and had an 18% reduction in length.

Results and Discussion

No radioactivity from [^{14}C] TBBPA was eluted from the soil column with 0.01 M $CaCl_2$ even after 11 pore volumes were displaced. Nearly 71% of the applied [^{14}C] TBBPA that was applied to the soil column was found to be retained in the soil column after combustion analysis. The top centimeter of the soil retained 16.1% of the applied [^{14}C] TBBPA and between 6 and 7% was retained in each of the next four centimeters. Generally, radioactivity in the remaining sections of the column appeared to taper off from four to two percent, except for a slight rise in activity (5%) 3- to 5 cm from the bottom (Figure 1). In the ASE extraction, only the toluene extract of each section contained ^{14}C, which was shown to be TBBPA by chromatography by TLC. Although it appeared that TBBPA was strongly sorbed to the soil because there was no radioactivity in the column effluent, there was significant redistribution of the TBBPA to a depth of 15 cm.

Figure 1 also shows the inverse fit of Eq. [1] to the observed data, which resulted in an r^2 of 0.67. The best-fit parameters of K_d and D were 207 cm^3 g^{-1} and 12.8 cm^2 min^{-1}, respectively. The very high K_d value indicates that TBBPA readily partitions to the solid phase.

A small amount of ^{14}C (4.5%) was found in the effluent of the sand column in the fractions between 0.86 to 1.4 pore volumes (260 to 430 ml). After 14 pore volumes had been displaced the sand column was eluted with methanol, which removed 73 percent of the [^{14}C] as TBBPA (Rf= 0.29). This may indicate that the TBBPA also sorbed to the mineral fraction of soil as well as the organic. Little ^{14}C was detected in sand from any of the four methanol rinsed sections (2.5% total, by Liquid Scintillation Counting).

Batch studies at 48h (Figure 2) showed that 97.9, 92.6, and 93.6% of the 0.025, 0.25, 2.5 _g ml^{-1} [^{14}C] TBBPA, respectively, were bound to the soil. Sorption of [^{14}C] TBBPA to the soil at the later time points was the same. The Freundlich isotherm fit well to the data, which was indicated by the r^2 value of 0.99. The K_d and n values were 657 cm^3 g^{-1} and 0.96, respectively. Again, the high K_d value indicates that TBBPA is highly sorptive to the soil material. The sorption process was essentially a first-order process, which was indicated by the n value approximately equal to one. The apparent first-order sorption also justified the use of the equilibrium sorption convective-dispersive model (Eq. [1]) to describe the transport experiment, instead of using a more complicated non-equilibrium sorption model. The independently estimated K_d values from batch and column experiments were similar; however, the column K_d values were lower which may indicate some type of mass transfer limitation. The mass transfer limitation may be caused by decreased lateral diffusion rates due to the nature of the flowing system.

These results show that TBBPA is sorbed extensively by loam soil and sand. This is consistent with an earlier report indicating that because of TBBPAs high partition coefficient (K_{ow} = 4.3 - 5.3) and low water solubility these sorption characteristics for TBBPA are not unexpected. Therefore, TBBPA, in the environment, would be expected to sorb largely to sediment and organic matter in the soil.

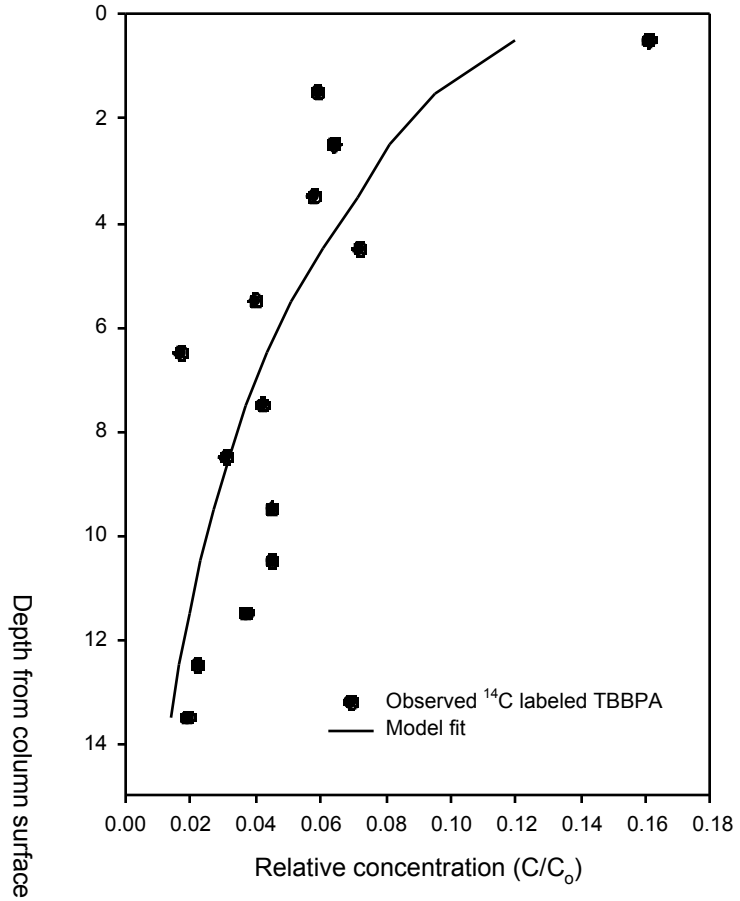

Figure 1. Observed ^{14}C labeled TBBPA relative concentrations with depth in the soil column, along with the inverse fit of Eq [1] to the data.

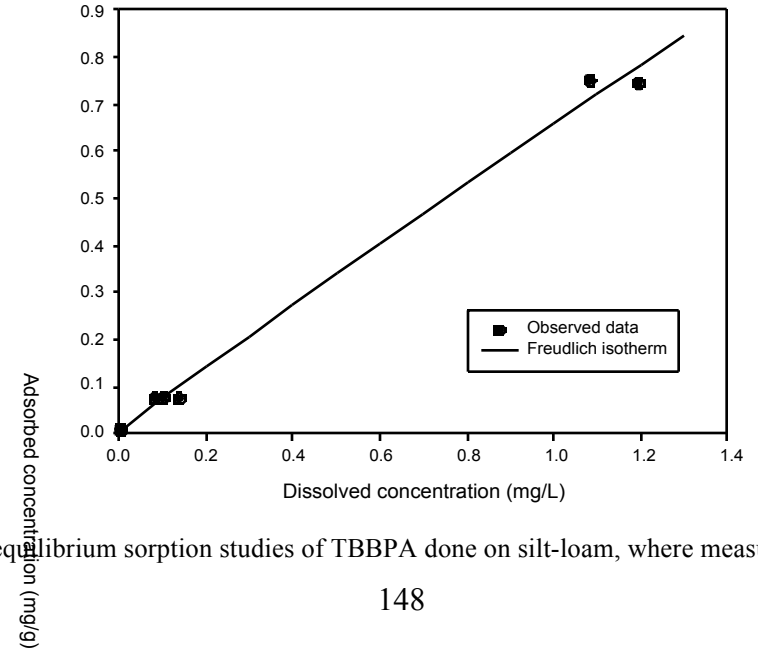

Figure 2. Batch equilibrium sorption studies of TBBPA done on silt-loam, where measured data was fit with

a Freundlich isotherm.

Xenopus laevis eggs harvested in the mid-blastula stage were exposed to 0.1, 1.0, 10, 100 and 500 ppb BPA and TBBPA in FETAX mineral media and S5511 mineral media. Table 1 summarizes the results in mineral media and S5511 media for BPA and TBBPA. The slight, statistically insignificant decrease in the embryo lengths obtained in S5511 media versus the standard FETAX mineral media is characteristic of S5511 and reflected in the presence of a slight residual cement gland in the embryos. However, under both conditions BPA and TBBPA displayed no activity in the FETAX bioassay (i.e. no effect on Xenopu*s* development, survivability of the embryos, the malformation rate, or the length of the embryos). Additional studies are necessary to determine whether these results are reflective of the narrow sensitivity of the FETAX bioassay or the compounds' biological activity.

A limitation of the FETAX bioassay is that it screens for biological activity during only the first 96 hours of embryo development when organ development occurs. As such, hormonally active compounds that only effect later stages of development appear to lack biological activity. In order to fully ascertain the biological activity of a compound it is therefore necessary to determine its activity on several organisms and during various stages of development.

Conclusion:

These experiments have shown that levels of TBBPA found in the environment or which may be excreted from mammalian systems are strongly sorbed to soil and effectively to sand. Further the biological/hormonal activity in the FETAX was shown to be negative. This indicates that environmental levels of TBBPA likely do not enter ground water if they are sorbed onto the organic or mineral components of soil.

Table 1. Biological Activity of BPA and TBBPA In The FETAX Bioassay

		conc.	Lethality	Malformation	length*
		ppb	%	%	mm
Mineral Media			9	3	9.5 ± 0.5 (100/2)
	+ 0.015% EtOH		8	7	9.7 + 0.5 (50/2)
	BPA	0.1	0	8	9.5 + 0.4 (50/2)
		1	1	3	9.6 ± 0.4 (50/2)
		10	12	8	9.4 + 0.5 (50/2)
		100	4	4	9.6 ± 0.3 (50/2)
		500	0	7	9.6 ± 0.5 (75/3)
	TBBPA	0.1	0	10	9.5 + 0.5 (50/2)
		1	1	4	9.7 ± 0.4 (50/2)
		10	5	6	9.7 + 0.4 (150/4)
		100	3	4	9.7 + 0.4 (50/2)
		500	4	6	9.6 ± 0.4 (150/4)
S5511			3	1	9.4 ± 0.4 (50/2)
	0.015% EtOH		1	3	9.4 ± 0.4 (50/2)
	BPA in S5511	0.1	4	4	9.3 + 0.5 (50/2)
		1	4	4	9.5 ± 0.2 (50/2)
		10	4	5	9.5 + 0.4 (50/2)
		100	2	1	9.4 + 0.3 (50/2)
		500	2	4	9.5 ± 0.4 (150/4)
	TBBPA in S5511	0.1	10	3	9.3 + 0.4 (50/2)
		1	7	3	9.4 ± 0.5 (50/2)
		10	5	4	9.4 ± 0.4 (150/4)
		100	7	4	9.2 + 0.5 (50/2)
		500	6	6	9.3 ± 0.4 (125/4)

* length is expressed as mm ± standard deviation (# embryos screened / # different clutches)

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Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others than may also be suitable.

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Biographical Sketch

Dr. Gerald Larsen has been with the USDA for 30 years and is the Research Leader of the Animal Metabolism-Agricultural Chemicals Research Unit. His research has focused on the elucidation of metabolic pathways, building, transport and disposition of chemicals in animals with regard to food safety. Along with his colleagues, he has shown that a source of dioxins in U.S. beef result from animals exposure to technical pentachlorophenol (i.e. treated wood). Most recently, his research has involved the metabolism and disposition of brominated flame-retardants and fate of estrogenic chemicals in the environment.

Occurrence of Antibiotics, Pesticides, and Pesticide Degradates in Agricultural Runoff, Stillwater River Basin, southwest Ohio

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Abstract

In a synoptic study to assess the occurrence of agricultural compounds (antibiotics, pesticides and nutrients) in spring storm runoff in the Stillwater River Basin, stream samples were collected from 30 watersheds, most of which drain less than 200 square kilometers. The Stillwater River Basin is one of the most intensely cultivated row-crop areas in western Ohio, but it also contains many poultry and swine feedlot operations. The 30 watersheds were chosen to represent a gradient of livestock-production intensity ranging from few to approximately 2,000 animal units per square kilometer (AU/km^2).

Surface-water samples were collected May 19-20, 2000, after a series of thunderstorms produced 25 to 50 mm of rainfall across the Stillwater River Basin. These storms were preceded by several weeks of generally dry weather that allowed most farmers to complete pre- and post-planting applications of manure, inorganic fertilizer, and pre-emergent herbicides. Liquid chromatography-mass spectrometry was used to analyze for 22 human and veterinary antibiotics. Reporting limits for antibiotic analyses were from 0.01 to 0.05 micrograms per liter (µg/L). Gas chromatography-mass spectrometry and high-performance liquid chromatography were used to analyze for 46 pesticides and 8 pesticide degradates; reporting limits for individual compounds ranged from 0.001 to 0.1 (µg/L).

One or more antibiotics were detected—generally at or near minimum reporting limits—in 10 of the 30 streams sampled. The maximum number of antibiotics detected in any single sample was three; two antibiotics were detected in four samples, and a single antibiotic was detected in five samples. The most commonly detected antibiotic was lincomycin (7 sites) followed by roxithromycin (3 sites), erythromcyin-H_2O, sulfadimethoxine, trimethoprim, tylosin (2 sites each), and tetracycline (1 site). No significant correlation was found between the intensity of animal production in a given watershed and detection or nondetection of antibiotics in a stream sample. In half of the 10 watersheds with moderately high levels of livestock production (animal densities greater than 200 AU/km^2) no antibiotics were detected, nor were any detected in three control watersheds with minimal animal production; however, antibiotics were detected in samples collected from two locations affected by wastewater effluent and septic-tank discharge.

Nitrates and high-use herbicides such as atrazine, metolachlor, acetochlor, alachlor, and simazine were detected in all 30 stream samples at concentrations that greatly exceeded spring-summer seasonal averages recorded at a downstream monitoring station on the main stem Stillwater River. High concentrations (generally greater than 3 µg/L) of selected degradates of atrazine, metolachlor, acetochlor, and alachlor also were detected at all 30 sites. The herbicide glyphosate was detected at 70 percent of the sites (0.1 µg/L reporting limit). Complex mixtures of pesticides and pesticide degradates were found in all 30 streams; the median number of compounds detected was 18 (maximum number detected was 23). Current drinking-water standards and aquatic-life guidelines are based on toxicity tests done on a single compound and little is known about the effects of mixtures of pesticides and their degradates on aquatic organisms or human health.

Results of this study indicate that aquatic biota in streams draining areas of intense row-crop production will be exposed to high levels of nutrients, pesticides, and pesticide degradates during post-application runoff events and that trace levels of antibiotics are also likely to found in runoff from areas of livestock production. The effects of acute short-term exposure of aquatic organisms to high levels of pesticides and pesticide degradates, as well as trace levels of antibiotics, is unknown. Factors that could affect the occurrence of antibiotics in storm runoff include (1) multiple human and veterinary sources of antibiotics in a watershed, (2) application of manure produced at feedlots outside the local watershed, (3) timing of manure application in the watershed, and (4) degradation or soil sorption of excreted antibiotics after land application.

Biographical Sketches

Gary Rowe has worked as a Hydrologist/Geochemist for the U.S. Geological Survey (USGS) since 1991. He received his Ph.D. in Geochemistry and Mineralogy from the Pennsylvania State University in 1991. His research has focused on various ground-water quality issues, the use of environmental tracers to age-date ground water, and the occurrence of pesticides and other anthropogenic contaminants in the environment. Currently he is the project chief of the Great and Little Miami River Basins NAWQA study.

Michael Meyer has worked as an Organic Geochemist for the U.S. Geological Survey (USGS) since 1988. He received his Ph.D. in Geology from the University of Kansas in 1994. His research has focused on the development of methods for the analysis of emerging organic contaminants (e.g. herbicide metabolites and pharmaceuticals) and determining their occurrence, fate, and geochemical transport in the environment. Currently he is the Supervisory Research Geochemist of the USGS, Florida District, Ocala Water Quality and Research Laboratory.

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Occurrence and Fate of Pharmaceuticals during Bank Filtration – Preliminary Results from Investigations in Germany and the United States

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Abstract

The growing need for clean drinking water worldwide has increased the interest in natural surface-water treatment techniques such as bank filtration. Bank filtration has been used for more than a century in drinking-water production. Historically, it was recognized as an efficient natural attenuation process ensuring sustainability of drinking-water supply. If bank-filtration facilities are designed and constructed properly, the groundwater used for drinking-water supply can be protected efficiently from contamination with microbial organisms and inorganic or organic pollutants. Recently, however, several new types of organic contaminants, such as pharmaceuticals, have been found in the aquatic environment. Some of the polar pharmaceuticals also have been detected in groundwater and drinking-water samples, especially when water from induced recharge is used for drinking-water production. In 2001, two projects were initiated at bank-filtration sites in Berlin, Germany, and in Nebraska, USA, to investigate the natural attenuation of pharmaceutical residues and several other environmentally relevant contaminants systematically under natural conditions. Preliminary results of the studies in Germany and the United States revealed that several polar organic compounds, especially several pharmaceutically active compounds, are relevant to drinking-water production at bank-filtration sites.

1. Introduction

At the end of the 19th century, water pollution issues were becoming more and more evident in growing metropolitan centers. The presence of anthropogenic substances, including pharmaceuticals, that potentially can affect the endocrine systems of aquatic organisms and human health through drinking water or aquatic recreational activities is creating a high level of concern among scientists and policy makers throughout the world (Ghijsen and Hoogenboezem, 2000). Treatment technologies for drinking-water supplies such as bank filtration, slow-sand filtration, ozonation, chlorination, granular activated carbon, or membrane filtration were developed to reduce or remove undesirable chemicals in drinking water and to provide sufficient amounts of quality drinking water for public-water supply.

More than 100 years ago, bank filtration was recognized as an efficient natural attenuation process ensuring sustainability of drinking-water supplies (Stuyfzand, 1989; Kruhm-Pimpl 1993; Mathys, 1994; Grischek, et al., 1997;Verstraeten et al., 1999; Brauch et al., 2000; Verstraeten et al., in press A and B). These researchers found that quality of bank-filtered water depends not only upon the contaminant itself, but also upon the hydraulic and chemical characteristics of the bottom sediment and the aquifer, the local recharge-discharge conditions, and biochemical processes. They also established that fate and transport of organic chemicals are complex and can include microbial degradation, adsorption/desorption, photolysis, oxidation, and transport from one medium in the hydrologic system, e.g. surface-water bodies and river banks, to another medium. Organic contaminants that are not readily biodegradable may occur in bank-filtered water, often in lower concentrations than those in the river (Verstraeten et al., 1999).

1.1 Importance of Bank Filtration for Drinking-Water Production in Berlin, Germany

In the metropolitan area of Berlin, Germany, 100% of public-water supply originates from groundwater with approximately 70% from bank filtration and artificial recharge (Verstraeten et al., in press B). At Lake Müggelsee in

Berlin, bank filtration was used as a method of drinking-water treatment about 100 years ago. Currently (2001), it is used at several locations in Berlin. Supply wells generally are drilled within short distances (as little as 600 m) from rivers, canals, and lakes (Pekdeger, 2001). The sewage treatment plants (STPs) often discharge their purified sewage water into the surface-water system upstream from well fields and their corresponding bank filtration or groundwater recharge facilities. Especially during low-flow conditions (primarily summer), the proportion of purified sewage in several waterways may be equal to or even greater than the natural surface-water discharge (SenSUT, 1999). Due to the progress in environmental analytical chemistry, increasing numbers of persistent organic compounds are being found in Berlin's surface water often released by municipal STPs (Heberer et al. 1998, 1999). Thus, monitoring of the quality of surface and bank-filtered water and understanding of local biochemical and hydraulic processes are needed to achieve and control a sustainable drinking-water supply.

1.2 Importance of Bank Filtration for Drinking-Water Production in the United States

In the United States, the growing need for quality drinking water has led to an increasing interest in the use of bank filtration as an inexpensive means of water-supply pretreatment. The interest of utilities in using bank filtration as part of their drinking-water treatment techniques recently has increased substantially, because utilities can request credit from their state environmental or regulatory agencies for removal of microorganisms through use of natural bank filtration as part of their drinking-water treatment process. Drinking-water systems using bank filtration may be classified as either groundwater, groundwater under the direct influence of surface water, or surface water (U. S. Environmental Protection Agency, 1989). To date, only two drinking-water utilities have received credit for virus and Giardia inactivation and/or removal by bank filtration from their respective State agencies. These utilities are Kearney, Nebraska, and Sonoma County, California (Schijven et al., in press).

In the United States, the number of utilities receiving bank-filtered water for drinking water is steadily increasing. For example, most cities in Nebraska historically have developed along the Platte River or its tributaries; as the population increases the dependence on this river is steadily increasing. In the United States, utilities have used bank-filtered water in states such as Iowa, Kansas, Kentucky, Nebraska, New Jersey, New York, Ohio, Texas, and Wyoming, to mention just a few, for a long time. In fact, bank-filtration sites probably exist along most major and some smaller rivers in the United States. Thus far, the use of bank-filtered water has not been quantified in the United States. In many cases, utilities do not recognize that their water substantially is derived from surface water, or, if they do, have yet to quantify how much of the water pumped from a well originated as surface water.

1.3 Relevance of Pharmaceuticals in Bank Filtration

Pharmaceuticals are used in large quantities in human and animal health care as well as in the fruit production industry. Drugs used in human and animal health care may enter the environment by discharges of municipal sewage or hospital effluents, sewage sludges, landfill leachates (waste disposal), domestic septic tanks, production residues, feedlot runoff, contaminated liquid manure, or direct introduction via aquacultures.

In human health care, the sales of drugs reached $210.7 billion worldwide from March 1999 through February 2000 (U.S. Environmental Protection Agency, 2001). The main therapeutic markets were cardiovascular, alimentary, antidepressants, anti-infectives, and respiratory. In 1997, the United States' per capita expenditure on drugs was one of the highest in the world ($319), after Belgium, Japan, and France ($321-351). About 1 to 2 percent of the sales in the United States were used for veterinary purposes. In the United States, although pharmaceutical sales amount to hundreds of kg (kilograms) per year, production of personal care products are an order of magnitude larger (U.S. Environmental Protection Agency, 2001).

Several pharmaceutical compounds, such as carbamazepine, diclofenac, and ibuprofen, are prescribed in quantities as large as 100 t/yr (tons/year) in Germany (Stan and Heberer, 1997). Over-the-counter pharmaceutical sales and hospital use of compounds such as aspirin are as much as 1 000 t/yr in Germany, which implies that the annual use of pharmaceuticals is similar to pesticide use (Verstraeten et al., in press B). Therefore, pharmaceutical residues could be present in surface water at concentrations similar to, or even greater than, those of the pesticide residues. Large concentrations of pharmaceutical residues may be found especially near urban areas, because they enter the surface water by discharges from municipal STPs, which are point sources for these compounds. In contrast, pesticides applied extensively on agricultural land tend to enter the streams as nonpoint sources. Nevertheless, pharmaceuticals also can enter the environment as nonpoint sources when manure containing traces of pharmaceuticals is applied to the fields, as is the practice in the United States (Meyer et al., 2000).

Data on the occurrence of pharmaceutical residues in the environment often have been only a random by-product from other investigations, such as studies of the occurrence of pesticide residues. To date, a substantial amount of data exists on the presence of antibiotics in the environment (Daughton and Ternes, 1999). The first detection of antibiotics in wastewater was reported in the 1970s in the United States (Daughton and Ternes, 1999), but these findings were ignored largely. In Europe, information on the presence of antibiotics in water has been gathered since the 1980s. Data on pharmaceuticals have been collected because of their potential effects on native biota and development of resistance in potential human pathogens.

Important factors affecting the occurrence of pharmaceutical residues in the environment include the amounts prescribed and the fate of the individual compounds both in the STPs and the aquatic environment. Some pharmaceuticals are not eliminated or degraded in the human body. They are excreted by the human body unchanged or only slightly transformed, mostly conjugated to polar molecules, such as the glucoronides. These conjugates are cleaved easily in the raw sewage or during sewage treatment. Thus, some pharmaceutically active compounds (PhACs) are discharged almost unchanged from municipal STPs into the receiving water (Stan and Heberer, 1997; Halling-Sørensen et al., 1998; Heberer and Stan, 1998; Ternes, 1998; Daughton and Ternes, 1999; Möhle et al., 1999; Wilken et al., 2000; Heberer, in press; Heberer et al., in press).

In 1990, after the reunification of East and West Germany, the Senate of Berlin commissioned monitoring of water collected from wells near the former Berlin wall. The purpose of this monitoring was to identify and quantify pesticide residues caused by the intensive use of these compounds along the Wall. As a result of these investigations, 2-(4)-chlorophenoxy-2-methyl propionic acid (clofibric acid) was first detected in Berlin groundwater samples in 1991 (Stan and Linkerhägner, 1992). Clofibric acid is the pharmacologically active metabolite of the drugs clofibrate, etofyllin clofibrate, and etofibrate, used as blood-lipid regulators in human health care. It was detected in an analytical method for the analysis of phenoxyacid herbicides. In fact, clofibric acid is a structural isomer of the herbicide mecoprop, frequently used as a pre-emergent herbicide in agriculture, and, initially, its occurrence in groundwater samples was not linked with its medical application. However, between 1992 and 1995, clofibric acid also was detected at concentrations at the µg/L-level in groundwater samples collected from former sewage irrigation fields near Berlin and in Berlin tap-water samples (Heberer and Stan, 1996; 1997). It became evident that these residues were caused by the infiltration of sewage effluents into the soil and that clofibric acid is a very mobile compound that is not substantially adsorbed in the subsoil and is leached easily into the aquifer. Clofibric acid was found even in samples collected from an aquifer at depths of more than 70 m (meters). In Germany, the first detections of clofibric acid in groundwater put focus on the presence of drug residues in the aquatic system as a new emerging issue (Umweltbundesamt, 1996) and researchers began to investigate the occurrence and fate of pharmaceutical residues in the aquatic environment, during drinking-water purification, and in drinking-water samples.

In 1994, by analyzing drinking-water samples collected from 14 utilities in Berlin (Heberer and Stan, 1996 and 1997), a relation was found between bank filtration, artificial groundwater recharge, and the level of drinking-water contamination by the drug clofibric acid. Several other polar PhACs also were identified as contaminants relevant to bank-filtration processes. Diclofenac, ibuprofen, propyphenazone, gemfibrozil, or primidone, also have been detected in groundwater wells from different bank-filtration sites in Berlin (Heberer et al., 1997; Heberer, in press). In a recent bank-filtration study by Brauch et al. (2000), the behavior of several selected polar organic contaminants, including diclofenac and carbamazepine, was studied in water from two utilities near the lower Rhine River. The transport of diclofenac, clofibric acid, and propyphenazone during bank filtration also has been studied at Lake Tegel in Berlin (Schyett et al., 2001). These studies indicate that PhACs are attenuated partially by bank filtration and that several of these compounds can be transported through the subsoil without any substantial attenuation.

2. Study of the Occurrence and Fate of Drug Residues along Transects in Berlin, Germany

The first detections of PhACs in groundwater samples from bank-filtration sites in Germany encouraged more intensive study of their behavior during bank filtration. The fate of organic residues, including PhACs, seven polar pesticides, two flame retardants, and a metabolite of an anticorrosive substance, currently is being investigated at two bank-filtration sites in Berlin, Germany. The bank-filtration research is carried out cooperatively by the Senate of Berlin (Senate Department of Urban Development, Environmental Protection and Technology) and the Hydrogeology Research Team of the Free University Berlin. Two transects ("Lieper Bucht" and "Lake Wannsee") are along the Havel River (figure 1), a river heavily impacted by municipal sewage effluent. Residues of PhACs have been detected in the water of the Havel River at individual concentrations of as much as 600 ng/L (nanograms

per liter) (Heberer et al., 1998). The objective of the current study is to evaluate bank-filtration processes and the transport and attenuation of contaminants such as PhACs under natural conditions. Beginning in April 2001, water samples collected monthly from the river and wells are analyzed for more than 40 polar organic contaminants to obtain representative data and to identify the effect of temporal (seasonal) variations, changes in environmental conditions, and hydraulic effects. The PhAC data will be put in context with the hydrogeologic framework being characterized with physico-chemical parameters and major-ion concentrations. The preliminary results obtained for samples collected in April 2001 from the Lake Wannsee transect are presented in a later section.

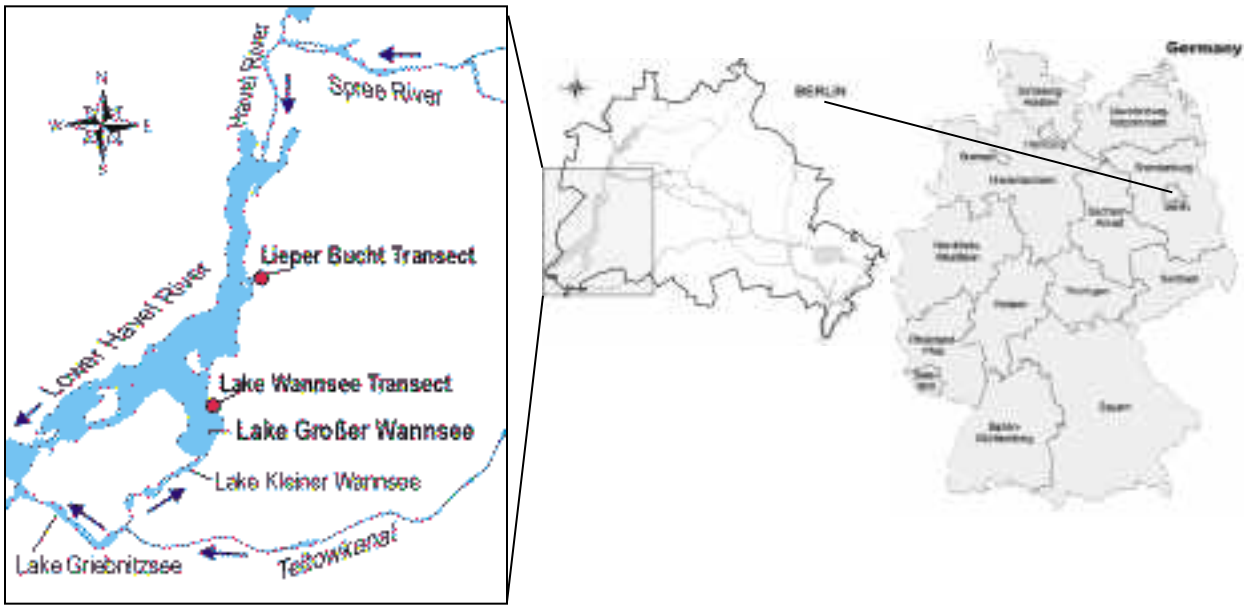

Figure 1: Maps showing the locations of both transects along the Lower Havel River (left), Berlin (middle), Germany (right).

2.1 Description of Lake Wannsee Transect

The transect at the east bank of Lake Wannsee (Großer Wannsee) is at a bank-filtration area of an active utility (Beelitzhof) of the Berliner Wasserbetriebe, which is using infiltrated water from the lake. The utilities in Beelitzhof use about 66% of bank filtrate in drinking-water production (Heberer, in press). Groundwater samples from seven monitoring wells and one water-supply well (WWK 4) drilled at various distances from LakeWannsee (figure 2) along a line of about 150 m were analyzed for a variety of compounds.

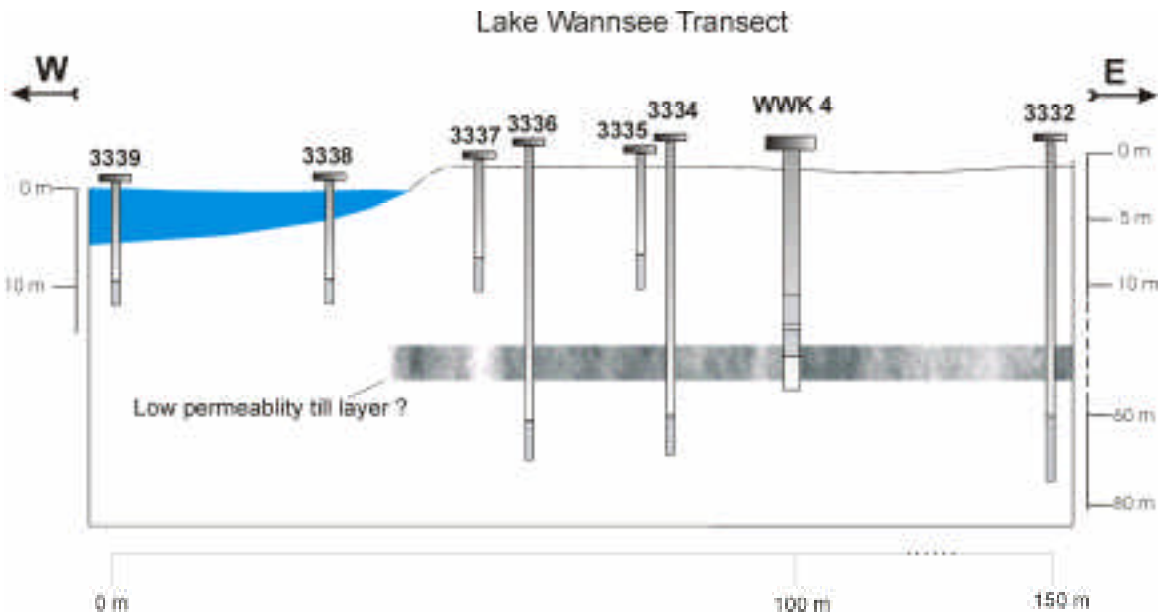

Figure 2: Preliminary schematic hydrogeologic section of the Lake Wannsee transect, location of monitoring wells (shallow and deep)and a water-supply well (WWK4) (Adopted and modified from Pekdeger, Workgroup of Hydrogeology at the FU Berlin, written commun., 2001).

The darker areas on the bottom of the wells are the individual screens.The shallow wells are screened in a shallow aquifer at an average depth between 10 and 15 meters. The deep wells are screened in a deep aquifer underneath a till layer at an average depth of about 70 meters. The small permeability of this layer and the discontinuity of this layer, commonly observed in the Berlin area, are a subject of these investigations. The water works wells, including water works well 4 (WWK 4) in figure 2, are usually screened at two depths (between 15 and 40 meters) in the intermediate aquifer. The second groundwater aquifer commonly is connected with the shallow (first) aquifer and may, thus, be influenced by contaminants that are mobile in the subsoil.

Lake Wannsee receives its water mainly from the Lower Havel River but also from the Teltowkanal, a canal in the south side of Berlin (figure 1). About half of the surface water from the Teltowkanal flows through Lake Griebnitzsee (figure 1) into the Lower Havel River. The remainder of the surface water flows through Lake Kleiner Wannsee into Lake Wannsee. The Teltowkanal carries substantial loads of treated sewage discharges (Heberer, in press). Sewage effluents are discharged into the canal by three STPs along the canal including the two largest STPs of Berlin (Stahnsdorf and Ruhleben). In autumn and winter, the effluents from the STPs in Ruhleben are discharged into the Spree River, which converges with the Upper Havel River. Thus, the Lower Havel River always carries loads of treated sewage from three STPs, and, during autumn and winter, also from STPs in Ruhleben, located in the north-western districts of Berlin.

2.2 Preliminary Results of Analysis of Pharmaceutical Residues and Related Organic Compounds

Water samples are being analyzed for 43 environmentally relevant organic contaminants. In samples collected in April 2001, 15 (13 of them are listed in table 1) of the 43 compounds were detected in surface-water and groundwater samples (table 1). Seven of these compounds, namely compound X (not identified because of potential legal ramifications), bezafibrate, carbamazepine, clofibric acid, diclofenac, primidone, and propyphenazone, were PhACs. They were detected only in the shallow wells, and the water-supply well but not in the deep monitoring wells. A significant decrease in concentrations along the transect away from the lake was observed for bezafibrate, a compound used as a blood-lipid regulator in human health care. About 10% of the bezafibrate concentration detected in surface water was found in water from shallow well 3339. It was not detected in water from shallow wells 3337 and 3335. Other PhACs, such as carbamazepine and primidone, both anti-epileptic drugs, were detected in all of the shallow wells. They also were found further away from the lake in water from well 3335 at concentrations similar to or even larger than those found in the surface water. The results for carbamazepine confirm soil-column experiments indicating that carbamazepine was not substantially adsorbed to sediments (TU Berlin, written commun., 2001). Diclofenac appeared to be attenuated during transport through the sediments (table 1).

This observation also confirms the results obtained by laboratory column experiments of Mersmann et al. (TU Berlin, written commun., 2001). In contrast to bezafibrate, diclofenac reached well 3337 and its concentrations decreased less from the surface water to well 3339 (only about 30% of the surface-water concentration) than those of bezafibrate.

Five of the PhACs also were detected in the samples from the water-supply well (table 1). Two of the PhACs were found at larger concentrations than those measured in the surface water or the shallow wells. This might be explained by seasonal differences and will be evaluated further using results of additional sampling. This phenomenon may also be explained by the history of this type of contamination. The use of the precursor compounds of clofibric acid in Germany has decreased during the last 10 years and clofibric acid is being substituted for other pharmaceuticals. The larger concentrations in the water-supply well may be because of larger concentrations of these compounds in sewage in the past. The concentrations of clofibric acid measured in the Berlin sewage effluents decreased during the last 5 years from about 1 to 0.5 µg/L. This phenomenon will be evaluated further when the contributing areas to wells and tracer velocities are determined as a result of the hydrogeologic studies that are being conducted at the study site.

In addition to the detections of PhACs in water samples, several pesticides were detected. Again, the results indicate that recent and past contamination of surface water is contributing to the concentrations of compounds found in ground water. The pesticides bentazone and MCPP are used frequently as herbicides in agriculture, but also in gardens, on sporting grounds, and on sealed surfaces. The largest concentrations of herbicides in Berlin's surface waters were detected during spring and early summer (Heberer and Reddersen, in press). Nevertheless, during all seasons, the concentrations of the pesticides measured remained substantially lower than the concentrations of PhACs (Heberer et al., 1998; Heberer and Reddersen, in press).

Table 1. Concentrations of selected organic compounds detected in water samples from Lake Wannsee transect, Berlin, Germany, April 18, 2001.
[ND: not detected; detection level, 1 to 10 nanograms per liter; data are reported in nanograms per liter]

Compound	Surface water	Shallow wells				Deep wells			Water-supply well 4*
		3339	3338	3337	3335	3336	3334	3332	
			Pharmaceutically active compounds						
Compound X**	200	155	350	185	135	ND	ND	ND	100
Bezafibrate	170	20	10	ND	ND	ND	ND	ND	ND
Carbamazepine	235	160	235	360	325	ND	ND	ND	20
Clofibric acid	50	20	60	20	ND	ND	ND	ND	70
Diclofenac	50	35	40	15	ND	ND	ND	ND	ND
Primidone	105	195	210	215	535	ND	ND	ND	15
Propyphenazone	280	170	145	10	70	ND	ND	ND	50
			Pesticides						
Bentazone	<10	10	30	<10	<10	ND	ND	ND	ND
MCPP	20	10	15	<10	ND	ND	ND	ND	ND
o,p'-DDA	<10	<10	10	ND	ND	ND	ND	ND	15
p,p'-DDA	15	25	35	ND	ND	<10	15	ND	20
			Other organic compounds						
TCEP	250	100	260	70	10	ND	ND	ND	105
TCIPP	510	440	1025	580	390	ND	ND	ND	180

* Water-supply well number 4 (WWK4) was out of use during the sampling series of April 2001; the given value was obtained in May 2001
** Compound X has been identified as a metabolite of a pharmaceutical substance originating from a source at the Upper Havel River outside of Berlin (Reddersen et al. (TU Berlin, written commun., 2001)). This compound is not identified because of potential legal ramifications (Reddersen et al. (TU Berlin, written commun., 2001)).

As reported by Meitzler et al. (1996), bentazone is very mobile in the subsoil. In the transect study, it was detected at trace-level concentrations from <10 to 30 ng/L in all samples from the shallow wells (table 1). Neither bentazone nor MCPP was found in the deep well. Based on the results in table 2, MCPP appears to be attenuated along the transect. It was not detected in water from well 3335 nor from the water-supply well. The compounds o,p'- and p,p'-DDA are the polar metabolites of the insecticide DDT. DDT was produced until 1986 by a former chemical production plant in the eastern part of Berlin. The release of DDT residues into the environment resulted in a DDT Superfund site at the eastern part of the Teltowkanal (Heberer and Dünnbier, 1999). DDA residues continue to be found in the surface water of the Teltowkanal down to the Lower Havel River (Heberer, TU Berlin, written commun., 2001). As described previously, the Teltowkanal provides water to Lake Wannsee. The DDT metabolites

in the water-supply well also may have originated from former applications of DDT, considering that they arevery persistent in groundwater (Heberer and Dünnbier, 1999). The detections of p,p'-DDA in two of the deep wells supports this hypothesis.

The flame retardants tris-(chloroethyl)-phosphate (TCEP) and tris-(chloroisopropyl)-phosphate (TCIPP) frequently are detected in municipal sewage effluents and surface-water samples in Berlin. In surface water, the concentrations of TCIPP generally are about twice as high as those of TCEP (Heberer & Reddersen, in press). Both compounds were found in high concentrations and in similar proportions in water from the shallow wells (table 1). In the deep wells, neither compound was detected; however, TCIPP (180 ng/L) and TCEP (105 ng/L) were detected in the water-supply well.

2.3 Preliminary Conclusions

The preliminary results of the Lake Wannsee bank-filtration project indicate that several polar organic compounds, especially several PhACs, probably are relevant to bank-filtration pretreatment near urban areas. Several compounds, such as bezafibrate, diclofenac or MCPP, seem to be removed effectively during bank filtration. But several other compounds, namely carbamazepine, clofibric acid, primidone, propyphenazone, compound X, bentazone, DDA, TCIPP, and TCEP, were present at concentrations between 15 and 180 ng/L in the water-supply well. Further study may clarify some unresolved questions about the origin of several compounds and their behavior (temporal and spatial) during the bank-filtration process. Investigators also will obtain information on the presence and fate of compounds other than the 41 compounds for which samples were analyzed already.

3 Investigations on the Occurrence and Fate of Drug Residues at Bank-Filtration Sites, Nebraska, United States

3.1 Background

In the United States, the use and eventual fate of antibiotics by humans and in livestock production are a concern. About 90 percent of the approximately 2.5 million kg of antibiotics sold in the United States are given as growth-promoting and prophylactic agents in sub-therapeutic doses instead of being used to treat active infections, thereby lowering the cost of animal care (Kolpin et al., 2000). Researchers report that antibiotics have been detected in the United States at trace levels (0.05-2.1 µg/L (micrograms per liter)) in surface water and groundwater (Thurman, written commun., 2001; Meyer et al., 2000) and that tetracycline and sulfonamide antibiotics have been detected in samples collected from groundwater and wastewater of (confined) animal feeding operations (AFOs/CAFOs) (Thurman and Hostetler, 2000; Lindsey and Thurman, 2000). Tetracycline and sulfonamide antibiotics are believed to occur at levels on the order of 100 _g/L in wastewater from CAFOs (Meyer et al., 2000; Lindsey and Thurman, 2000). Meyer et al. (2000) reported that, in the United States, tetracyclines were the most frequently detected class of antibiotics, followed by sulfonamides, macrolides, and beta-lactams, in liquid waste from hog wastewater lagoons, with concentrations of as much as 700 _g/L. Therefore, a concern exists in the United States that these contaminants can be transported at trace levels from lagoon discharges or after application of manure and compost onto the land, into a riverine environment, and ultimately into drinking water. Siler (1999) reported that caffeine and pharmaceuticals in water from wells could be used to trace wastewater.

Little is known about the presence of pharmaceuticals in the aquatic environment in the United States. A national study has been conducted to evaluate the presence of antibiotics and other organic compounds in surface water (Kolpin et al., in press). These investigators report that pharmaceuticals, hormones, or other emerging contaminants were found in the surface water in the United States. At this time, almost no information is available on the presence of pharmaceuticals in bank-filtered water and drinking water produced from bank-filtered water. A preliminary data collection effort was conducted by the U.S. Geological Survey (USGS) at the City of Lincoln bank-filtration site (Verstraeten et al., in press B) near Ashland, Nebraska. Water samples were collected from surface water, a water-supply well (bank-filtered water), raw water (before chemical and physical treatment) and treated drinking water (finished water) at this site on September 9, 1999. Samples were analyzed for 24 antibiotics. Sulfamethoxazole and trimethoprim were detected at concentrations of less than 1 _g/L in bank-filtered and raw water. Both compounds are components of the Bactrim antibiotic. No traces of pharmaceuticals were detected in the treated drinking-water sample. Previous studies indicated that herbicide concentrations such as atrazine decreased from 10 to 60 % by bank filtration depending on how the well field is managed (Verstraeten et al., in press A).

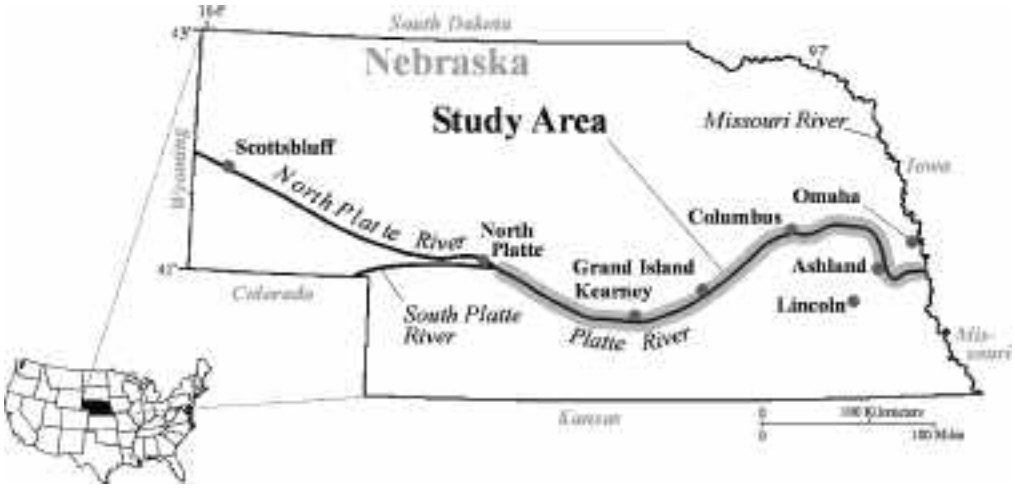

Figure 3: Generalized location of study area and unidentified bank-filtration sites along the Platte River, Nebraska, United States.

Recently (2001), the USGS initiated a study in cooperation with U.S. Environmental Protection Agency (USEPA) and the City of Lincoln to evaluate the transport of pharmaceuticals and other endocrine disrupting compounds, including hormones and herbicides, from the Platte River, through an alluvial aquifer into a municipal water supply in Nebraska. This study will collect and interpret data in support of the development of drinking-water treatment guidance pertaining to bank-filtration efficiency. Data will be compared to similar studies conducted in the United States and other nations, including Germany.

In this paper, preliminary results of another study are presented. Samples were collected from surface water, bank-filtered water, combined untreated water from all active water-supply wells of a municipal well field, and drinking water at three bank-filtration sites (the sites are not identified on figure 3 at the request of the utilities) along the Platte River in Nebraska, United States (figure 3). At the bank-filtration sites, drinking water is produced for cities and towns with population from about 30,000 to almost 500,000 people.

3.2 Description of sites along the Platte River

Three unidentified bank-filtration sites are along the Platte River, in Nebraska, United States (figure 3). At its mouth, the Platte River has a drainage area of about 218,000 km^2 (square kilometers). The mean annual discharge of the Platte River is about 200 m^3/s (cubic meters per second) near its confluence with the Missouri River.

During low discharges in the summer months, the Platte River becomes braided into multiple channels with exposed sandbars. At times and at places, its flow consists almost entirely of wastewater from STPs (Patrick O'Brian, Nebraska Department of Environmental Quality, written commun., 2001). The Platte River and its tributaries receive municipal waste from most cities along their courses, except the City of Omaha, which releases its wastewater into the Missouri River near and above its confluence with the Platte River. Along the Platte River and its tributaries, there are an estimated 200 STPs that release treated or untreated wastewater at rates from about 0.4 ML/d (million liters per day) to about 200 ML/d (Ronald Ash, Nebraska Department of Environmental Quality, oral commun., 2001). In addition, an estimated 7 000 or more AFOs, including about 1 000 CAFOs, exist in Nebraska (Dennis Heitmann, Nebraska Department of Environmental Quality, oral commun., 2001). They vary in size from more than 1 to almost 100 000 cattle and pigs. During runoff events or chronic wet periods (several rainfalls within 1 month leaving the soils very saturated and standing water in collection pits high), discharges occur from these AFOs into nearby streams. Nebraska was ranked second in 1997 in the production of cattle and calves in the United States with more than 14million, or, about 6.8% of the national production (U.S. Department of Agriculture, tbl36.pdf, accessed June 28, 2001). Moreover, Nebraska was ranked seventh in 1997 and sixth in 2000 in the production of pigs in the United States with about 3.5 million, or 5.7% of the national production (U.S. Department of Agriculture, 2001a; U.S. Department of Agriculture, 2001b)

30 m and varying in distance from tens to hundreds of meters from the river. The vertical water-supply wells typically produce as much as 5 ML/d. The other well is a horizontal collector well on an island in the Platte River

less than 30 m from the banks of the river. The collector well consists of a main vertical caisson and seven horizontal laterals, totaling more than 400 m in length. It is completed at a depth of less than 30 m. The collector well is equipped with three large pumps and has a capacity of 45 ML/d.

3.3 Preliminary Results for Pharmaceutical Residues

Representative water samples were collected from the Platte River (representative integrated samples), from water-supply wells, and at the drinking–water treatment plants including raw (a combination of bank-filtered water from as many as 50 water-supply wells) and treated water following USGS sampling protocols to prevent cross contamination. The physical and chemical treatments used at the three bank-filtration sites include filtration, chlorination, or ozonation but do not include the use of ultra-violet and granular-activated-carbon treatment. The samples were collected June 4-6, 2001, from the three sites when the Platte River had a moderate discharge (28 to 240 m^3/s). The samples were analyzed at the site for physical parameters and in the laboratory for (1) endocrine disrupting compounds and wastewater indicators, including 63 compounds and 4 nonionic detergent metabolites, with 23 compounds having known or suspected endocrine-disrupting potential (Zaugg et al., in press); (2) 24 pharmaceuticals derived from cattle and other animals (Kolpin and others, in press; Meyer, U.S. Geological Survey, written commun, 2001); and (3) 22 over-the-counter and prescription pharmaceuticals used by humans, and their metabolites (Kolpin and others, in press). The analyte concentrations reported for the wastewater indicators are for filtered water and may greatly underrepresent the total amount of some analytes present in whole surface-water samples for low-solubility analytes that strongly sorb to suspended material. This effect is suspected to be smaller for ground water and treated drinking water.

In general, dissolved-oxygen concentrations in water from water-supply wells were as much as 4.0 mg/L (milligrams per liter) and were less than 1 mg/L in water from two of four water-supply wells, suggesting potential reducing conditions may exist at some sampling sites. The pH of the river exceeded 8.0. Based on previous studies in Nebraska (Steele and Verstraeten, 1999; Verstraeten et al., 1999; Verstraeten et al., in press A), and based on water temperature and pH of water in the water-supply wells measured during this investigation, there is sufficient evidence that these wells receive bank-filtered water, except water-supply well W3 (Table 2). Previous studies established that, at times, water in the collector well sampled consisted of more than 50% surface water under similar conditions to those used during this investigation (Steele and Verstraeten, 1999).

Preliminary results of the analyses of water samples from the bank-filtration sites for selected endocrine-disrupting compounds and wastewater products (table 2) suggest that endocrine disrupting compounds, including herbicides, can occur in river water, well water, and drinking water, further suggesting that transport of these compounds can occur through bank filtration. Transport of contaminants would depend upon the proximity of contaminant sources, the concentrations of compounds occurring in the river, the efficiency of bank filtration, the characteristics of the compounds (hydrophobic versus hydrophilic, vulnerability to microbial degradation, etc.), and local drinking-water treatment methods. Twelve of the 63 compounds for which the samples were analyzed were detected in the water samples. They were more often detected in river water (six compounds) and water from the water-supply wells (10 compounds), than in drinking water (two compounds, one a drinking-water treatment by-product and the other, a herbicide). Two of the 12 compounds detected were fecal indicators. Two plant sterols were found at one of the three bank-filtration sites in the river and well water. Caffeine was detected in one raw-water sample at one bank-filtration site (43 ng/L).

Table 2. Selected results from the analysis of water samples at three bank-filtration sites, Nebraska, United States, June 4-6, 2001.

[BF, bank filtration; *, where shown, the level reported is not the method reporting level but the method detection level; RV, water from the Platte River; W, water from water-supply well; UW, untreated water combined from all active wells in the drinking-water treatment plant; DW, treated drinking water; ˚ C, degrees Celsius; mg/L, milligrams per liter; H, herbicide; ND, not detected; NA, not analyzed; E, estimated because concentrations are below the method reporting level; ng/L, nanograms per liter]

Parameter	Reporting units	Method reporting * level	BF Site A			BF site B					BF site C			
			RV1	W1	DW1	RV2	W2	W3	UW	DW2	RV3	W4	UW2	DW3
Physical parameters														
Temperature water	˚ C	0.5	18.5	16.5	16.5	18.0	14.0	4.5	17.5	16.0	17.0	17.5	18.0	17.0
pH	Units	0.1	8.2	7.8	7.8	8.2	7.8	7.8	7.5	7.6	8.9	7.6	7.2	8.3
Dissolved oxygen	mg/L	0.01	6.10	1.75	2.84	6.54	0.88	3.82	1.30	5.14	7.13	0.41	1.29	3.11
Selected endocrine-disrupting compounds including pharmaceuticals and other wastewater indicators*,														
Caffeine	ng/L	500	ND	ND	ND	ND	ND	ND	ND	NA	ND	ND	E 43	ND
Phenol (Disinfectant)	ng/L	500	ND	E 330	ND	ND	E 0.27	ND	E 490	NA	ND	ND	ND	ND
Prometon (H)	ng/L	500	E 29	E 21	ND	ND	ND	ND	ND	NA	ND	ND	ND	ND
Bromacil (H)	ng/L	500	E 54	ND	ND	ND	ND	ND	ND	NA	E 140	E 73	E 47	ND
Metolachlor (H)	ng/L	500	E 79	E 10	E 22	E 48	E 160	ND	E 130	NA	E 340	E 220	E 270	E 280
Bisphenol A	ng/L	1 000	ND	ND	ND	ND	ND	ND	ND	ND	ND	E 88	ND	ND
Triphenyl phosphate	ng/L	500	ND	ND	ND	ND	ND	ND	ND	ND	ND	E 120	ND	ND
3-_-coprostanol (Fecal indicator)	ng/L	2 000	ND	E 200	ND	ND	ND	ND	ND	NA	ND	ND	ND	ND
Cholesterol (Fecal indicator)	ng/L	2 000	E 680	E1 700	ND	ND	ND	ND	ND	NA	E 510	ND	ND	ND
_-sitosterol (Plant sterol)	ng/L	2 000	E 710	2 100	ND	ND	ND	ND	ND	NA	E 1 100	ND	ND	ND
1,7 Dimethylxantine (derivative of caffeine?)	ng/L	19*	ND	ND	ND	E 5.8	ND	ND	ND	ND	ND	ND	ND	ND
Sulfamethoxazole (antibiotic, Bactrim compound)	ng/L	23*	E 7	E 2	ND	ND	ND	ND	E 9	E 6	ND	ND	E 9	ND

Note: Data on endocrine disrupters other than pharmaceuticals and wastewater indicators are presented but not discussed in detail in the text.

The concentration of a compound was not always less in the water from the water-supply well than in the river, as one would expect because of attenuation by processes such as adsorption-desorption, photodegradation, or microbial degradation. The sampling scheme did not take into account the travel time of water from the river into the well or area of contribution to this well. All samples at a bank-filtration site were collected within 4 hours of each other. Therefore, the same aliquot of water from the river was not collected from the water-supply wells and the potential for removal of a compound cannot be determined from the data collected during this study.

One of 27 pharmaceutical compounds used by humans (sulfamethoxazole) was detected. Sulfamethoxazole was detected in river, well, and treated drinking water. Because the same aliquot of water was not collected at a bank-filtration site, no attempt to interpret the degree of attenuation during bank filtration was made for this compound. However, smaller concentrations of this antibiotic were detected in well and drinking water than in river water, except at bank-filtration site C.

Samples were not analyzed for ethylenediaimetetreacetate (EDTA), which is thought to be a very good indicator of wastewater contamination that occurs in large concentrations throughout the year. As mentioned previously, a larger study evaluating these temporal variations and actual transport mechanisms of these compounds recently has been initiated by the USGS in the United States. This study and other studies in the United States, Germany, and other nations will provide more answers to some of the questions raised herein.

3.4 Preliminary Conclusions

The data collected during this reconnaisance indicate that transport of wastewater and endocrine-disrupting compounds from a river through groundwater into drinking water may occur. However, the data collected during this reconnaissance do not identify the potential temporal variations in detections and concentrations of the compounds analyzed. When lower discharges occur and surface water is dominated by wastewater effluent, or during and immediately after a rain event, when STPs cannot treat all of their water, one might detect more compounds in river water, water from water-supply wells, and treated drinking water. At these times, more

compounds could be detected in water at concentrations that can be an order of magnitude different (smaller or larger) than those found during this study.

Conclusions

The investigations in Germany and the United States revealed that several polar organic compounds, especially several PhACs, are relevant to drinking-water production at bank-filtration sites. In Germany, several compounds, such as bezafibrate, diclofenac or MCPP, seem to be removed effectively during bank filtration, but other compounds, such as carbamazepine, clofibric acid, primidone, propyphenazone, compound X, bentazone, DDA, TCIPP, and TCEP, are not. The latter were detected at concentrations between 15 and 180 ng/L in the water-supply well of the Lake Wannsee transect in Berlin, Germany. Endocrine disrupting compounds including herbicides and one pharmaceutical were detected in surface water, water-supply wells, or drinking water at three bank-filtration sites in Nebraska, United States. Further investigations are needed to answer questions about the origin of several compounds and their behavior during the bank-filtration process.

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The Qualitative and Quantitative Evaluation of Estrogen and Estrogen-Mimicking Substances in the South African Water Environment: a 1996-1997 Perspective *

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Abstract

Background: Recent reports indicate that many chemical compounds which enhance the quality of life (detergents, agricultural products, additives to plastic, dyestuffs, paint components, and pharmaceuticals, to name but a few) possess endocrine-disrupting effects. These chemicals mimic estrogen, androgen and thyroid hormones. Reports of reproductive abnormalities and abnormal hormone levels are well documented in both human and wildlife (Elliot *et al*., 1989, Best, 1994, Hileman, 1994, Hose, 1995, Mellius, 1995). The aims of this study were (1) to identify estrogen and estrogen-mimicking substances (EMS) in the South African water environment and (2) to quantitate levels of these substances in the water.

Materials and Methods: Water samples were obtained from the Pretoria, Witwatersrand, Vereeniging (PWV) area and included industrial effluent (n=5), dam (n=4), river (n=1) and drinking water (n=4). Standard HPLC and/or GC-MS analysis were conducted by the SABS and the Council for Scientific and Industrial Research (CSIR) to measure amounts of the following chemicals: nonylphenol, o′p′-DDE, p′p′-DDE and o′p′-DDD, lindane, and two polychlorinated biphenyls (PCBs) - Arochlor 1254 and Arochlor 1260. The Jockey Club Laboratory of Southern Africa tested for ethinylestradiol (EE), while the PU for CHE tested for atrazine using HPLC.

Results: The following levels of EMS were found: nonylphenol: 2-5µg/l, 11µg/l; o′p′-DDE, p′p′-DDE and o′p′-DDD: <1µg/l; lindane: 6.7µg/l; Arochlor 1254: 2 & 5 of the 6 isomers and Arochlor 1260: 3 & 8 of the 9 isomers and atrazine: <0.25µg/l-1.193µg/l and 2 and 10µg/l. EE could not be detected by the methods used. No EMS were detected in the drinking water tested.

Conclusions: These data show contamination of river, dam and industrial effluent with EMS. Many of these substances undergo bioaccumulation. Their presence in river and dam water, which becomes the source of drinking water, should be a matter of concern. Future studies are planned to re-assess the South African water environment on a continual basis.

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Introduction

The issue of environmental estrogens and putative estrogen-mimicking substances has been a subject of emotional controversy in recent years, especially because these chemicals are accused of wreaking havoc with human and animal reproductive system function.

Much concern has been raised about possible adverse consequences arising from release into the environment of substances with estrogenic properties. Possible effects on humans include reproductive disorders such as reduction in sperm count and quality, cryptorchidism, testicular cancer, male breast cancer, infertility, and abnormalities in sperm. Environmental effects such as the occurrence of the female yolk protein vitellogenin in male fish and developmental abnormalities in reptiles have also been observed and there is evidence that these effects can be elicited by estrogenic chemicals released into the environment. The effect that these substances

have on the reproductive systems of both human and animal life is well documented in the literature (Bergeron *et al.,* 1994; Best *et al.,* 1994; Bishop *et al.,* 1991; Bull, 1980; Carlsen *et al.,* 1992; Colborn, 1991; Cooke, 1973; Cooper, 1991; Davison & Sell, 1974; Deeming & Ferguson, 1988; Dibb, 1995; Dutta *et al.,* 1994; Elliot *et al.,* 1989; Espir *et al.,* 1970; Faber & Hickey, 1975; Faber & Hughes, 1993:35-39; Facemire *et al.,* 1995; Falck *et al.,* 1992; Fox, 1992; Fry & Toone, 1981; Giesy *et al.,* 1994; Ginsburg *et al.,* 1994; Giwercman & Skakkebaek, 1992; Giwercman *et al.,* 1993).

Chemicals implicated in causing estrogenic effects, or general endocrine disrupting effects, include many substances commonly used in industrial and household products which enhance the quality of life. Such substances include some detergents, agricultural pesticides, additives to plastic, dyestuffs, paint components and pharmaceuticals.

Detailed lists of the chemical names and classification of the various estrogen and estrogen-mimicking substances, the various usages and applications of, and some structures of estrogen and estrogen-mimicking substances, have been compiled by the authors. This information was generated during a previous study conducted in 1996-1997.

Millions of women are using the oral contraceptive pill but little is known about the fate of the excreted material. In general, there is a paucity of information regarding the organic chemical content of sewage effluent and raw and potable waters in South Africa. Specifically, there is a lack of information regarding levels of estrogen-mimicking substances such as nonylphenol and polychlorinated biphenyls in raw sewage, sewage effluent and potable water in South Africa. This information will assume more significant proportions as South Africa prepares for increased water recycling and reclamation.

When seen in this light, an investigation was necessary to evaluate the current situation of the South African water environment, as very little is known about the occurrence of these substances in water.

This project was not aimed at examining any specific cause and effect relationships. It was rather aimed at establishing which of the alleged estrogen-mimicking substances were likely to occur in the water environment, what the possible sources were and how the source(s) could be reduced if found to be present in unacceptable concentrations. If these facts indicate a direct cause and effect they could further be evaluated by the water industry for cost effective treatment.

Materials and Methods

(a) Literature suvey. A comprehensive literature survey was undertaken to identify substances reported to be estrogen-mimicking. More than 600 references were found, and 142 substances identified and classified in groups according to their use. This list was reduced to those substances that were most likely to be found in the South African water environment, and resulted in a short list of substance to be investigated. The following elimination procedures were applied to reduce the list of 142 substances: **(a)** Personal visits were undertaken to Industrial companies, and the Institute for Water Quality Studies (Pretoria) for guidance as to which of these substances were most likely to be found in South Africa, and especially in the water environment. **(b)** Personal communications were made to various institutions over a period of 2_ months to determine which of these substances were already found in South Africa, what their specific location was as well as at what concentration levels they were found. **(c)** An investigation of the imported quantities of some of the estrogen and estrogen - mimics was made but with little success because of lack of co-operation. This data would have been useful as to see which of the substances are used more than others in terms of import and export values. **(d)** A data search through Chemdata was undertaken to determine which of these substances are manufactured locally. This would ensure that only relavent chemicals that were most likely to contaminate the South African environment, were studied. **(e)** Elimination by means of the chemical characteristics (water solubility & melting points) and some toxicological data (LD_{50} & LC_{50} values). **(f)** Eliminating from the list substances that were too vaguely described such as “some PCB’s” and “organochlorinated hydrocarbons” which covered a too wide spectrum of substances as well as substances that were highly unlikely to pollute the water environment in a harmful manner: e.g. “boron”, “musk ketone and scent”, “Clone ID5”, “carboxylic acid” and “chicken manure”. **(g)**

Eliminating from the list those substances that were not able to be determined and analysed in South Africa due to reasons such as inadequate methods or equipment as well as due to the high cost involved in analysing these substances - as was the case with the dioxin determination. The above resulted in a list of estrogen and estrogen-mimics that could be tested and analysed for at specifically allocated areas in South Africa. The above mentioned list included the following substances: atrazine, chlordane, DDT (total), dicofol, endosulfan (total), ethinylestradiol, lindane, malathion, methoxychlor, nonylphenol and the PCBs: Arochlor 1254 and 1260.

(b) Estrogen and estrogen-mimicking substance sampling. Water samples were obtained from Pretoria, Witwatersrand and Vereeniging areas and included industrial effluent (n=5), dam (n=4), river (n=1), and drinking water (n=4). The reasoning behind the locations selected were as follows: **(a)** The Vaalriver (monitored at Vereeniging) was sampled at varying sites taken from the Barrage because of the large number of factories neighbouring or situated around it. Taking the samples at intervals 0, 10 and 24 km from the Barrage made it possible to assess what the concentration levels were at different distances from the Barrage). **(b)** The effluent of chemical manufacturing companies were inspected because of their local manufacturing of estrogen-mimics such as nonylphenol, DDT, DDE, DDD, methoxychlor, toxaphene, endosulfan, malathion, chlordane, dicofol which could thus lead to unintentional spillage into the water environment.

These samples were collected in the following manner: **(a)** 2.5 L Amber glass bottles were pre-rinsed with distilled water and left to dry (glass bottles were chosen because most of the samples contained organic compounds). **(b)** The bottles were then closed and clearly labelled, containing the name of the sampling area. **(c)** The bottles were transported to the sampling area, where the sampling took place. **(d)** The sampling was done as follows: in the case where industrial effluent was taken, the sampling was done by lowering a bucket into the manhole at the discharge site. The water was then transferred into the supplied bottles. This was done either before the effluent entered the river or at intervals 0, 10 and 24 km from the Barrage. Dam and river samples were sampled by lowering the bucket approximately 40cm under the water and allowing it to fill. The water content was then transferred to the bottles. The drinking water samples were taken directly from the water taps in the following areas of investigation: Windhoek, Pretoria, Johannesburg and Vereeniging. Samples were fozen until analyzed. Three drops of analytical grade chloroform were added to each sample.

(c) Analytical methods

Determination of ethinylestradiol in water. Extraction was carried out by the Potchefstroom University for Christian Higher Education; the Jockey Club of Southern Africa Laboratory assisted with detection of ethinylestradiol in sewage effluent by means of Gas Chromatography/Mass Spectrometry (GC/MS). Samples were extracted by means of solid phase extraction (SPE) followed by a further clean-up procedure using immunoaffinity column chromatography (IAC). *The solid phase extraction* was as follows: SPE columns (3 cc/500 mg Bond Elut$^{\hat{a}}$, C_{18} columns, Varian, Harbor City, CA) were conditioned with 6 ml of methanol, followed by 6 ml of water. To the 1 liter sample, 2 ml of orthophosphoric acid was added, and the sample was put through a 0.7 µm glass fiber filter (Whatman GF/F, Whatman International Ltd., Maidstone, England). The sample was then put through the columm by means of a large volume extraction tube under vacuum. The SPE columns were rinsed with 6 ml of water and dried by suction. Elution was carried out with 4 ml of methanol. The eluant was placed in a 47° C water bath and dried with a stream of nitrogen gas. After addition of 2 ml of a washing solution the sample was analysed by IAC. *Immunoaffinity column extraction (IAC)*: Immunoaffinity gels were purchased from the Laboratory of Hormonology, Marloie, Belgium. Gel specifications were as follows: (1) ethinylestradiol gel: matrix, Sepharos® CNBr4B; antiserum: anti-ethinylestradiol; capacity as determined with tritiated tracer: >50 ng/ml of gel. Crossreaction of the antibody: ethinylestradiol, 100%; norethindrone, 10%; 17-β-estradiol and estrone, 0%. (2) diethylstibesterol gel: matrix: Sepharos® CNBr4B; antiserum: anti-diethylstilbesterol; capacity as determined with tritiated tracer: >50ng/ml of gel. Crossreaction of the antibody: trans-diethylstilbesterol, 100%; transdithylstilbesterol di-n-propionate, 9.7%; hexestrol, 3.3%; dienestrol, 1%; trans-diethylstibesterol-dimethylether, 0.4%; benzestrol, 0.1%. *IAC extraction procedure:* 1 ml of gel containing ethinylestradiol and the internal standard was transferred to a column. The column was washed with 2 aliquots (5 ml each) of PBS (0.05 M, pH 7.5). The sample was applied after gel equilibration with 3 washes of 3 ml distilled water. The column was washed 3 times with 3 ml of washing solution and eluted with 3 ml of elution solution. The eluate was dried in a 47°C water bath with a stream of nitrogen gas; 50 µl of methanol were added, and the solution vortexed, and transferred to a vial for analysis. The columns with

gel were washed with 1ml of elution solution, regenerated with 2 x 5ml of PBS solution and stored at 4°C in a PBS solution for re-use.

Determination of alkylphenolic compounds in water. Analyses were performed at the CSIR's Food Science and Technology division in Pretoria, which is an accredited analytical facility. Standard methods of extraction were used, and analysis was performed by means of HPLC on a reversed phase column with fluorescence detection.

Determination of organochlorine pesticides in water (all o′p′ and p′p′ forms of DDD, DDE and DDT, methoxychlor, α and β isomers of endosulfan, chlordane, and dicofol). Analyses were carried out by the General Chemistry division of SABS, an accredited analytical facility, with a Hewlett Packard Series 6890 instrument; injection volume was 1 µl. The following conditions were used for the specific analyses: (1) chlordane: column 1, HP-5 5% phenyl methyl siloxane, 0.25 µm, 30 m x 320 µm (Hewlett Packard, Palo Alto, CA), flow rate, 1.7 ml/min; column 2: HP-1 methylsiloxane, 2.65 µm, 30 x 530 µm (Hewlett Packard, Palo Alto, CA); flow rate 4 ml/min; run time, 30 min; column temperature, ambient; detector, front: ECD with nitrogen gas, back NDP with nitrogen gas. (2) α- and β-endosulfan: column HP-5 5% phenyl methyl siloxane, 30 x 25 µm (Hetlett Packard, Palo Alto, CA); flow rate: 0.8 ml/min; run time: 29 minutes; detector: MSD Transfer Line Heater; retention time α-endosulfan, 20.49 min, β-endosulfan 22.13 min. (3) DDT and methoxychlor: column, HP-5 5% phenyl methyl siloxane, 0.25 µm, 30 x 25 µm (Hewlett Packard, Palo Alto, CA); flow rate, 0.8 ml/min; run time, 29 min; detector, MSD Transfer Line Heater; retention time, p′p′-DDE 21.08 min, p′p′-DDD 22.39 min, o′p′-DDT 22.50 min, p′p′-DDT 23.80 min, methoxychlor 26.74 min. *Extraction procedure:* Two 500 ml aliquot of each sample was removed after shaking; extraction was carried out in duplicate. Pesticides were extracted according to the Association of Official Analytical Chemists' method number 990.06 (Sawyer *et al.,* 1995). Final analyses were carried out by gas chromatography employing electron capture, flame photometry (nitrogen/phosphorus selective), or by mass selective detection. Recovery determinations done at 1µg/l were: α-endosulfan 120%, β-endosulfan 113%, lindane (γ-BHC) 91%, chlordane 90%, methoxychlor 133%, p′p′-DDE 128%, p′p′-DDD 134%, o′p′-DDT 126%, p′p′DDT 129%. The lower limit of detection was 1µg/l.

Determination of organophosphate pesticides in water (malathion). Analyses were carried out by the General Chemistry division of SABS with a Hewlett Packard Series 6890 instrument. Analytical conditions were: column, HP-5 5% phenyl methyl siloxane, 0.25µm, 30 x 25 µm (Hewlett Packard, Palo Alto, CA); flow rate, 0.8 ml/min; run time, 29 min; column temperature, ambient; detector, MSD Transfer Line Heater; retention time 18.69 min. *Extraction procedure:* Two 500 ml aliquots of each sample were removed after shaking; extraction was carried out in duplicate. Pesticides were extracted according to the Association of Official Analytical Chemists' method number 990.06 (Sawyer *et al.,* 1995). Final analyses were carried out by gas chromatography employing a mass selective detector. Recovery determinations gave a mean value of 132%; the lower limit of detection was 1 µg/l.

Determination of polylchlorinated biphenyls (PCBs) in water. Analyses were carried out by the General Chemistry division of SABS with a Hewlett Packard Series 6890 instrument. Analytical conditions were: column 1, HP-5 5% phenyl methyl siloxane, 0.25µm, 30 x 320 µm (Hewlett Packard, Palo Alto, CA), flow rate 1.7 ml/min, column 2, HP-1 methyl sioxane, 2.65µm, 30 x 530 µm (Hewlett Packard, Palo Alto, CA); flow rate 2.4 ml/min; run time, 30 minutes; column temperature, ambient; detector, front: ECD with nitrogen gas, back: NPD with nitrogen gas. *Extraction procedure:* Two 500 ml aliquots of each sample were removed after shaking; extraction was carried out in duplicate. Pesticides were extracted according to the Association of Official Analytical Chemists' method number 990.06 (Sawyer *et al.,* 1995). Final analyses were carried out by gas chromatography employing electron capture and nitrogen/phosphorus selective detection. Recovery determinations carried out at a level of 1 µg/l gave the following mean values: arochlor 1245, 92%; arochlor 1260, 72%. The lower limit of sensitivity was 1 µg/l.

Determination of triazine herbicides in water. (atrazine). Analyses for atrazine determination in dam water were carried out by the Potchefstroom University for Christain Higher Education with a Hewlett Packard Series 1050 instrument equipped with an autosampler, gradient pump, UV detector and HP Chemstation data handling and control system; column, Hypersil ODS (Phenomenex); 5µm, 250 x 4.6 mm, internal standard: terbutylazine

(TBA); flow rate, 1 ml/min; injected volume, 100 µl; run time, 15 min; column temperature, 40° C; detector, UV at 220 nm; retention times, atrazine 5.96 min, terbutylazine 8.99 min; mobile phase, 50:50 acetonitrile:0.01 M ammoniumdihydrogen-orthophosphate, final pH 6.5. *Extraction procedure*: 2 ml of a 0.50 mg/l solution of TBA and 5 ml 6N ammonium hydroxide solution were added to a 500 ml sample. The sample was then shaken and extracted with 3 aliquots of dichloromethane (30 ml each) and centrifuged at 3000 rpm for 5 min. The top layer was removed and discarded; the sample was placed in a 47°C waterbath and dried under a stream of nitrogen gas. After drying, 5 ml of mobile phase was added to the sample and placed in an ultrasonic waterbath; the sample was then transferred to a vial and injected into the HPLC. The lower limit of detection was 0.25 µg/l. Extraction efficiency was 78.22% for 1µg/l, 8.37% for 5 µg/l, and 75.27% for 1 µg/l. *Atrazine determination in industrial effluent*. This analysis was carried out by the General Chemistry division, SABS using a Hewlett Packard Series 5880A GC instrument; column, Megabore 15m x 0.53 mm; column temperature, 150° C; detector, nitrogen phosphorus (NPD).

Results

(a) Literature survey

From the literature 142 substances were identified as estrogen-mimicking substances. These substances were classified according to their use. The results are shown in Table 1. A literature survey and personal survey to determine which of estrogen and estrogen-mimicking substances were already found in the South African environment (water and in animal tissue) was also conducted. The results of this survey indicated that the South African water environment was contaminated with estrogen-mimicking substances, as early as 1974, but at the time of the analysis, were not known to be estrogen-mimicking (van Dyk 1978, Grobler *et al.,* 1996, Grobler 1994, Hassett *et al.,* 1987, Coetzee & Vahrmeijer, 1986, Pick *et al.,* 1981, de Kock & Randall, 1984, Gardner, 1984, de Kock, 1985, Lötter & Bouwman, 1995, Peakall & Kemp, 1976, de Kock & Boshoff, 1987, Cockroft *et al.,* 1989, Mason & Rowe-Rowe, 1992, van Dyk *et al.,* 1977, Roux *et al.,* 1994, Hassett *et al.,* 1987, de Kock & Lord, 1989, Bouwman *et al.,* 1990, Butler *et al.,* 1983). Most of these substances were from agricultural and industrial origin. The following substance were found: endosulfan (α and β), 2,4-D, 4-nonylphenol, 4-octylphenol, aldrin, alkylphenolic ethoxylates, arochlor 1254, atrazine, BHC (total), bisphenol A, butylated hydroxytoluene, chlorinated pesticides (excluding atrazine), chlorophenolic compounds, chlordane (α and χ), DDE, dieldrin, ethylhexylphtalate, heptachlor epoxide, lindane, methoxychlor, n-butyl phtalate, n-butylbenzene, o'p'-DDD, p'p'-DDD, p'p'-DDE, p'p'-DDT, PCBs, PDDFs, phenols, poly aromatic hydrocarbons, toluene, toxaphene. These substances were illustrated on a South African map to indicate their prevelance to in specific.

Table 1 Categories of chemicals reported to be estrogen-mimicking

CATEGORY	N*
Herbicides	5
Pesticides/Insecticides	21
PCBs	3
Plasticisers	10
Industrial	9
Pharmaceutical	14
Naturally occurring substances	10
Natural estrogens	6
Phenols	13
Plant substances	17
Miscellaneous	34

* Number of substances identified

(b) *Estrogen and estrogen-mimicking substance detection*

Ethinylestradiol The analytical method, did not identify ethinylestradiol. Extraction efficiencies varied between 10-30% at the 5ng/l level and repeatability was poor. At 1ng/l and lower, no peaks were observed in extracted samples.

Alkyl phenolic compounds such as nonylphenol No nonylphenol was found in the drinking water of Johannesburg, Windhoek, Vereeniging or Pretoria (table 2). However, it was found in the Vaalriver at 0km

Table 2 Estrogen-mimicking substances found in water

SAMPLE IDENTIFICATION	SUBSTANCES TESTED FOR	CONCENTRATION
Vaal River – 24 km upstream from Barrage Vaal River – 10 km upstream from Barrage Vaal River at the Barrage Industrial effluent from local industry	Nonylphenol Nonylphenol Nonylphenol Nonylphenol	2 µg/l 3 µg/l 3 µg/l 1 µg/l
Industrial effluent from other local industries Industrial effluent from other local industries	o'p'-DDE, p'p'-DDE and o'p'-DDE Lindane (χ-BHC)	<1 µg/l 6 µg/l
Effluent from various industries Site 1 Site 2 Site 1 Site 2 Vaal River samples	Arochlor 1254 Arochlor 1254 Arochlor 1260 Arochlor 1260 Arochlor 1254 & 1260	Indicated 2 of the 6 isomers Indicated 5 of the 6 isomers Indicated 3 of the 9 isomers Indicated 8 of the 9 isomers None found
Roodeplaat Dam	Atrazine	0.42 µg/l
Hartbeespoort Dam	Atrazine	1.19 µg/l
Vaal River 10 km upstream from the Barrage	Atrazine	1.18 µg/l
Vaal River at the Barrage	Atrazine	0.60 µg/l
Vaalharts Irrigation Scheme	Atrazine	0.38 µg/l
Boskop Dam	Atrazine	<0.25 µg/l
Local industrial effluent	Atrazine	Apparent level of 2 and 10 µg/l
Johannesburg drinking water Pretoria drinking water Windhoek drinking water Vereeniging drinking water	Organochlorines, Atrazine, p-nonylphenol, Organophosphates and PCBs	No residues found above the detection limit Organochlorines <1 µg/l Atrazine <2 µg/l p-Nonylphenol <1 µg/l Organophosphates <10 µg/l PCBs <5 µg/l

(2.0 µg/l), 10km (4.0µg/l), and 24km (5µg/l) from the Barrage and in local industry plant effluent (10µg/l). The decreasing concentration of nonylphenol with the increasing distance from the Barrage is due to the dilution of the effluent as it flows along the river. The nonylphenol found in the effluent correlates well with information that was accumulated during the course of this investigation, namely that the industry manufactures nonylphenol.

Organochlorine pesticides No organochlorine substances (total DDT, methoxychlor, total endosulfan, chlordane and dicofol) were found in the Vaalriver samples, and industrial effluent or in the drinking water of Johannesburg, Windhoek, Vereeniging or Pretoria (limit of detection: <1 µg/l). o'p' DDE, p'p' DDE and o'p' DDD were found in a sample of a local industry with a concentration of < 1µg/l. A lindane (χ-BHC) concentration of 6.7 µg/l was also found in an industrial sample.

Organophosphate pesticides No malathion was found in any of the samples taken. The lowest limit of detection for the drinking water tested was: < 10 µg/l.

Polychlorinated biphenyls (PCBs) No PCB content was found in the drinking water of Johannesburg, Windhoek, Sasolburg or Vereeniging. The lowest limit of detection for the drinking water tested was: < 5µg/l. The industrial effluents were analyzed for two PCB's: Arochlor 1254 (6 isomers) and Arochlor 1260 (9 isomers). One industrial effluent sample contained five isomers of Arochlor 1254 and eight isomers of Arochlor 1260 while another contained only two isomers of Arochlor 1254 and three isomers of Arochlor 1260. No PCB content was found in the Vaalriver samples, or in industrial effluent tested.

Triazine herbicides such as atrazine:. *Atrazine in dam water:* Table 2 shows the levels of atrazine in dam water. It can be seen that atrazine was found in all of the dams sampled at concentrations varying from a high of 1.193 µg/l (Hartebeespoort dam) to as to a low of < 0.25 µg/l (Boskop dam).

Table 3: Atrazine found in water

SAMPLE SOURCE	CONCENTRATION (µg/l)
Roodeplaat dam	0.416
Hartebeespoort dam	1.193
Vaalriver - 0 km from the Barrage	0.603
Vaalriver - 10 km from the Barrage	1.179
Vaalharts Irrigation Scheme	0.383
Boskop dam	< 0.25

Atrazine in industrial effluent: Atrazine was detected at a level between 2 and 10 µg/l in two industrial effluent samples. Due to high residues of nitrogen containing compounds present in these samples, quantitative analysis was not possible. *Atrazine tested for in drinking water.* No atrazine was found in the drinking water of Johannesburg, Vereeniging or Pretoria. The lowest limit of detection was: < 2µg/l. The levels found for all of these substances were within the µg/l range found internationally.

Discussion and Conclusions

Contamination of water supplies by man-made chemicals is an area of major concern throughout the world. During the past 30 years attention has been directed to identification and quantitation of residues of pesticides that persist in the environment. Many of these chemicals have been found to be toxic and to have carcinogenic or mutagenic potential and thus to pose a threat to the health of aquatic species and man.

Recently it has been shown that many of those previously studied chemical pollutants also have the ability to mimic the activity of estrogenic hormones, and to disrupt function of the endocrine system in a wide variety of species. The literature survey conducted as part of this study identified 142 man-made chemicals which have been found in the environment at specific locations and which have endocrine-disrupting effects. Investigators have directed particular attention to studies of PCBs, Arochlor 1254, Arochlor 1260 and DDT, since the lipophilic nature of these compounds results in their accumulation in the body. The reported effects on the endocrine system of the chemicals studied include, among others, hermaphrodism, cryptorchidism, abnormal sperm, and abnormal ratios of estrogen:testosterone in the male. The potential seriousness of the action of these chemicals has been discussed extensively (Aherne & Briggs, 1989, Ando *et al.,* 1985, Anon., 1995, Best *et al.,* 1994, Bouwman *et al.,* 1990, Espir *et al.,* 1970, Falck *et al.,* 1992, Carlsen *et al.,* 1992, Giwercman & Skakkebaek, 1992.)

Very little information exists regarding the presence of estrogen-mimicking chemicals in the water of underdeveloped countries. The analyses carried out in the study reported here provide the first comprehensive information about the level of contamination of critical components of the South African water supply by chemicals with this type of bioactivity.

The levels of these contaminants were not detected in drinking water, and appear to post no threat to human health at this time. Since chemicals with this type of activity were detected in other water samples, it appears that current water purification procedures remove these substances effectively. It will be important to monitor this situation carefully during the next several years, as the population and industrialization increase.

The contamination of industrial effluent with DDE, DDD, Arochlor 1254 and Arochlor 1260 is of concern, as is the contamination of dam and river water with PCBs. These water sources are accessible to rural communities. Some of these communities have little access to purified water, and thus there is a great temptation to use the more easily obtained river water.

The situation in South Africa is representative of that in the other underdeveloped nations of Africa, where there are widely divergent living conditions among the urban and rural populations. Since South Africa has a

position of leadership among the other sub-Saharan countries, the information from this study has the potential for a providing a critical impact on governmental water policies throughout Southern Africa.

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Biographical Sketch

Liesl is from in Newcastle, Kwazulu Natal, in South Africa, where she completed her school career and matriculated in 1990 as one of the top 20. She pursued a career in Pharmacy and studied at the Potchefstroom University for Christian Higher Education an obtained her BPharm degree in 1996. She then focused on the Undertaking of a Masters degree in Pharmaceutics from 1996-1997. Her thesis title was *"The qualitative and quantitative evaluation of estrogen and estrogen-mimicking substances in the South African water environment"*. She completed this degree and graduated Cum Laude in 1998. It was during her Masters degree that she became aware of the environmental and health issues surrounding endocrine disruptors. Currently she is working on her PhD with the focus on pharmaceuticals (and endocrine disruptors) found in the South African water environment. During this study she will also pay attention on *in vivo* and *in vitro* effects of these substances on the immune and reproductive system. Since September 1999 she has been a Senior Lecturer at the School of Pharmacy, Medical University of Southern Africa.

The Toxicity of Three Pharmaceutical Agents to Soybean Plants

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Abstract

Substantial quantities of chloroquine, quinacrine, and metronidazole that are used for therapeutic purposes may end up in the environment. The potential effect of these compounds on plants and on the protozoa in soil was assessed. The growth of soybean plants was negatively affected by increasing concentrations of all of the three compounds. Both the plants and soil protozoa were particularly sensitive to low concentrations of metronidazole (0.5 mg metronidazole g^{-1} soil). The implications of these results in agricultural systems are discussed.

Introduction

The fate of pharmaceutical compounds in humans and animals has been widely researched. However, the activity of these compounds in the environment is only beginning to capture interest. Just like pesticides and herbicides, human and veterinary therapeutic agents are designed to be biologically very active chemicals. The quantities of therapeutic compounds that are used per annum in some countries are in the same range as those of pesticides (Hirsch et al., 1999). Figure 1 shows the major routes through which pharmaceutical compounds get into the environment. Excretion, feedlot refuse (for livestock), and deliberate disposal of expired or leftover medicines are the three most common means of introducing therapeutic agents into the biosphere. Thus, manure, runoff septage, groundwater and surface water eventually get inoculated. These inoculation routes could subsequently expose plants and microorganisms directly or indirectly to therapeutic agents (Migliore et al., 1998; Kuhne et al., 2000; Steur-Lauridsen et al., 2000). Once in the environment, the effects of these compounds to soil microbiota, marine organisms, and plants is still largely unknown.

Chloroquine, metronidazole and quinacrine are extensively used therapeutically against protozoa infections such as *Giardia* sp., *Plasmodium* sp., *Trichomonas* sp., and in the case of metronidazole, against some anaerobic bacteria pathogens. Each of these anti-protozoan agents and their metabolites have been detected in urine, feces, and/or livestock manure. Metronidazole is known to be persistent in the environment but the degradation of both chloroquine and quinacrine in the environment has, to the authors's knowledge, not been investigated (Table 1). However, considering the complexity of their chemical structures of both chloroquine and quinacrine, they are likely to be persistent. Compounds that are structurally similar to chloroquine and quinacrine, such as quinoline, are not readily biodegradable (Thomsen et al., 1999).

Some antimicrobial compounds negatively affected the growth and development of plants, the effect varying between plant species (Batchelder, 1981 and 1982). Information about how chloroquine, metronidazole and quinacrine might affect the soil biota and plants once they get into the environment is lacking. Since all of the three compounds are designed to mainly control protozoa in humans and other animals, they are likely to be potentially hazardous to protozoa in soil when manure, sludge or wastewater that contains their residues is applied to soil. Thus, it is likely that soil protozoa would serve as a reasonable bio-indicator of the effects of any of these compounds in the environment. Preliminary studies were conducted to determine the potential effects of chloroquine, metronidazole and quinacrine on soybean (*Glycine max*) plants and on the associated protozoa in the rhizosphere.

2. Materials and methods

Soil and pharmaceutical compounds

A sample of Palmyra gravelly silt loam (pH 6.6, 4.9% organic matter content, and 34% field capacity) was collected from the top 30 cm at a site near Cortland, NY. The soil was sieved through a 2-mm mesh prior to use.

Aliquots of soil that are equivalent to 15 g (dry weight) were introduced into pyrex test tubes (2 cm dia, 15 cm long). Chloroquine, quinacrine dihydrochloride, and metronidazole, purchased from Sigma Chemical Company (St. Louis, Missouri, USA), were, prior to adding to soil, dissolved in deionized water at concentrations of 60, 20 and 7.5 mg ml^{-1}, respectively. The molecular structures are shown in Figure 2. All of the compounds are readily soluble in water.

Toxicity of the pharmaceutical compounds

To determine the phytotoxicity of chloroquine, metronidazole and quinacrine dihydrochloride, soybean (cultivar A4045) was tested in triplicate test tubes (2-cm dia, 15-cm depth) containing the soil. To ensure that the test plants were of uniform stature, the soybean seeds were surface sterilized, pre-germinated, and then introduced into the tubes containing the soil (Jjemba and Alexander, 1999). Each tube contained one soybean seedling. Aliquots of the pharmaceutical compound were added to the respective triplicate tubes to attain the desired concentration of the compound in soil. The phytotoxicity of chloroquine was tested at concentrations of 2, 4, 8, and 16 mg g^{-1} soil, whereas that of metronidazole was tested at 0.5, 1, 2, and 4 mg g^{-1} soil. Quinacrine dihydrochloride concentrations were at 2.6, 5.3, and 10.6 mg g^{-1} soil. A set of triplicate tubes without any pharmaceutical compound was also included. The moisture of the soil in each tube was adjusted to 4 bars and the plants were maintained in the growth chamber with 22-26°C and 12 h light (34 :mol photons m^{-2} s^{-1}).

The appearance of the plants was recorded at 13 days after planting. At the end of this period, the soil from one of the randomly selected triplicate tubes at the respective concentrations that had a plant in each of the three replicate tubes, was uprooted to enumerate the protozoa in the rhizosphere. Both the roots and the attached soil that remained adhered to the roots after slightly shaking the uprooted plant (i.e., rhizosphere soil) was suspended in 2 ml sterile phosphate buffer (0.2M KH_2PO_4 and 0.06M Na_2HPO_4, pH 6.5). The protozoa in the rhizosphere soil were enumerated using the ring method of Singh (1955) with some modifications (Jjemba, 2001). The final population of soil protozoa was then derived following the most-probable number technique (Halvorson and Ziegler, 1933).

Results and discussion

The pre-germinated soybean seedlings that were selected for use in the polluted soil were quite uniform in appearance. All of the three pharmaceutical compounds are toxic to soybean plants but the sensitivity of the plants depends on the type and concentration of the pharmaceutical compound present (Table 2). Concentrations of 8 mg chloroquine or 10.6 mg quinacrine dihydrochloride g^{-1} soil were toxic to soybean. The plants were especially sensitive to metronidazole since levels as low as 0.5 mg g^{-1} soil were toxic. The number of protozoa in the rhizosphere was increased in the presence of both chloroquine and quinacrine dihydrochloride. However, in the presence of only 0.5 mg metronidazole g^{-1} soil, the density of protozoa was reduced by a tenfold.

Metronidazole is weakly adsorbed to soils (Rabølle and Spliid, 2000; Ingerslev and Halling-Sørensen, 2001) and this could have enabled most of the added compound to remain in the soil solution, making it readily available for uptake by the plants. Of the three antimicrobial agents tested, chloroquine is least toxic to soybean whereas quinacrine dihydrochloride is only moderately phytotoxic.

Until recently, interest about pharmaceutical compounds has focused on their direct adverse effects to the end-user. However, they can be introduced to arable land with manure, sludge or through irrigation with wastewater. The environmental risks associated with applying manures, wastewater or sludge to arable land has, in the past focused on the potential spread of pathogens and heavy metals in soil, ground water and on the existing vegetation (US EPA, 1992; Walker et al., 1997). However, the presence of therapeutic agents in these materials is becoming an increasingly recognized environmental risk as well (Halling-Sørensen et al., 1998; Hirsch et al., 1999; Jørgensen and Halling-Sørensen, 2000). Losses in crop production after application of these biosolids has almost always been suspected to be due to high heavy metal concentrations (Harrison et al., 1999).

The preliminary results obtained during this study arouse interest because some of the losses encountered after applying sludge, manure or wastewater may be attributable to non-degraded pharmaceutical compounds. A wider range of crops and pharmaceutical compounds need to be tested. The most common methods of treating sewage include anaerobic and aerobic digestion, composting, and liming. There is a need to investigate the physical-chemical properties of most pharmaceutical agents in manure, sewage, and wastewater as well as the degradability of these compounds during routine treatment of sewage and processing of manure. The concentrations of compounds used in this preliminary study were arbitrary and there is a need to survey manures, sludge, groundwater as well as surface waters for concentrations of pharmaceutical compounds that are typically encountered in the environment. The fact that pharmaceutical compounds have been used for decades is no reason for complacency in evaluating their impact on plants.

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Biosketch:
Patrick Jjemba holds a Masters degree in Soil Science and a Ph. D. in Soil Microbiology. He is currently a Director of Laboratory at Environmental Associates Ltd. in Ithaca, NY where he routinely addresses issues that relate to the quality of wastewater and biosolids.

Table 1: Excretion and biodegradability of the three therapeutic compounds used.

Compound	Excretion (%)	Reference	Degradability	Reference
Quinacrine	10	Kulda and Noh&nkovà, 1995	Persistent?	
Metronidazole	40	Kümmerer et al., 2000	Persistent	Richardson and Bowron, 1985
Chloroquine	70	Goldsmith, 1992	Persistent?	

Table 2. Toxicity of three pharmaceutical compounds to soybean and indigenous soil protozoa in the rhizosphere at 13 days after planting.

Test compound	Concentration (mg g^{-1} soil)	Plant appearance 13 days after planting[a]			Num
		Replicate 1	Replicate 2	Replicate 3	
None	0	++	++	++	78
Chloroquine	2	++	++	++	2700
	4	++	++	+	5400
	8	+	+	-	ND[b]
	16	+	+	+	ND
Quinacrine dihydrochloride	2.6	++	++	++	180
	5.3	++	++	++	120
	10.6	+	-	+	ND
Metronidazole	0.5	++	+	+	8
	1	-	-	++	ND
	2	-	-	-	ND

4	-	-	-	ND

[a]++, seedling growing normally; +, poor (stunted) growth compared to the treatment with no test compound; -, seedling dead 13 days after planting. [b]Not determined

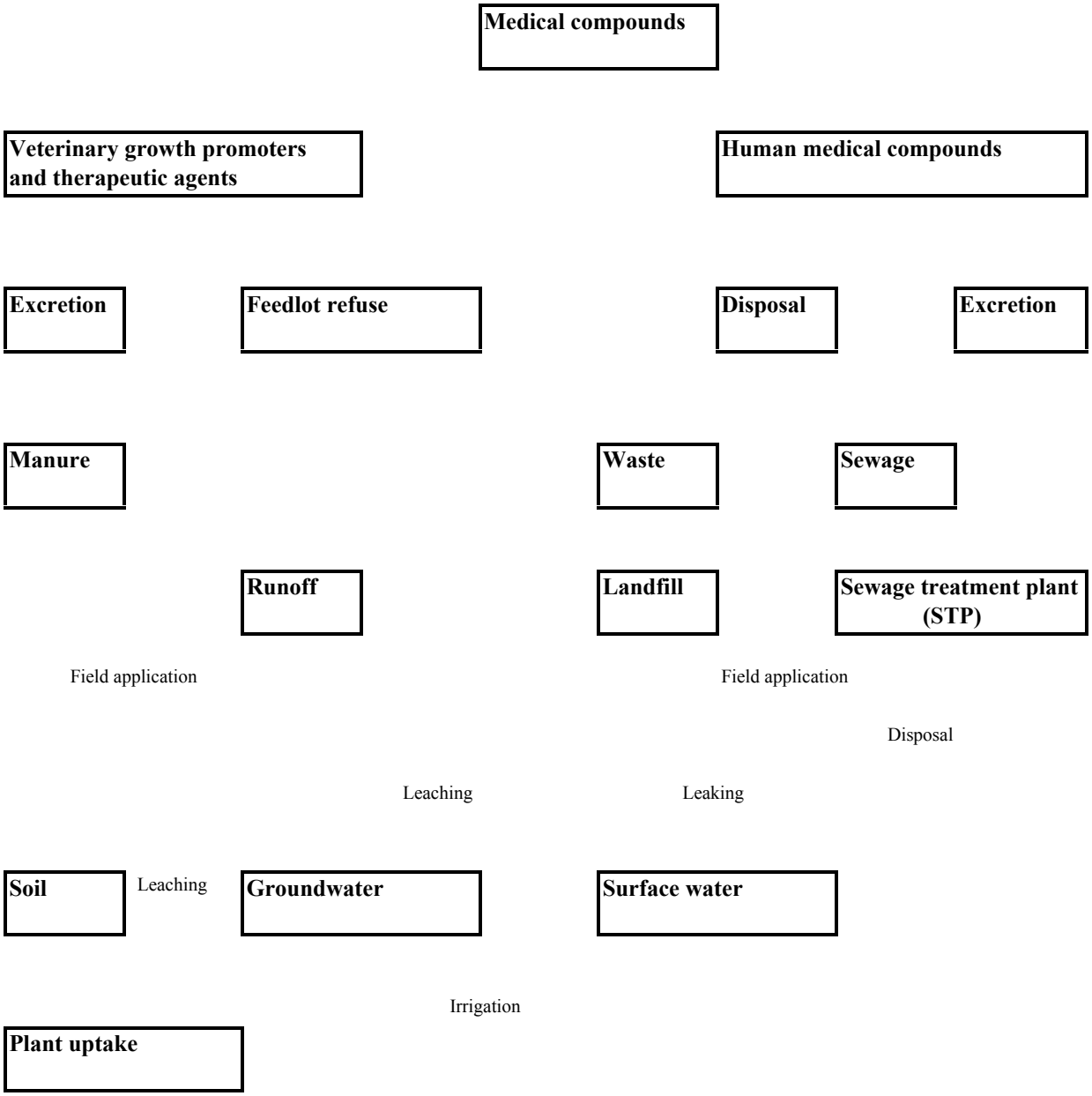

Figure 1: The fate of pharmaceutical agents in the environment.

The Use of DNA Macroarrays to Evaluate the Effects of Environmental Estrogens on Wildlife

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Abstract

During the mid-1990s, several investigations in the United States and United Kingdom showed that wild fish of several species collected downstream of sewage treatment plants or industrial discharges presented expression of estrogen-responsive genes, or phenotypic sex reversal. Subsequently, numerous studies have shown up-regulation of vitellogenin and choriogenin proteins in male fish after exposure to natural estrogens, or estrogenic pharmaceutical, industrial and agricultural chemicals. In this study we have employed DNA macroarray technology to demonstrate a characteristic expression of several genes, including estrogen receptor _ , vitellogenin _ and _, choriogenins _ and _, and transferrin in sheepshead minnows exposed to the natural estrogen, estradiol-17_.

Introduction

During the last decade, several studies in the United States (Folmar et al. 1996, 2001a, Goodbred et al. 1997, Nichols et al. 1999) and United Kingdom (Purdom et al. 1994, Harries et al. 1996, 1997, Lye et al. 1997) demonstrated that wild fish collected downstream from sewage treatment plants (STPs) exhibited a variety of physiological and pathological abnormalities. Chemical analyses of surface waters downstream from both urban (STPs and industrial discharges) and agricultural areas have identified a variety of natural estrogens and estrogenic pharmaceutical, industrial and agricultural chemicals (Desbrow et al. 1998). Both *in vivo* and *in vitro* laboratory studies have demonstrated that these chemicals are capable of up-regulating estrogen receptors (ER) (Yadetie et al. 1999), egg vitelline envelope proteins (choriogenins) (Arukwe et al.1997), and the egg yolk precursor protein vitellogenin (VTG) (Sumpter and Jobling 1995), as well as, causing vitellogenin-specific related pathologies (Wester et al. 1985, Herman and Kincaid 1988, Folmar et al. 2001b), hermaphrodism and reversal of phenotypic sex characteristics (Gimeno et al. 1996, Gray et al. 1999).

The presence of VTG in the plasma of male fish has been broadly accepted as a measure of exposure to natural or anthropogenic estrogens. This measure is frequently thought of as a single metric of exposure and not necessarily a reflection of the physiological status of an individual animal nor predictive of population level effects. To illustrate that physiological responses are considerably more diverse than simple VTG induction, we have employed an emerging technology in the environmental sciences (DNA Macroarrays) to analyze livers from sheepshead minnows (*Cyprinodon variegatus*) exposed to the natural estrogen, estradiol-17_ (E2). Further, we demonstrate how this prototype array technology can be used to monitor for estrogens or other pharmaceutical chemicals in the aquatic environment.

Materials and Methods

Total hepatic mRNA was extracted from 4 adult male sheepshead minnows (SHM) receiving an aqueous exposure to 65 ng/L (measured) of estradiol-17_ (E2) dissolved in triethylene glycol (TEG) for five days (Hemmer et al. 2001). Minipreps of 54 cDNA clones derived from differential display analysis were PCR amplified using primers specific to the M13 sequence of the cloning vector (pGEMT-Easy, Promega, Madison, WI). The PCR products were then purified in spin columns (Qiagen, Chatsworth, CA) and concentrated in a speed-vac. The cDNA samples were denatured with NaOH, heated to 65° C for 10 min, and then immediatedly quenched on ice. A 20X SSC solution (3M NaCl, 0.3M sodium citrate, pH 7.0) that contained 0.01mM bromphenol blue was then added to the samples to yield a final concentration of 0.3M NaOH, 6X SSC and 100 ng/_l cDNA template. The samples were then robotically spotted

(Biomek 2000, Beckman Coulter, Fullerton, CA) in duplicate onto neutral nylon membranes (Fisher Scientific, Pittsburg, PA) using 100 nL pins. The membranes were UV cross-linked and then stored under vacuum at room temperature until hybridized. Various controls, which provide information about the cDNA labeling efficiency, blocking and non-specific binding of the arrays, were also spotted onto the array. Those controls included three *Arabidopsis thaliana* cDNA clones, Cot-1 repetitive sequences, poly A sequence (SpotReport 3, Stratagene, La Jolla, CA) and a M13 sequence (vector but no cDNA insert).

The cDNA probes were generated by random primer labeling DNase-treated (Ambion, Austin, TX) total RNA with [_-^{33}P] dATP (Ambion). The blots were prehybridized and hybridized with ultraArray hybridization buffer (Ambion) at 64° C, and then exposed to a phosphor screen (Molecular Dynamics, Piscataway, NJ) at room temperature for 48 hours. The blots were quantitatively evaluated using a Typhoon 8600 imaging system (Molecular Dynamics).

Results

To insure comparability between membranes, we first hybridized aliquots of identical RNA onto two separate membranes. A scatter plot correlating the intensity values between the two membranes is illustrated in Figure 1. The data points clustered along a slope of one ($R^2 = 0.95$) indicating very little variability between membranes. Slightly more variability occurred at the lower intensity values, an observation also reported in previous studies (Richmond et al. 1999).

The gene transcripts used for this preliminary evaluation were selected from Differential Display (DD) analyses of livers from sheepshead minnows exposed to E2 (65 ng/L), or the carrier solvent TEG in an aqueous flow-through exposure. From the DD gels we selected 54 cDNA clones to be spotted on the array. Fifteen of those genes appeared to be up-regulated, 32 genes were considered constitutive and 7 genes were considered down-regulated in the E2-treated fish. The patterns of expression were similar between the two methods, demonstrating significantly up-regulated expression of the ER, VTG and choriogenin genes and a consistent down-regulation of the iron transport protein, transferrin in the E2-treated fish (Fig. 2B) compared with the TEG control (Fig. 2A). The constitutive genes were uniformly expressed between the two treatments. In the array, constitutive genes had intensity values within the range of 1.27 to 0.83 log intensity units. Based on these values, any cDNA clone in macroarray with an intensity value greater than 1.27 was designated as E2 up-regulated, and any cDNA clone with a value below 0.83 was considered to be down-regulated by the E2 treatment. Figure 3 illustrates the intensities of each gene displayed on the array.

Discussion

In order to insure comparability in our DNA evaluations, we conducted a comparison of two randomly selected membranes from our initial production batch using identical RNA samples (Fig. 1). The strong correlation of the data points between the two membranes ($R = 0.95$) supports comparability of results for the individual membranes.

Although the genes for the array were selected from preliminary DD evaluations, a similar response of the arrayed genes was not guaranteed. We found the induction patterns of the E2-responsive genes on our array (Figs. 2 & 3) to be very similar (up-regulated, down-regulated and constitutive genes) with that observed by DD analyses (data not shown). Figure 2 shows the actual responses on the membrane, while Fig. 3 is a quantitative representation for each of the arrayed genes. In Figs. 2 & 3, letters a - f represent estrogen receptor _ (position #14), vitellogenin _ (position #22), choriogenin _ (position #28), choriogenin _ (position #32), vitellogenin _ (#41), and transferrin (position #54), respectively. The up-regulation of genes (a-e) is consistent with modes of action recognized for estrogenic chemicals. The reason for down-regulation of transferrin (an iron-binding) plasma protein by estradiol is unclear at this time. We also identified at least 15 other bands on our DD gels that appeared to be up-regulated by the E2 treatment. We are using those clones to probe a recently created cDNA library for the sheepshead minnow liver to obtain the identities of those genes.

Our results show that directed macroarray technology can be an effective method to demonstrate broad physiological responses to pharmacological or environmentally relevant doses of estrogenic chemicals. The smaller directed assays containing specific, pre-selected genes avoid problems and costs inherent to large-scale commercially available (pre-manufactured) microarrays or cDNA clone sets.

Conclusion

Once specific DNA is available, from DD or other sources, targeted gene macroarrays provide a rapid assay which is easily evaluated and quantitated. We are currently field validating our array with male sheepshead minnows collected from a variety of locations, that tested positive for vitellogenin in the plasma. We are also evaluating other genes associated with growth and development for addition to the array.

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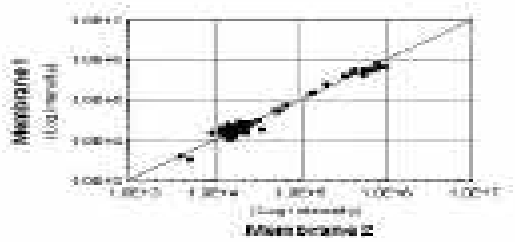

Figure 1. Scatter plot of self-self hybridization. Aliquots of identical RNA samples were hybridized onto two independent arrays. For each cDNA clone, the values from each duplicate spot were averaged and then subtracted from the general background of each membrane. The values were then normalized to the average value of 17 cDNA clones that appeared to be constitutive based on previous Differential Display analyses.

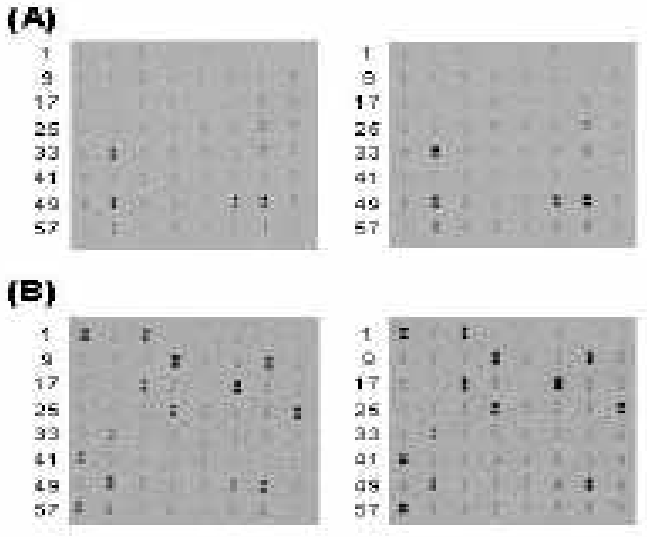

Fig. 2.

Figure 2. The panels in this figure are arrays that were hybridized with RNA from TEG control (A) and E2-treated (B) fish. The numbers to the left of each array represent positions where genes were spotted. No genes were applied to positions #7 and #8. Examples of up-regulated genes include position #14 (estrogen receptor _), position # 22 (vitellogenin _), position #28 (choriogenin _), position #32 (choriogenin _), position #41 (vitellogenin _) and position #54 (transferrin).

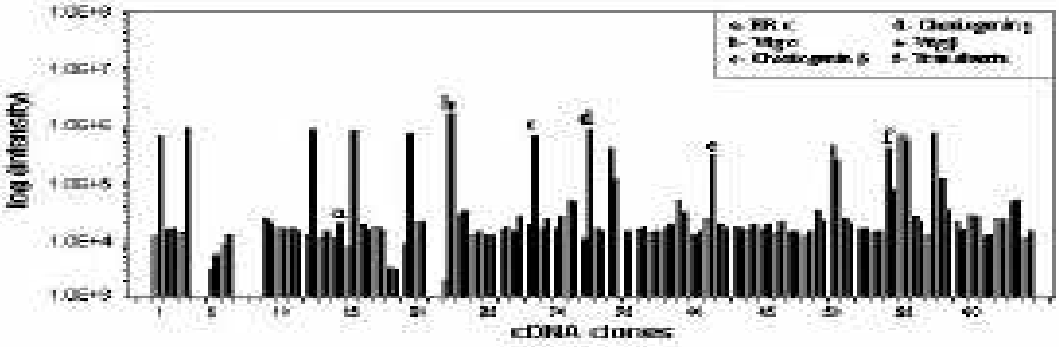

Fig. 3.

Figure 3. This is a graphic representation of the arrays pictured in Figure 2. The bar for each gene is depicted in log intensity. The grey bar represents the intensity of the gene from the TEG control fish at each location and the black bar represents the intensity of the same gene from the E2-treated fish. Positions 7 and 8 are blank because no genes were spotted. Positions # 4 (poly A control) and #19 (no template PCR negative control) were negative after subracting background. Specific genes identified include: a) estrogen receptor _ (position #14), b) vitellogenin _ (position #22), c) choriogenin _ (position #28), d) choriogenin _ (position # 32), e) vitellogenin _ (position #41), f) transferrin (position #54).

Drugs in Canadian Surface Waters: Distribution and Effects on Fish

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Abstract

A variety of prescription and non-prescription drugs have been identified in the effluents of sewage treatment plants (STPs) and in rivers and streams near discharges from STPs. Chronic exposure of aquatic organisms to drugs in surface waters could induce biological effects that may alter their physiology, behavior or reproductive capacity. In a survey of the effluents from STPs in 14 Canadian cities, analgesic/anti-inflammatory drugs such as Indomethacin, Ibuprofen and Naproxen, as well as the metabolite of acetylsalicyclic acid, salicylic acid, were often detected in final effluents at µg/L concentrations. The lipid-regulating drugs, Bezafibrate and Gemfibrozil were detected in some samples of influent and effluent. The neutral anti-epileptic drug, Carbamazepine was detected in all effluent samples at concentrations as high as 2.1 µg/L. Comparisons between concentrations of drugs in influent and final effluent samples are consistent with efficient removal of most drugs during sewage treatment, but some drugs, such as Carbamazepine may be relatively resistant to degradation.

Samples of surface water were collected in the summer and fall of 2000; at open water sites in the lower Great Lakes (i.e. Lake Ontario and Lake Erie) and at sites near the STPs of the cities of Burlington, Ontario and Windsor, Ontario. At sites near the Little River STP in the city of Windsor, concentrations of acidic drugs were relatively constant at 6 sites located 100 meters apart, downstream of the STP. Since the effluent from this STP contributes approximately 50% to the total flow of Little River, high concentrations of drugs in these surface waters were expected. In contrast, in samples of surface water collected downstream of the West Windsor STP, which discharges directly into the Detroit River, and at sites within Hamilton Harbour., concentrations of acidic drugs declined to concentrations near or below detection limits within a few 100 meters of the STP discharges. These preliminary data indicate that drugs are detectable only close to the point of discharge from STPs, unless effluents are discharged into small rivers.

Various molecular and biochemical tools were developed to study the biological effects of Gemfibrozil and Carbamazepine on goldfish. When goldfish were injected with Carbamazepine and Gemfibrozil (5 mg/kg), Carbamazepine stimulated the release of growth hormone into the blood, but Gemfibrozil had no effect. However, Carbamazepine had little effect on the expression of mRNAs for growth hormone. On the other hand, Gemfibrozil suppressed expression of the gene for the secretory protein, Sg-II by approximately 3-fold. These preliminary data demonstrate that both Gemfibrozil and Carbamazepine are biologically active modulators of the endocrine system in goldfish. Work will continue to study the impacts of these drugs on the hormonal and metabolic systems of the goldfish.

Biographical Sketch

Chris Metcalfe is a Professor in the Environmental and Resource Studies Program and Director of the Water Quality Centre at Trent University in Peterborough, Ontario, Canada. He is also the Dean of Research and Graduate Studies at Trent. His research interests include analysis of the distribution and biological effects of organic contaminants in aquatic ecosystems.

Possible Effects of Endocrine Disrupting Compounds on Walleye, *Stizostedion vitreum*, Near the Metro Sewage Treatment Plant, Saint Paul, Minnesota

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Abstract

Recent studies in the United Kingdom and the United States have reported the presence of estrogenic compounds, natural and synthetic, in treated domestic and industrial sewage effluent. The presence of vitellogenin (VTG), an egg yolk protein, in male fish is used as a bio-indicator of exposure to environmental estrogens. This research is attempting to determine if endocrine disrupting chemicals are having a significant effect on the gonadal histopathology of male and female walleye (*Stizostedion vitreum*) exposed to sewage treatment plant (STP) effluent. In 2000, male walleye collected from the Metro STP effluent channel had elevated VTG levels, no expressible milt and one case of intersexed testes, while upstream the males had low VTG concentrations and expressible milt. Female walleye were found with atretic ovaries at the Metro STP and the reference site. The frequency of gonadal abnormalities appearing in walleye collected in the Metro STP effluent channel suggests that the effluent is having a pathological effect on this species. These preliminary findings address the need for further investigation into the effects of the effluent on gonadal histopathology.

Keywords: Environmental Estrogens, Walleye, Histopathology, Sewage Effluent

Introduction

Municipal sewage treatment plants (STPs) throughout the world may serve as pathways for the introduction of estrogenic compounds into the aquatic environment (Desbrow *et al.*, 1998, Ternes *et al.* 1999) and these compounds may reduce the reproductive potential of exposed fish. Evidence of exposure to estrogenic compounds has been verified by the presence of elevated levels of the female yolk protein precursor vitellogenin (VTG) in male fish (Purdom *et al.* 1994, Harries *et al.* 1996, 1997, Folmar *et al.,* 1996, 2001, Routledge *et al.,* 1998). Male fish, although capable of synthesizing VTG, typically only do so when exposed to chemicals with estrogenic properties (Mommsen and Walsh, 1988, Routledge *et al.* 1998). VTG can build up to high concentrations in the blood of male fish before it is degraded by the liver (Mommsen and Walsh, 1988); therefore, VTG induction in males is a widely accepted bio-indicator of exposure to estrogenic chemicals (Sumpter and Jobling, 1995).

Estrogenic compounds may reduce the reproductive potential of fish populations by causing intersex, decreased gonad size, and altered sex ratio. Intersex, a condition in which both male and female gonadal tissue is present in the same gonad has been found in fish exposed to sewage effluent (Goodbred *et al.*, 1997, Jobling *et al.*, 1998, Vigano *et al.*, 2001, Nolan *et al.*, 2001). Sexual differentiation in fish is plastic and can be altered by exposure to estrogens or androgens. Such exposure may cause an intersex condition to occur or cause a preponderance of males or females depending on whether the exposure was to an androgen or estrogen (Baldwin and Li, 1945, Yamazaki, 1976, Kobayashi *et al.,* 1997, Gimeno *et al*. 1998a, 1998b). STP effluent may also cause a decrease in the size of the gonad (Harries *et al.,* 1997, Jobling *et al*, 1998,).

In the present study, we investigated the effects of the Metro STP effluent on the reproductive potential of walleye (*Stizostedion vitreum)* by evaluating VTG induction along with gonadal size, sex ratio, and histopathology. We chose walleye because Folmar *et al.*, (2001) found elevated levels of VTG and abnormal sex steroid levels in walleye exposed to the effluent from the Metro STP but did not determine effects on reproductive potential of those walleye. Walleye are of considerable economic importance as a highly sought after game fish, and a reduction in their reproductive potential could affect population numbers and thus yield to the fishery.

Materials & Methods

We collected walleye from the Metro STP effluent channel located in Mississippi River Pool 2 (MRP-2) (Fig. 1). Previous studies at this site have documented the presence of VTG in carp (*Cyprinus carpio*) and walleye males (Folmar *et al.* 1996, 2001). A second site, located below Lock and Dam 1 on the Mississippi River approximately 20 km upstream of the STP channel, served as a reference site. The dam is the upper limit of MRP-2 (Figure 1). Although the reference site is not impacted by the effluent discharge from the Metro STP, upstream sewage treatment plants and non-point source pollutants may affect fish at that site.

The Metro STP is a large, modern facility serving the greater metropolitan area of Minneapolis and St. Paul, Minnesota. Domestic sewage from a population of 1.8 million people comprises 92% of the wastewater treated at this plant while 7% is from industrial sources and 0.7% from landfill run-off collections. The Metro STP uses a tertiary treatment process year round and chlorinates and de-chlorinates the effluent from April to October. The plant discharges approximately 800 million liters per day of treated effluent into a natural side channel of the Mississippi River (Figure 1). This input is the primary water source for the channel during normal to low water stages of the Mississippi River

At both study sites we collected walleye using an electrofishing boat with pulsed DC current from a Smith Root Type VI-A electrofisher. Upon capture, we anesthetized the fish with tricaine methane sulfonate (MS222) (Argent Chemical, WA), measured and weighed them, took scales for aging, drew blood for analysis of VTG, and determined the presence of milt by applying pressure to the abdomen. Prior to gonad extraction we killed the walleye with an overdose of the anesthetic 2-phenoxy ethanol (Sigma, MO) or MS222.

We collected 1/2 to 1 ml of blood from the caudal vasculature of each fish with a 23 gauge needle and 3 ml syringe. The blood was stored on ice until centrifuged to separate the serum from the blood cells (90sec., 13,600 x g). Serum was pipetted off and frozen at –20ºC until further analyses. We shipped the serum on dry ice to the Protein Chemistry and Molecular Biomarkers Laboratory at the University of Florida where quantification of VTG was performed using an enzyme linked immunosorbant assay according to a standardized protocol described by Folmar *et al.* (2001).

We calculated the gonadosomatic index (GSI) for male walleye by extracting the gonads, taking the wet weight of the right gonad prior to preservation and using the following equation: GSI= [2x weight of right gonad / total weight of fish with intact gonads] x 100.

We preserved the right gonad in 10% buffered formalin, dehydrated in increasing concentrations of ethanol (10%, 30%, 50% and 70% ethanol for a minimum of 2 h. each), and stored in 70 % ethanol. Further processing included cutting a 4-6 mm thick tissue sample from the anterior, middle and posterior regions of the right gonad, dehydrating to 100% ethanol in two steps of ethanol (80%, 95%) for 2 hours each, clearing in xylene followed by paraffin embedding and sectioning. Sections were cut 6-8µm thick, mounted on glass slides, stained with Harris haematoxylin and counter-stained with alcoholic eosin. We examined the slides under a light microscope at 100-400x and photographed structures of interest with a Nikon CoolPix 950 digital camera, and optimized images using Adobe Photoshop 5.5 (Macintosh).

Results

During the 2000 spawning season of March and April, we collected fish from the STP effluent channel and the reference site, but in 2001 we only collected fish from the STP effluent channel prior to the spawning season, November 2000 through March 2001 (Table 1). Due to spring flooding no collection was possible during the 2001 spawning season. Our intention was to collect only male fish, based on the presence of expressible milt or eggs and body shape. Very few fish had expressible milt or eggs, and we subsequently learned from histological examination of the gonads that our ability to differentiate males from females by body shape was very inaccurate. Neither of the two histologically confirmed male walleye collected from the effluent channel in 2000 had expressible milt, nor did any of the 4 males from the 2001 collection. All five males from the reference site had expressible milt in 2000.

Histological analysis revealed that only two of 20 walleye collected at the STP effluent channel in 2000 and four of 16 in 2001 were male, giving a combined sex ratio of 1:5, male to female. The sex ratio for the reference site is unknown because a number of known (by expression of eggs) or suspected (by body shape) females were released without histological examination of the gonads.

Male walleye collected at both the reference site and the effluent channel during the 2000 spawning season were in late stage spermatogenesis with predominantly free spermatozoa present, but the four males collected on March 16 and 26, 2001 were in an earlier stage of spermatogenesis with relatively few free spermatozoa (Figure 2A). One of the males collected in 2001 was intersexed with a multifocal type of ovo-testes with male and female gonadal tissue in close proximity (Figure 2B). Spermatogonia and spermatozoa were found in the histological sections of the ovo-testes, but the middle stages of spermatogenic development, spermatids and spermatocytes, were not seen. Additionally, degenerate oocytes were interspersed throughout the tissue and an abundance of connective tissue was apparent in all sections of the ovo-testes.

Table 1. Summary of fish collected during the 2000 and 2001 field season and frequency of gonadal abnormalities at the Metro sewage treatment plant (STP) site and a reference site.

	Reference 2000	Metro STP 2000	Metro STP 2001	Totals
Number of Males (Intersex)	5 (0)	2 (0)	4 (1)	11
Number of Females (Atretic)	4 (2)	14 (9)	6 (0)	24
Immature Females	n/a	4	6	10
Totals	9	20	16	45

Female walleye sampled at both sites were in various stages of sexual development, ranging from having primary oocytes, to ovulated eggs (Figure 3A). Of the four females taken at the reference site, two were ovulating. The additional two females from the reference site, and nine collected from the effluent channel in the 2000 field season, showed varying degrees of atresia or degradation of the oocytes (Figure 3B). Ten immature female walleye, externally indistinguishable from male walleye, were collected during the two field seasons at the Metro STP. One fish, captured in 2000 in the Metro STP channel, was found to have parasite cysts, probably trematodes, interspersed throughout its gonadal tissue (Figure 4).

Serum VTG concentrations above the detection limit (0.03 mg/ml) were found in males from both collection sites in the 2000 field season, but the two from the effluent channel had VTG levels greater than the highest concentration found in any of the five males from the reference site (Figure 5). Also, three male walleye had higher VTG concentrations than two female walleye (Figure 6). The two females with the lowest VTG concentrations (0.16 and 0.18 mg/ml) had atretic ovaries. There did not seem to be a relationship between the presence of atretic oocytes and VTG concentrations or the collection site; however, most females collected at the STP site had higher concentrations of VTG than females from the reference site (Figure 6). VTG data for the fish collected in 2001 are not yet available.

The GSIs of male walleye from the STP effluent channel during the two field seasons were not significantly different ($P > 0.05$). Therefore, we compared the pooled sample of fish from the effluent channel to fish from the reference site. The GSIs of the pooled sample of STP site fish were significantly lower than for the reference site fish (unpaired t-test, $P<0.05$).

DISCUSSION

The higher serum vitellogenin concentrations in male walleye captured in the effluent channel compared to walleye from the reference site provides good evidence that the fish were exposed to estrogenic chemicals. This finding is consistent with Folmar *et al.* (2001). VTG induction in male fish has been linked to the presence of estrogenic chemicals in sewage effluent in the United Kingdom (Desbrow *et al.*, 1998) and is a bio-indicator of the presence of estrogenic chemicals in the aquatic environment (Sumpter and Jobling, 1995). Additionally, the reduced GSI in male walleyes from the STP site is also suggestive of estrogen exposure (Jobling *et al.*, 1996, Christiansen *et al.,* 1998).

The small number of males that we collected makes it very difficult to determine if exposure to estrogenic chemicals in the Metro STP effluent is reducing the reproductive potential of the walleye population. However, our failure to capture adequate numbers of males was due to the highly biased sex ratio in favor of females which, in itself, may be evidence for endocrine disruption and reduced reproductive potential. Estrogen exposure can cause a biased sex ratio in juvenile and adult male carp (Gimeno *et al*. 1998a, 1998b). Although the presence of VTG in male walleye from the effluent channel confirms their exposure to estrogenic compounds, potentially capable of affecting sex ratios, the bias in favor of females may also have resulted from the natural migratory patterns of walleye prior to spawning. Males may have migrated out of the effluent channel prior to our sampling.

Despite the fact that male walleye at the reference site expressed milt and those from effluent site did not, no differences in the histological appearance of the testes was apparent. Mature males at approximately the same latitude as the Metro STP are likely to have expressible milt by late January (Malison et. al, 1994). Environmental estrogens may prevent the expression of milt even though maturation and development of the testes are not affected because, at least in goldfish and carp, the endocrine system has direct control over milt production (DeFraipont and Sorensen 1983, Stacey *et al.* 1987, 1994). In the same manner, hormonal cues in females could be disrupted causing atresia or early regression of the oocytes. Atretic oocytes and a lack of expressible milt could also be linked to the winter temperature of the effluent channel, 17°C ± 1.3°C (mean ± SE) which is greater than the Mississippi River. The related yellow perch (*Perca flavescens*), and perhaps walleye, need a period of cold temperature during the winter for proper gonadal development (Hokanson, 1977, Jones *et al.*, 1977). During the 2001 field season, we tagged 68 walleye and returned them to the effluent channel over 12 sampling dates between November 15, 2000 and March 26, 2001. Only five were recaptured, suggesting that the walleye did not spend the entire winter in the effluent channel.

The large number of pathological changes in gonadal structures of the walleye from the Metro STP site could have resulted from exposure to endocrine disrupting chemicals. One male walleye was intersexed with large quantities of female gonadal tissue present in the testes. Jobling *et al*. (1998) and Goodbred *et al*. (1997) found intersexed fish in sewage effluent; however, the occurrence of one intersexed fish in our small sample is insufficient to attribute its occurrence to endocrine disruption. The percentage of females with atretic oocytes, particularly at the reference, site seems high. Walleye move extensively in MRP-2 (Gangl *et al.,* 2000), and it is conceivable that atretic females from the reference site were exposed to the effluent from the Metro STP at an earlier point in time.

The combination of the presence of high levels of VTG in males, a highly skewed sex ratio biased towards females, lack of milt production in males, and frequent gonadal abnormalities in both sexes suggests a likely exposure of walleye in MRP-2 to endocrine disrupting chemicals. However, water temperature in the Metro STP channel and the movement patterns of walleye could account for some of these effects. While the small sample size in the present study does not allow a determination of the factors responsible for these reproductive deficiencies they clearly warrant further study.

Acknowledgements

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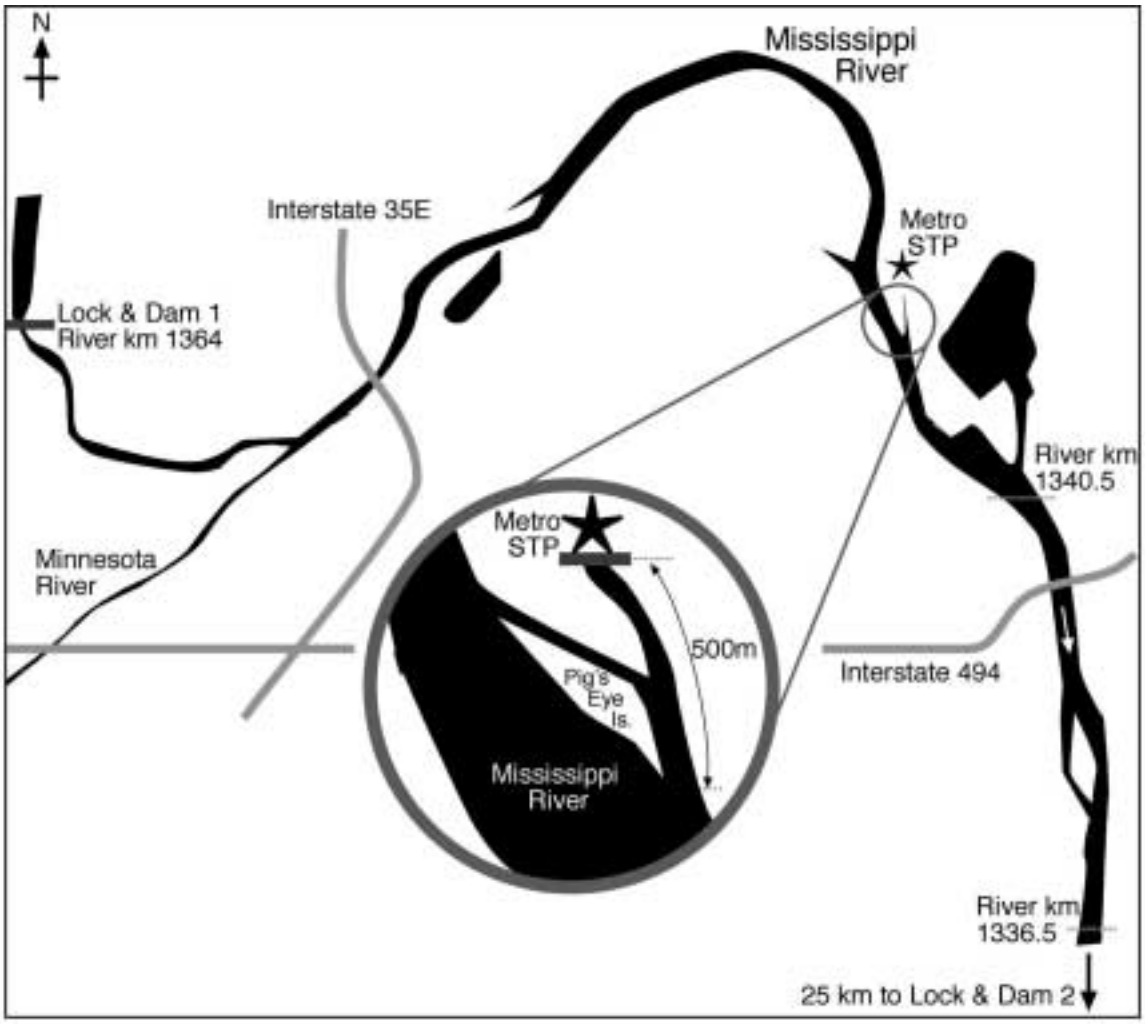


Figure 1. Map of the Upper Mississippi River, Pool 2, near St. Paul MN. The effluent channel of the Metropolitan Sewage Treatment Plant (Metro STP) is magnified in the inset and served as the study site. The reference site for this study was located below Lock and Dam 1, approximately 20km upstream of the effluent channel.

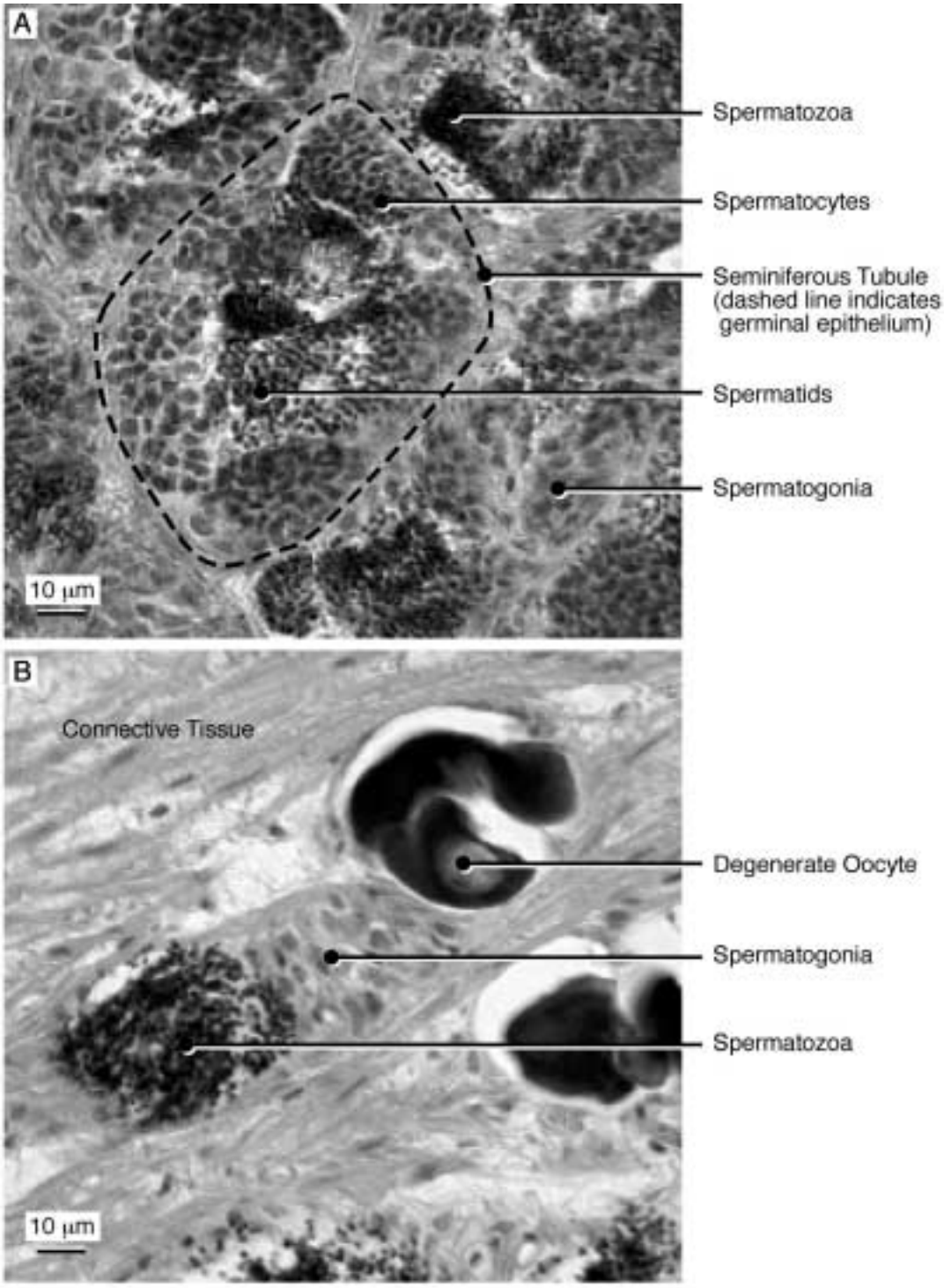
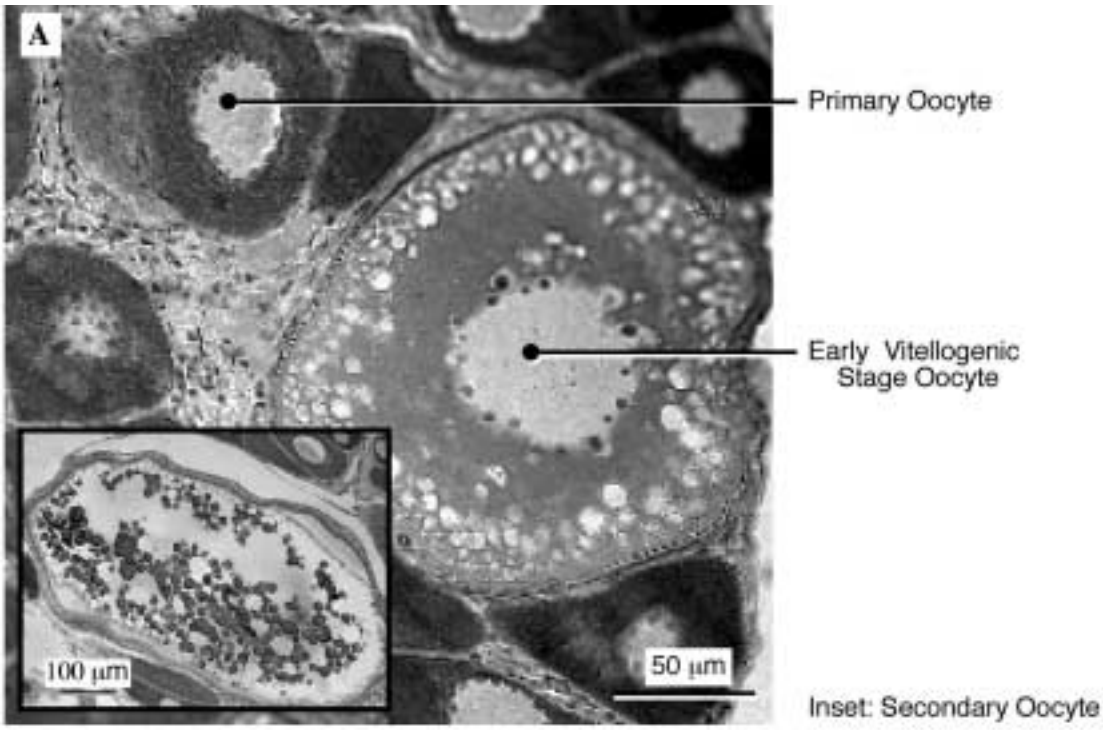


Figure 2. Sections through the testes of male walleye collected at the Metro sewage treatment plant effluent channel. (A) Section through the testes of a maturing male walleye with all stages of spermatogenesis present. (B) Section through the testes of an intersex male walleye showing degenerate oocyte and sperm in close proximity.



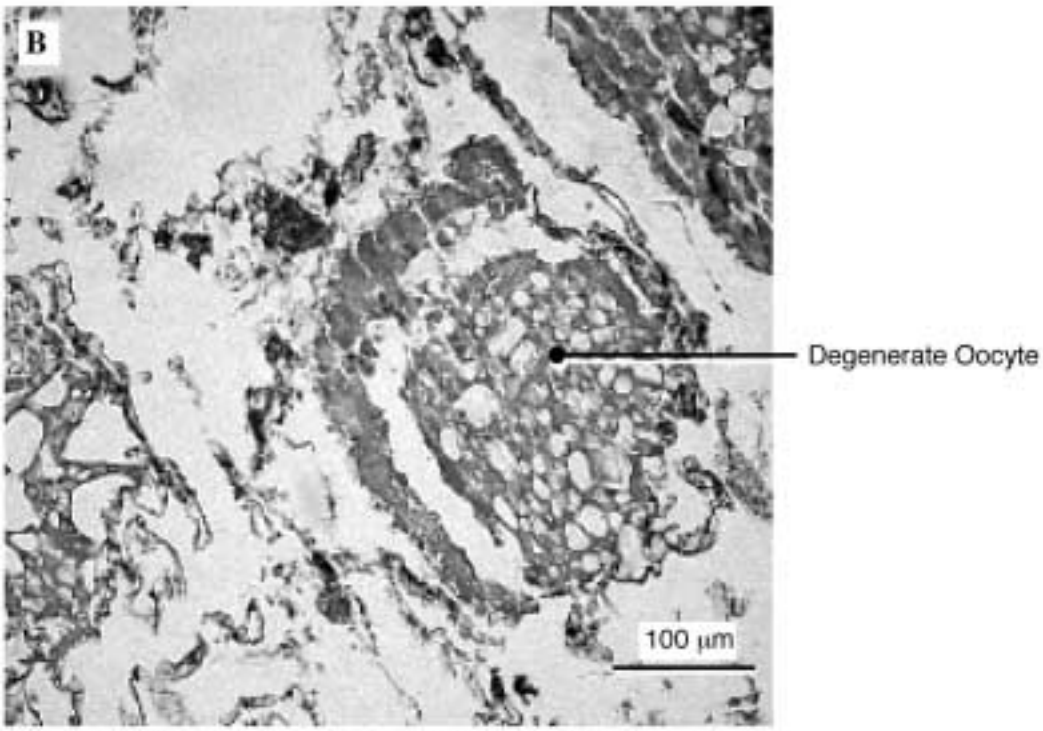


Figure 3. Section through the ovaries of female walleye collected at the Metro sewage treatment plant effluent channel. (A) Normal development of oocytes showing both primary and secondary oocytes in the same ovary. (B) Degenerate oocyte typical of many walleye sampled from both study sites in Mississippi River Pool 2.

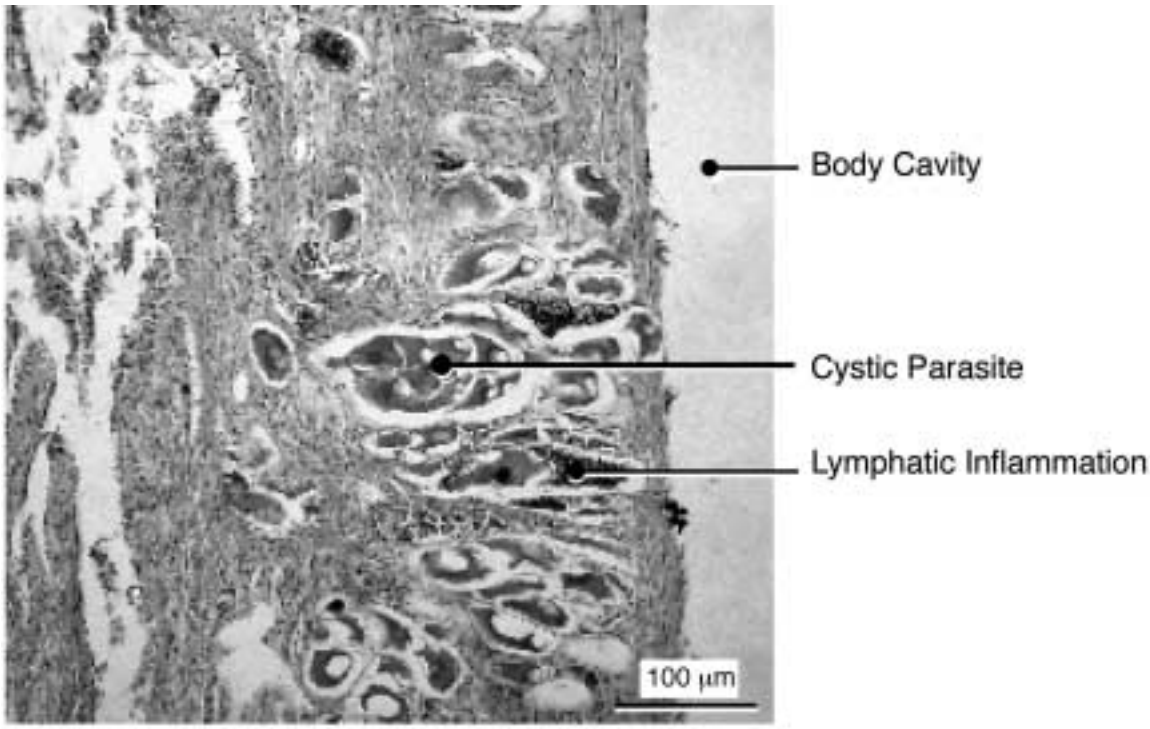


Figure 4. Histological section through the ovary of a female walleye collected at the Metropolitan Sewage Treatment Plant effluent channel. Infestations of cystic parasites and widespread inflammation is apparent throughout the specimen.

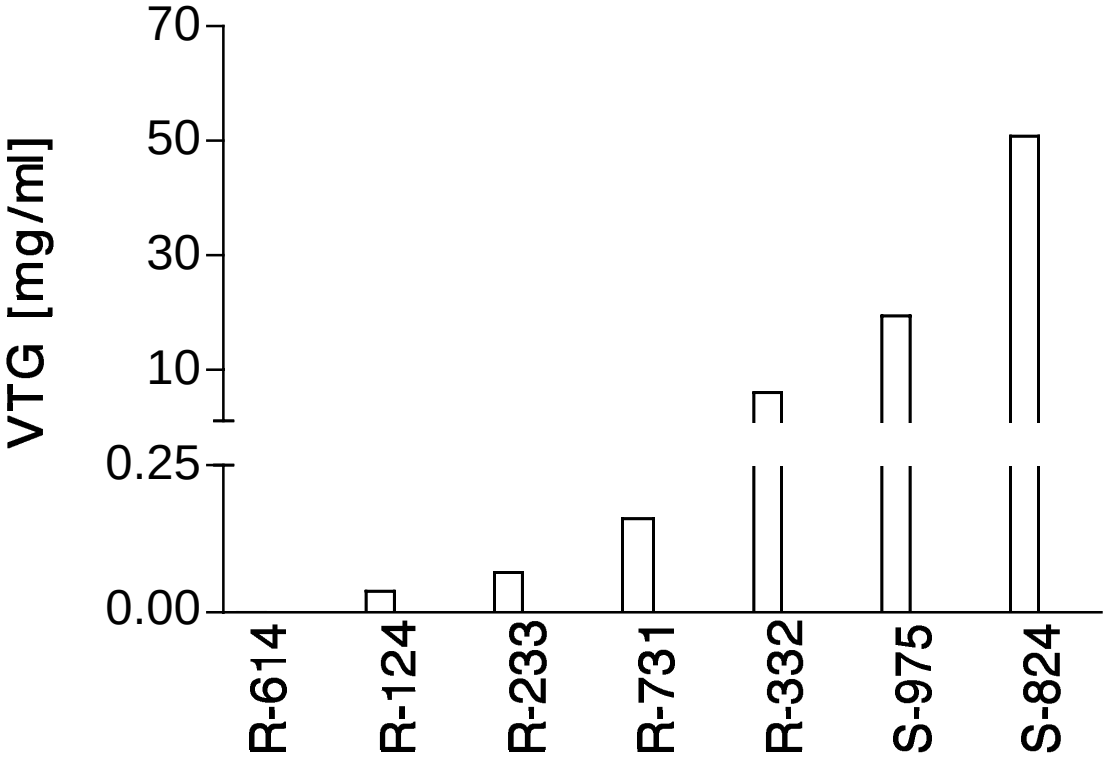


Figure 5. Serum vitellogenin (VTG) concentrations in male walleye collected from the Metro sewage treatment plant effluent channel (S) and from a reference site (R) during the 2000 field season.

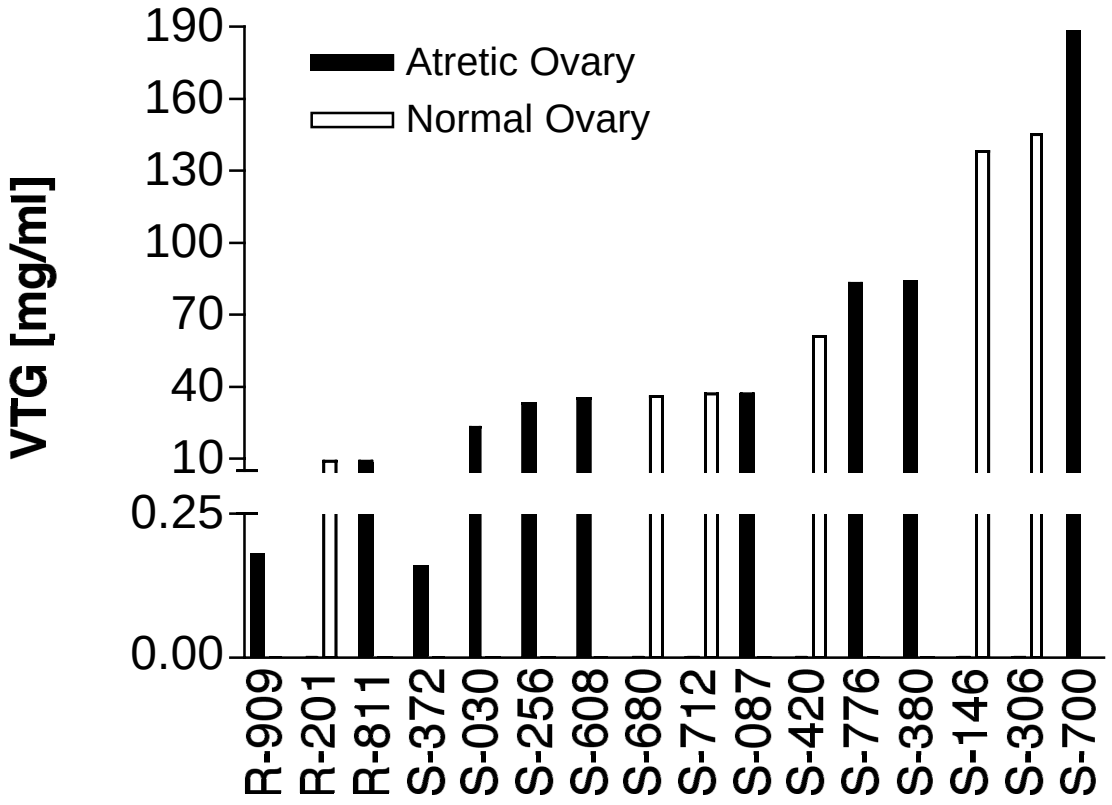


Figure 6. Serum vitellogenin (VTG) concentrations in female walleye collected from the Metro sewage treatment plant effluent channel (S) and from a reference site (R) during the 2000 field season.

Data from an Estrogen Receptor-based Biosensor Correlates with Evidence of Frog Malformation and Demonstrates a Differential Response of hER-α & β to Beneficial and Harmful Estrogenic Compounds

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Abstract

A novel evanescent-field, fluorometric biosensor using recombinant human estrogen receptor (hER-α and/or hER-β) as the detection agent has been developed as a screening tool for detection of estrogen mimics in water samples. The biosensor detects the presence of estrogenic compounds that interact with the human ER-α or β and facilitate or antagonize complex formation between the estrogen receptors and the estrogen response elements (ERE). Two models of the sensor instrument have been developed, a hand-held model that is suitable for obtaining measurements in the field and a benchtop model for more detailed studies.

Field application of the sensor to the problem of frog malformations

The presence of hormonally active agents has been proposed as one possible explanation for the occurrence of frog malformations in Minnesota. Data is presented demonstrating the use of the hand-held version of the instrument to assess whether water from ponds and lakes in Minnesota associated with the occurrence of frog malformations exerted an inhibitory effect on binding between estrogen receptor and an estrogenic compound on the fiber sensor surface. Sensor data was compared to indicators of frog malformation including the number of malformed frogs observed in a survey of the pond, whether the frog population was declining in that pond, and the results of the Frog Embryo Teratogenesis Assay - Xenopus (FETAX).

Water from the three sites (DAR, WAS, IND), which reported no evidence of malformed frogs, produced ≤10% inhibition of binding between estrogen receptor and an estrogenic compound attached to the sensor fiber. Water from five ponds (HIB, NEY, ROI, ROI2, MHL2) produced ≥ 27% inhibition and were associated with either survey evidence showing frog malformation or population decline, or FETAX evidence of malformation, lethality or developmental delay. One pond (CWB) showing frog abnormalities produced 17% inhibition. One site without evidence of frog malformations (BOE) produced inhibition of 16%. Thus if 10% is considered to be a discriminator, sensor data correlated with frog malformations 90% of the time.

Screening estrogen mimics based upon effect on ERE binding to hER-α & β

The benchtop model of the sensor was configured to compare binding of hER-α and hER-β to the ERE in the presence of several estrogenic compounds including 17β-estradiol, estrone, 4-OH-tamoxifen, genestein, daidzein, diethylphthalate and diethyl stilbestrol. This instrument provides real time kinetic data on association and dissociation between receptor and ERE. Of particular interest is the observation that in the presence of genestein, a phytoestrogen believed to be beneficial, hER-β showed more rapid association with ERE than did hER-α, while in the presence of diethylphthalate, a xenoestrogen believed to be associated with reproductive abnormalities, hER-α showed rapid association with and slow dissociation from the ERE, while hER-β_was not affected. This suggests the possibility that a differential response between hER-α and hER-β in the kinetics of association with and dissociation from the ERE could play a role in determining whether an estrogenic compound exerts a beneficial or a harmful effect. The sensor provides a rapid means for evaluating these responses.

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Background

Environmental Estrogen Disruptors

Correlation between reproductive and developmental abnormalities in wildlife and the presence of endocrine mimicking compounds in the environment has been suggested by several studies.[1-6] In particular, estrogen mimicking compounds have been reported to induce developmental abnormalities.[7, 8] This, taken together with the documented developmental adverse effects of diethylstilbestrol on children of women given DES during pregnancy suggests the possibility that wildlife may be sentinels for the human population; that their developmental abnormalities and several disturbing trends in human health might find a common origin through environmental endocrine disrupting compounds. Whether environmental endocrine disrupting chemicals are producing the observed abnormalities remains highly controversial. Although it is difficult to establish cause and effect, certain evidence points in that direction. Endometriosis, once thought to be a rare condition, has become the most common diagnostic entity in gynecology today.[9] Exposure of rhesus monkeys to dioxin has been shown to induce severe endometriosis.[10] The incidence of female breast cancer is now 1 in 8.[11] The pesticide o,p-DDT stimulates cell proliferation in cultures of human breast cancer cells.[12] A retrospective analysis of data since the 1940's revealed that the sperm count of men all over the world has dropped by 50%.[13] A recent reanalysis of sperm count data suggests that the rate of sperm density decline in Western industrialized nations is greater than that which is observed in non-Western countries.[14] A review of numerous subsequent analyses of sperm count, and condition, presents a picture of geographic variation and a possible role of estrogen mimics in the severity of the decline.[15] At the same time testicular cancer rates have tripled and prostate cancer rates have doubled.[13] Increased incidence of undescended testes, urogenital tract abnormalities and reduced semen quality resemble the effects observed on male offspring of mothers treated with the estrogenic compound diethylstilbestrol during pregnancy.[16] More recently, considerable attention has been focused on the potential reproductive and developmental impact of certain phthalate esters.[17] Moreover, elevated urinary phthalate levels have been detected in the human populations,[18] and in Puerto Rico elevated levels appear to be associated with increased incidence of premature thelarchy.[19]

In response to a Congressional mandate, which grew out of such concerns, the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) was created by the EPA and has issued recommendations for a multi-tier screening program for testing some 87,000 chemicals known to be present in the environment to determine their potential for producing endocrine disruption. Tier 1 screening is based upon the concept of functional toxicology.[20] This involves assessment of the effects of test compounds on molecular interactions which play critical roles in the biological function of interest. In the case of processes involving the endocrine system, signal transduction begins with binding between a receptor and its cognate ligand. Hence the most fundamental *in vitro* method is measurement of binding affinity between a receptor and a test compound. This is classically done by means of competitive radioligand binding assay. Typically this type of assay uses a fixed concentration of receptor and radio-labeled estradiol-17ß with several concentrations of a competitor to generate a Scatchard or Hill plot.[21] These assays provide information on affinity under equilibrium conditions, but cannot reveal anything about the kinetics of association and dissociation.[22] Because the realistic situation of a cell responding to a hormonal stimulus is unlikely to be one of stable equilibrium, kinetic measurements of receptor binding and dissociation provides a measurement which is more reflective of biological reality. Evanescent biosensors using purified receptor reagent provide a rapid kinetic means for assessing binding between receptor and a test compound as well as the effect of ligand on binding between receptor and response element.[23] Because such biosensors provide kinetic rather than equilibrium measurements and do not require maintenance of cell cultures or use of radioactivity they are convenient and rapid, providing data in roughly 10 minutes. In addition, the size of some of these instruments is small enough for testing samples in the field.

In the work reported here, biosensor instrumentation was obtained from *Threefold Sensors,* Ann Arbor, MI. Biosensors using recombinant human estrogen receptor as the recognition agent were developed to assess whether water from certain Minnesota ponds, having deformed frogs, contains something that could function as an estrogenic endocrine disruptor. Water samples were obtained from sources where frogs were observed to be normal and from sources where frog malformations or developmental abnormalities were observed.

The Estrogen Receptor and Gene Expression

The primary initiating event by which an endogenous endocrine-active substance exerts an effect is through binding to specific receptors associated with target organs. The binding between these receptors and their hormonal cognate ligands mediates control of differentiation, growth and metabolic homeostasis.[24, 25] This is accomplished through the functioning of these receptors as transcription factors and modulators of gene expression in target cells.[25, 26] The details of subsequent events differ somewhat for the various endocrine-responsive tissues. Because this study focuses upon estrogenic endocrine disrupting compounds, the mechanisms characteristic of those tissues possessing estrogen receptor are now described.

The estrogen receptor (ER) belongs to the steroid family of nuclear receptors, which include receptors for androgens, estrogens, glucocorticoids, mineralocorticoids and progestins.[27] These receptors possess certain structural characteristics in common.[28] The amino-terminal A/B domain interacts with the DNA so as to initiate transactivation of gene expression. Two zinc-finger structures, which are found in the C-domain, are essential to receptor specific DNA-binding and receptor dimerization. The ligand binding domain is found in the carboxy-terminal E/F region. This region plays a role in nuclear translocation, receptor dimerization, and the coactivator, corepressor recruitment needed for modulation of target gene expression.[29, 30]

Following binding of cognate ligand to the carboxy-terminal ligand binding domain, estrogen receptors undergo a conformational change resulting in their dissociation from complexes with heat shock proteins hsp90 and hsp70[31]. This allows homodimerization and binding to a portion of DNA known as the estrogen response element (ERE).[32] The response element shows minor variations among the different estrogen-responsive tissues, but contains a particular consensus sequence of bases which is highly conserved. This consensus sequence comprises the 13 bp sequence 5'-GGTCAnnnTGACC-3' where *n* = any nucleotide.[33] Upon binding, coactivators are recruited to the promoter region of the gene bearing the ERE and transcription is initiated, resulting in synthesis of the appropriately coded protein.

There are at least two types of estrogen receptors, which exhibit significant differences in binding affinity with respect to estrogenic ligands.[34, 35] The form of ER which was cloned in 1986[36] was, until the last decade, believed to be the only ER. Following the cloning of a different form of ER in 1996[37-40] the old form has been designated as ER-α and the new form as ER-β. Sequence homology between hER-α and hER-β is 96% in the DNA-binding region, but only 58% in the ligand binding region.[39] Although both receptors demonstrate similar binding affinity for 17β-estradiol, ER-β shows a 20-fold higher affinity for genestein, a beneficial phytoestrogen.[35] This suggests the possibility that beneficial estrogenic compounds might be distinguished from harmful ones, based upon relative binding affinities for the two types of ER. Data presented here provides some support for this idea.

Many of these characteristics of estrogen receptor mechanisms have been utilized in developing the fiber-optic sensor instruments and reagents used to obtain the data reported herein.

Technologies for Endocrine Disruptor Screening

For evaluating whether water contains endocrine disrupting compounds, the use of receptor molecules which mediate biological endocrine responses has been employed in several types of assays including bioassay, radio-receptor assay, cell based bioassay and fluorescence polarization assay in addition to evanescent sensor methods.[23] The classical method for investigating the effects of compounds is bioassay. In the end, bioassay presents the bottom line of biological impact of a compound. There are several types of bioassay based upon specific estrogen-mediated biological effects. Examples include production of vitellogenin, a precursor to egg yolk found commonly in female fish, but not in males.[6] All bioassay methods require long periods of time.

The gold standard for measurement of binding affinity between a receptor and a test compound is competitive radioligand binding assay. Typically this type of assay uses a fixed concentration of receptor and radio-labeled estradiol-17ß with several concentrations of a competitor to generate a Scatchard or Hill plot. From this, an affinity constant may be derived and the number and cooperativity of binding sites on the receptor determined.[21] These assays provide information on affinity under equilibrium conditions, but cannot reveal anything about the kinetics of association and dissociation.[22]

Binding between receptor and response element can be observed using DNA band shift and super shift assay performed under non-denaturing conditions. Quantification can be challenging and kinetic information cannot

be obtained, but the different conformations that may result from interactions between receptor and response element emerge as distinct bands.[41]

A recent method that has been applied to high throughput screening is the cell-based bioassay, which mimics the normal biological sequence of events. Typical, two plasmids are inserted into yeast cells. One plasmid contains an estrogen receptor gene fusion, and the other plasmid contains an ERE linked to a reporter gene (e.g. ß-galactosidase). Estrogenic stimulation is reflected in the ß-galactosidase activity of the yeast cell.[21] This method is well adapted to the demands of Tier 1 screening, but it has the potential to produce misleading results deriving from the presence of yeast rather than human co-activators and co-repressors. Another confounding factor in the use of cell-based assay is the transport of the test compound into the yeast.

Polarization hER-α and β competition assays for single sample (Beacon™) and high throughput screening (CoreHTS™) have also been developed by PanVera. However, this assay technique requires a 2-hour incubation time and is not suitable for measuring kinetics.[42]

Evanescent sensing instruments employing surface plasmon resonance (SPR) rather than fluorescence have been previously used to examine the effects of estrogen receptor modulating compounds on binding between ER and ERE.[23] The size of the SPR instrument used for that study, and the exquisite sensitivity of SPR to refractive index changes make this type of evanescent sensor ill-suited to field applications. However, it did establish the utility of evanescent sensing for evaluating the presence of estrogen mimicking compounds at low concentrations.

Evanescent Fiber-Optic Biosensors

There are several characteristics that make evanescent fiber-optic sensing ideally suited to field applications such as ground water analysis. (1) Because the instrument measures kinetic binding and dissociation rather than equilibrium binding, tests can be performed in minutes rather than hours. (2) Key biological molecules (e.g. estrogen receptor) can be used as reagents in the sensor system to provide a response that bears a reasonable relationship to biological effect. (3) Evanescent sensors can be as sensitive as immunoassay methods. (4) The instrument is small enough to be easily carried to a site.

Evanescent sensors can make kinetic measurements because they do not require separation of bound analyte from unbound analyte before a measurement can be taken. This is achieved by virtue of an evanescent field that is generated by total internal reflection of incident light within a waveguide, such as an optical fiber or a glass plate. This field falls off exponentially with distance from the fiber surface. Consequently, fluorescent molecules bound to the surface of the sensor will be stimulated by the evanescent field to produce fluorescence that radiates into the optical fiber. Fluorescence is not stimulated in molecules that are present in the surrounding liquid because they are not within the range of the evanescent field. They, therefore, do not present a background that obfuscates measurement of binding to the fiber surface. Since kinetic measurement of binding and dissociation is made possible in evanescent sensors, the need for binding components to come to equilibrium in order for binding affinity to be evaluated is eliminated. This makes assessment of complex binding events possible within minutes.

Materials and Methods

Instrumentation and Components Used in These Studies

The instrument utilized for studies on pond water from Minnesota is a field portable evanescent fiber-optic fluorescence sensor from *Threefold Sensors*, Ann Arbor, MI. The instrument measures roughly 8”x10”x5”. Fibers were sensitized so as to bind fluorophore-labeled ER in solution to estrone-3-glucuronide on the fiber surface. Estrogen mimics present in the test sample competed with estrone-3-glucuronide on the fiber surface with the result that binding between ER and the fiber sensor is less rapid than it was prior to inclusion of the sample. For these studies, recombinant human ER-α was produced in yeast culture by Dr. James Wittliff of the University of Louisville according to previously described methods.[43] Receptor was labeled with Cy5, a fluorophore having A_{max} =650 and E_{max}=668 and used in the studies at a concentration between 2-4 x 10^{-10}M.

A different instrument was employed for studying the effect of estrogen mimics on binding between ER-α or ER-β and ERE. This instrument was used because ER-β lost activity when fluorophore-labeled. An instrument was, therefore, needed which would measure kinetic binding between ER and the sensor fiber without having to

label ER-β. This is possible using the TG01 sensor instrument, also from *Threefold Sensors*. This instrument is not field portable, but is well suited to detailed kinetic studies. The TG01 utilizes a nitrogen laser. This results in a larger instrument than the HH01, which employs a laser diode. Detailed descriptions of the instruments are provided in the materials and methods section of this report.

For studies where the operative question is one of effect on binding between ER and ERE, sensor fibers were treated so as to possess a nucleotide sequence corresponding to the consensus 35-mer of fish vitellogenin. Both binding and dissociation curves were studied because receptor-modulating compounds can influence the two kinetic rates differentially. Estrogen modulating compounds create conformational changes in ER which can either increase or decrease the binding affinity for ERE. Fiber surfaces were created which possessed the ERE in a relationship to a fluorescent binding matrix such that binding between the ERE and the ER resulted in modulation of the fluorescence seen by the sensor. This is shown in figure 1.

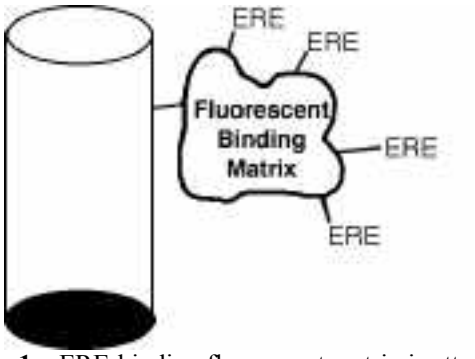

Figure 1: ERE-binding fluorescent matrix is attached to the fiber surface.

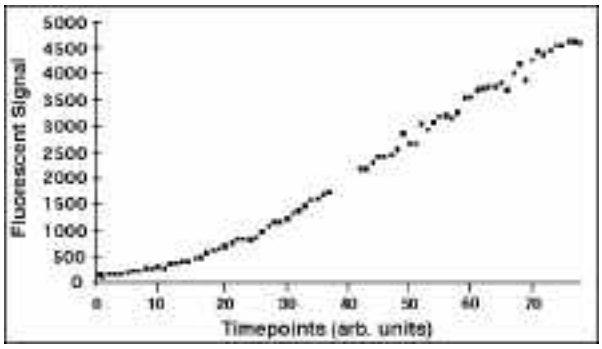

Figure 2: Fluorescence is recorded as sensor's binding matrix is preloaded with fluorescence.

Prior to use, sensor fiber fluorescence was maximized as is shown in Figure 2.
These fibers were used along with 10^{-14} moles of unlabeled hER-α or hER-β. hER-α was obtained from Dr. Wittliff. hER-β was obtained from *PanVera*, Madison, WI. The sensor cartridges were used repeatedly by flowing through them between samples, buffer in the absence of estrogenic ligand. This transformed high affinity ligand-receptor-ERE complexes to low affinity receptor-ERE complexes that washed off within minutes.

Biosensor Fluorometer Instrumentation

HH01 Biosensor Fluorometer

The HH01 Biosensor Fluorometer (See Figure 3) utilizes a AlGaInP diode laser operating at 637 nm to excite Cy5 fluorophores attached to the fiber-optic biosensor surfaces. A computer controller modulates this laser's output so that low level biosensor fluorescence can be more easily distinguished from background noise. To improve the fluorometer's ultimate signal to noise ratio (SNR), the frequency spectrum of the laser beam is first narrowed to remove the low-level (typically 10^{-4} of the peak power) fluorescence sidebands typically present in the output of diode lasers. To do this, the nearly collimated beam is first reflected from a grating that spatially spreads it in wavelength around the central laser wavelength. This beam then passes through a limiting aperture that strips the fluorescence side-bands from the beam. The remaining laser light is injected into the fluorometer beam path using a small turning mirror and is focused off axis through the focusing optics into the beam annularizing optics used to connect the fluorometer to the fiber-optic biosensors.

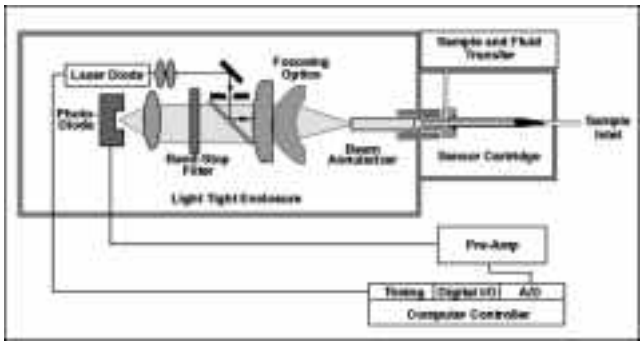

Figure 3: The optical layout of the HH01 Biosensor Fluorometer.

Because evanescent coupling of the laser light to surface bound fluorophores is strongest for light propagating within the biosensor at the critical angle, annularizing optics first convert the cylindrical beam of laser light into a conical bundle of rays propagating within the annularizer at the desired critical angle.[44-46] In the HH01 fluorometer, this is accomplished by injecting the narrow diameter laser beam off-axis into a length of fiber-optic cable having the same diameter as that of the fiber-optic biosensor. The laser beam traversing through the annularizing fiber undergoes many reflections and in so doing is spatially redistributed such that it emerges from the end of the annularizing fiber in a thin, hollow conical beam with substantially all light energy contained within a thin ring located at the outside of the cone and propagating at the same angle with respect to the axis of the annularizing fiber. The use of the annularizer for coupling fiber-optic biosensors to the fluorometer greatly increases the instrument's sensitivity over previous evanescent biosensor instrument designs because 80-90% of the light from the laser can injected into the sensor within 1-2° of the critical angle as compared to ~10% for illumination systems utilizing the focusing optics full numerical aperture.

The emerging cone of light at the distal end of the annularizer fiber is butt coupled with minimal losses to the proximal end of the fiber-optic biosensor. Finally, by adjusting the angle at which the laser beam is first injected into the annularizing fiber, the angle of light injected into the fiber-optic biosensor can be matched exactly to the critical angle required for maximal evanescent coupling to surface-bound fluorophores on the biosensor.

Fluorescence from surface-bound Cy5 fluorophores on the biosensor surface radiates in a broad band centered about 670 nm. This broadband fluorescence is captured by the fiber-optic biosensor and propagates back through the annularizing fiber, is captured with focusing optics matched to the numerical aperture of the annularizing fiber, collimated, and directed through a narrow band holographic filter that rejects a narrow band of wavelengths centered at 637 nm by a factor of over 10^6, while passing, with minimal loss, all the fluorescence above 650 nm. This fluorescence is detected using a Si photodiode and the photodiode's output is amplified using a low-noise amplifier.

The computer controller uses a National Instruments DAQcard 1200 to modulate the laser output and to digitize the voltage signal from the photodiode amplifier. Software averaging and filtering of the signal with and without the laser turned on is used to enhance signal to noise in a manner similar to that used by a lock-in amplifier. Data are automatically acquired by computer at specified intervals, typically every 4 seconds.

The TG01 Time-Gated Biosensor Fluorometer

The TG01 Time-Gated Biosensor Fluorometer shown in Figure 4 is used with fiber-optic biosensors incorporating near-IR lanthanide fluorophores having long fluoresence lifetime. Since these fluorophores require the excitation wavelength to be in the ultra violet (UV), the TG01 uses a pulsed nitrogen laser operating at a wavelength of 337 nm. Like the HH01 Biosensor Fluorometer, the TG01 Time-Gated Biosensor Fluorometer utilizes annularizing optics to provide strong evanescent coupling between the exciting laser light and the fluorescent label. However, because UV radiation is used, completely different illumination and fluorescence measurement optical systems are required to achieve both high sensitivity and a high signal to noise ratio.

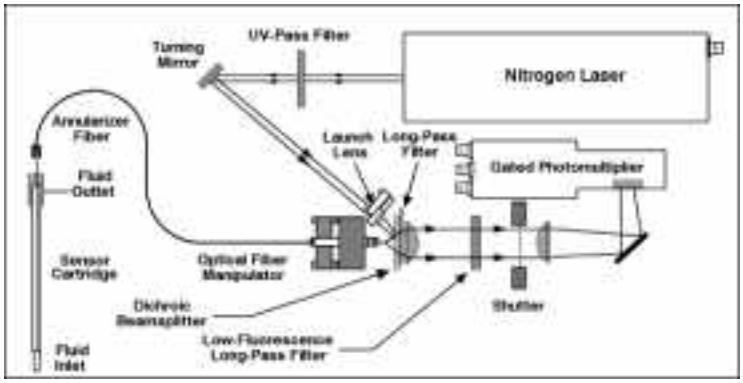

Figure 4: The optical layout of the TG01Time-Resolved Biosensor Fluorometer.

The TG01 fluorometer's optics are designed to eliminate or minimize fluorescence caused by the UV radiation passing through or reflecting from optical elements. This is done in several ways. First, non-UV radiation emitted by the gas discharge of the nitrogen laser is blocked using a UV pass filter. Second, the fluorometer uses non-fluorescent, UV transmissive optics that are made from quartz or fused silica. Third, the UV beam is injected off-axis into the annularizer fiber in a manner such that it does not pass through the large numerical aperture of the focusing doublet (which can not be made out of fused silica) used to collect fluorescence from the sensor. Finally, long wavelength bandpass filters minimize residual UV radiation or fluorescence below 600 nm from reaching the detection system.

Unfortunately, these steps to minimize fluorescence sources can not by themselves provide the TG01 with as high a SNR as that of the HH01 which achieves its high SNR by employing a holographic notch filter to block the exciting laser radiation propagating back to the photodiode detector by a factor of over 10^6. Since UV holographic notch filters do not yet exist, totally blocking fluorescence from sources other than the fluorophore label requires the TG01 to employ time-gated photon counting.

The fluorophore's fluorescence peaks at 613 nm and has a long-lived decay time of hundreds of microseconds whereas the fluoresence lifetimes of optical component and most organic compound fluorescence is at least 1000 times shorter and is emitted at wavelengths below 600 nm. Thus, by measuring only fluorescence occurring after any biological and optical fluorescence has decayed to zero, it is possible to measure, with very high discrimination, only fluorescence from the fluorophore label.

To measure only long-lived fluorescence, the TG01 uses time-gated photon counting. Computer software controls the timing and gating of the TG01 laser and data acquisition system using an IEEE serial bus. Prior to triggering each laser pulse, control software disables the Hamamatsu R4949, red-extended photomultiplier and the SRS SR400 Dual Channel Time-Gated Photon Counter. To compensate for pulse-to-pulse variation in the nitrogen laser's output, a beam splitter and a second Hamamatsu R4949 photomultiplier are used to count the number of UV photons delivered in each laser pulse. This allows the biosensor's response vs. time to be normalized to a constant laser output value. At each laser pulse, a delay generator is triggered, which, after the delay time set by the control program, generates a pulse to gate on the photon counter and the photomultipliers. After a specified time has elapsed, the computer acquires the photon counting results.

Fiber-Optic Biosensor Preparation

A roll of 400 µm step indexed, multi-mode, fused silica fiber was cleaved using a York Electronic Fiber Cleaver to yield 10.5 cm pieces with optically acceptable end faces. Portions of the ends of the fiber were covered with a polyimide sheath to protect the cladding from chemical and physical disruption. The region of the fiber between the polyimide sheaths was chemically stripped of cladding and treated to inhibit non-specific binding of proteins. Silanization of fibers was performed in a Teflon fiber processing unit which was designed for the purpose of performing chemistry on a batch of fibers. Procedures were performed in a glove box under nitrogen to prevent hydrolysis, using a method deriving from that reported by Bhatia, et al.[47] The longitudinal surfaces of the fibers were surrounded by a 2% solution of 3-(mercaptopropyl)-trimethoxysilane in dry toluene for 2 hr at room temperature, creating a glass surface bearing thiol groups. After rinsing in toluene, the thiol groups on the fibers were reacted with the maleimido moiety of the heterobifunctional agent, γ-maleimidobutyric acid-N-hydroxysuccinamide ester (GMBS) by incubating them in a 2mM solution of GMBS in reagent alcohol for 1 hr. The succinimide ester of the GMBS was then coupled to hexane diamine. 1-ethyl-3-

(3-dimethylaminopropyl) carbonyldiimide (EDAC) was used to condense carboxylates of the next reagent to the amine-studded fiber surface. In the case of fibers used in pond water studies this was estrone-3-glucuronide. For studies on effects of estrogen mimics on binding between ER and ERE, this reagent was an ERE-linked fluorophore. Reagents were obtained from Sigma, St. Louis, MO.

Procedures for Ground Water Testing

Samples of water from several ponds and lakes in Minnesota were collected by Dr. Eric Garber of the USDA, Fargo, ND. Blind samples were shipped to IA, Inc. and stored frozen until assayed. Samples were prepared for injection into a HH01 portable evanescent sensor by mixing the water 1:1 with assay buffer (0.1M phosphate / 0.1M NaCl / 3.0mM EDTA / 20mM Na_2MoO_4 / 20% glycerol / 2mM DTT / 1mM PMSF, pH 7.4) containing either $2x10^{-10}$ M or $4x10^{-10}$ M Cy5-hER-α. A 7.5cm sensor cartridge was assembled using a sensor fiber having estrone-3-glucuronide of its surface. The sensor was mounted in the instrument and conditioned by a 10-minute injection of assay buffer. Solutions were then passed through the sensor cartridge (volume 80 ul) for 3 minutes at a flow rate of 100 µl/min in the order: control sample followed by Minnesota water sample followed by control sample. A fresh sensor cartridge was used for each control-sample-control series. Buffer was washed through the sensor for three minutes between control and sample flows. Data was processed using software from *Threefold Sensors*. The parameter of interest for kinetic measurements is the rate of change of fluorescence (y axis) with respect to time (x axis). As receptor binds to the fiber, a real-time sensorgram is produced by the instrument. The slope of the sensorgram becomes the parameter that is used to compare controls with sample. Depletion of sites on the sensor fiber is compensated mathematically by predicting that the slope of the sample should occur as the average of the two controls. The difference between this prediction and the actual slope produced by the sample is then divided by the predicted value and multiplied by 100 to obtain a percent inhibition or activation.

Procedures for Studying the Effect of ER Modulators on ER Binding to ERE.

The estrogenic compounds 17β-estradiol, estrone, estriol, 4-hydroxytamoxifen, genestein, diethylphalate, and diethyl stilbestrol were obtained from *Sigma Chemical* and dissolved in ethanol to a concentration of 10^{-4}M. Subsequent 10-fold dilutions were performed using DI water then assay buffer to create samples having 10^{-6} M concentrations of the estrogenic compound. Prior to use, receptor preparation was chromatographed on a P10 column to remove EDTA which was present and which might possibly block fluorescence. In order that the receptor not be lost on the column, ovalbumin was added to hER preparations so as to produce receptor solutions containing 0.1% ovalbumin prior to chromatography. The receptor peak emerged from the column in roughly 10 times its original volume. This was divided into 50µl aliquots and snap frozen. A snap frozen aliquot of heparin column-purified alpha receptor was diluted 1:6 in a fashion which produced concentrations of fluorophore, ovalbumin and MOPS identical to that of the assay buffer, (50 µM fluorophore in 1% ovalbumin MOPS-buffered saline (MBS) at pH 7.1.) The snap-frozen aliquot of beta receptor was similarly diluted 1:2. ERE fibers were mounted in flow-through cartridges inserted into the TG01 sensor instrument and equilibrated with a flowing solution of assay buffer. Ten minutes prior to each test, individual aliquots of receptor were thawed and made 10^{-6} M with the estrogen being tested. Once the fluorescent counts had stabilized, data was collected for one minute in the above buffer, then 300 µl of the receptor-estrogen sample was flowed through the sensor, and data collected for 3-4 minutes, followed by 5-7 minutes of flowing through the assay buffer containing the estrogen but not the ER. For this preliminary work, only 10^{-6} M concentrations of the various estrogens were tested. In order to calculate actual dissociation constants, K_d, and kinetic constants, k_a and k_d, several concentrations of each estrogenic compound must be used. It was not necessary to carry out such calculations because the data shown below provides a fairly clear picture of the differences in the effects of the various estrogens, without calculation of the precise constants. The values for the y-axis, % maximum bound, were calculated as 100 * [1- (sample fluorescence / initial fluorescence)].

Results

Biosensor Groundwater Measurements

An example of a sensorgram obtained by flowing, first a control solution, then a sample of water which probably contains an estrogen-mimicking substance, and then another control solution, is shown in Figure 5. The top and bottom traces are produced by the control solutions. The flatter trace in the middle is produced by

a water sample that probably contained an estrogen mimicking substance of unknown origin. The source of this sample was the pond designated ROI2 which is described in the accompanying paper.[48]

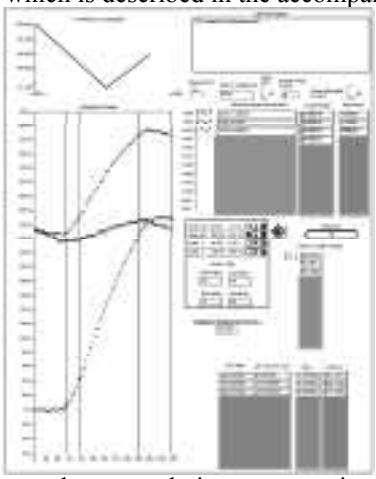

Figure 5: A real time sensorgram shows an obvious suppression of binding between estrone-3-glucuronide on the sensor surface and Cy-5 ER-α on the sensor fiber.

The slopes from sensorgrams of this type were obtained for a dilution series of diethylstilbestrol in order to establish the response of the sensor to a known estrogen mimic. The dose-response curve for DES that results from plotting the slope of the sensorgram trace vs. DES concentration is shown in Figure 6. In this experiment, a single sensor cartridge was used repeatedly with different concentrations of DES. Buffer was flowed through the sensor between samples.

Water samples from ponds in Minnesota were collected and coded so that persons performing sensor measurements were blind as to the nature of the sample. Included in the coded samples was a sample of DI water that had been spiked with $9x10^{-9}$M 17β-estradiol. That this sample fell in the proper place on a sensor calibration for 17β-estradiol is seen in Figure 7.

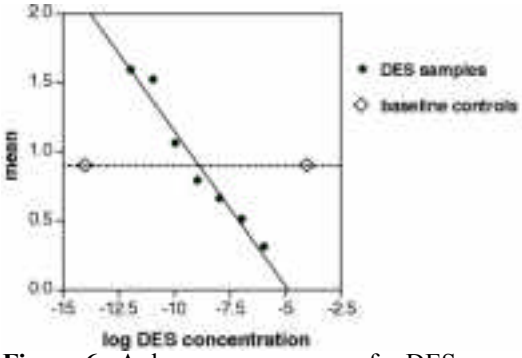

Figure 6: A dose-response curve for DES was obtained by injection of serial concentrations of DES into the sensor cartridge. The diamond symbols represent sensor response to control buffer before and after the series. This establishes that the DES data is not an artifact of progressive depletion of sites on the sensor.

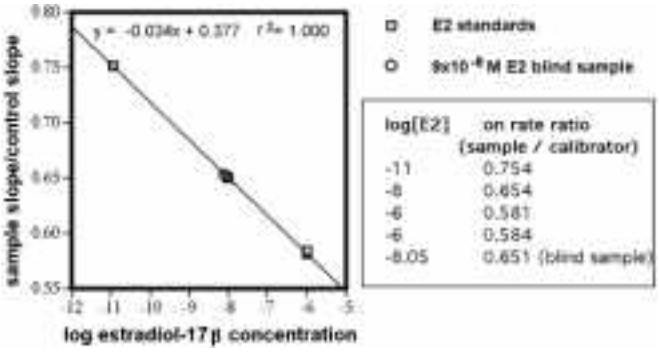

Figure 7: Sensor response to estradiol-17ß is log linear in the region between 10^{-11} M and 10^{-6}M. A sample containing distilled water spiked with $9x10^{-9}$ M estradiol-17ß was included with the pond water samples as a blinded control. This sample is indicated by the circle.

The most striking differences among the ground water samples are seen by comparing ROI, ROI2 and WAS, all of which are in the vicinity of one another, but which differ in reports of the presence of malformed frogs. The details regarding the sites and the FETAX procedures and results can be found in the accompanying paper.[48] Figures 8a and 8b show real time data for binding and dissociation between Cy5-ER and estrone-3-glucuronide fibers. It can be seen that the binding is slower for ROI than for WAS and is considerably slower still for ROI2. Off rates are the most rapid for ROI2 and the least rapid for WAS. This is consistent with hypothesis that there exists a compound in the ROI2 and to some extent in the ROI water that competes aggressively for ER in the samples.

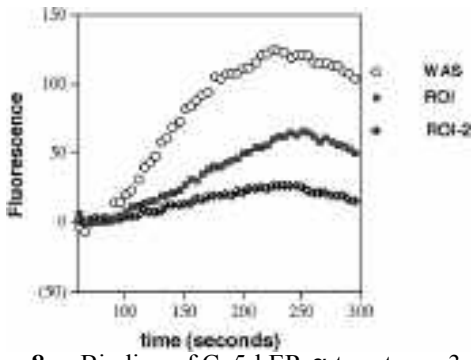

Figure 8a: Binding of Cy5-hER-α to estrone-3-glucuronide fibers in the presence of three water samples suggests that ROI and ROI2 contain estrogen mimicking compounds to varying degrees. The WAS sample does not produce significant suppression of binding.

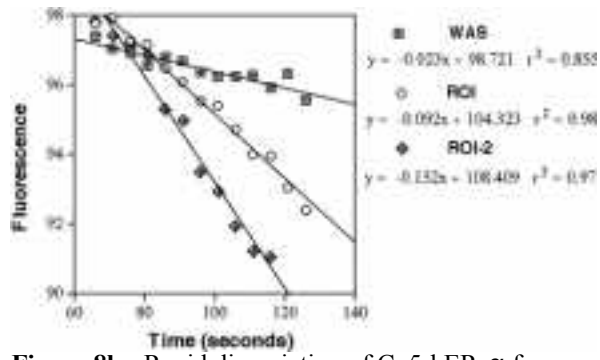

Figure 8b: Rapid dissociation of Cy5-hER-α from estrone-3-glucuronide fibers in seen in the presence of water from ROI2 and to a lesser extent ROI. The WAS sample does not produce this effect.

Table 1 summarizes data obtained from several Minnesota water sites along with an indication of whether or not some type of adverse effect on the frog population is suggested. Details of frog status appear in the accompanying paper.[48] Percent inhibition is the average of several readings obtained for each sample. Readings were normalized across several batches of fiber cartridges and receptor preparations using the inhibition created by a 10^{-6}M sample of 17β-estradiol as a standard. Establishment of a cutoff value where a sensor reading >10% indicates possible presence of an estrogenic endocrine disrupting compound would correctly place all samples with the exception of BOE. The variation in readings from a ground water sample taken from a given pond was about 10%, thus samples, CWB, and BOE could be misplaced on the basis of a single reading. ROI-2, ROI, HIB, NEY and MHL2, where adverse impact has been observed, are clearly higher than DAR, IND and WAS, where frogs appear normal.

CODE	Evidence of Adverse impact on Frogs	% inhibition
D water control 1	N/A	8%
ROI-2	yes	83%
ROI	yes	66%
HIB	yes	89%
NEY	yes	43%
MHL-2	yes	27%
CWB	yes	17%
BOE	no	16%
WAS	no	3%
IND	no	5%
DAR	no	0%

Table 1: A relationship exists between sensor inhibition and frog status in Minnesota ponds

In order to pursue the nature of the component present in two of the ponds having significant inhibition, samples of water from ROI-2 and from HIB were extracted into hexane. The hexane layer was removed and dried down and the remains were taken up in ethanol and diluted with water to create a sample having 10% ethanol. In the case of ROI2, the volumes were adjusted so that the component of interest was dissolved in the same volume of fluid at the end as it was at the beginning. In the case of HIB, the component from the organic extract was concentrated five-fold over the original concentration. The aqueous phases were measured using the sensor as before along with the extracted samples. Results of this procedure are shown in Table 2. Since the percent inhibition is calculated as 100 times the actual value divided by the projected value in the absence of inhibition, values greater than 100% inhibition are not unreasonable. They do not mean that the signal from the sensor becomes less than zero. Whatever is producing the sensor inhibition is at least partially extracted into hexane and can be concentrated by extraction and evaporation followed by redissolution in a smaller aqueous

volume. Loss of some but not all of the ability to inhibit the sensor occurred in the aqueous phase remaining after extraction. No inhibition was produced by simply drying down hexane and treating the tube in the manner of a sample. There appears to be something in the water of ROI2 and HIB that is more soluble in organic phase than in aqueous phase and which binds to ER-α. This does not imply that these water contain the same ER-interactive compound.

Sample description	% sensor inhibition
original ROI-2 water	123%
aqueous ROI2 after extraction	44%
ROI-2 hexane extract/EtOH	67%
original HIB water	77%
aqueous HIB after extraction	45%
5x (2%) EtOH resuspension of hexane HIB	264%

Table 2: The component producing inhibition of ER binding can be extracted into hexane.

Several possible alternative explanations for sensor inhibition were investigated. In case the effect was related to a sodium or potassium deficiency, FETAX salts were added to the sample and sensor response measured as before. There was essentially no change. In case the high iron content of the water was to blame, a sample that showed little inhibition (WAS) was spiked with iron so as to be equivalent to ROI2. This did not increase inhibition of sensor response. In order to eliminate the possibility that interaction with the sensor that was unrelated to binding between estrone-3-glucuronide and estrogen receptor might account for the observations, a fiber that was treated with streptavidin was used in place of the estrone-3-glucuronide fiber. When Cy5-hER was injected into the sensor according to the protocol, there was not a significant response from the sensor.

The data presented support the hypothesis that estrogen-mimicking compounds are present in water from certain sources of groundwater in Minnesota and that there is a correlation between the presence of such compounds and observations of frog abnormalities. The mechanism by which the correlation results is not at all clear at this point. Until a compound is isolated from the water and shown to produce abnormalities cause and effect cannot be drawn. The sensor has shown itself to be a useful device for tracking the presence of the component of interest as various manipulations such as extractions are performed.

Biosensor Studies on the Effects of Estrogen Mimics on ER binding to ERE

Comparison of effects of estrogenic compounds on binding and dissociation between fiber-sensor-bound ERE and unlabeled hER-α or hER-β in solution was carried out using one fiber cartridge repeatedly as test compounds and buffers were washed through. The resulting sensorgrams are shown in Figures 9-12. On these graphs, a vertical line defines the moment when the sample was changed from one containing both hER and estrogen to one containing only estrogen. Figure 9 demonstrates a fundamental difference between hER-α and hER-β with regard to ERE binding in the absence of estrogen. Whereas hER-α forms a loosely bound complex, hER-β does not form an ERE complex in the absence of estrogen.

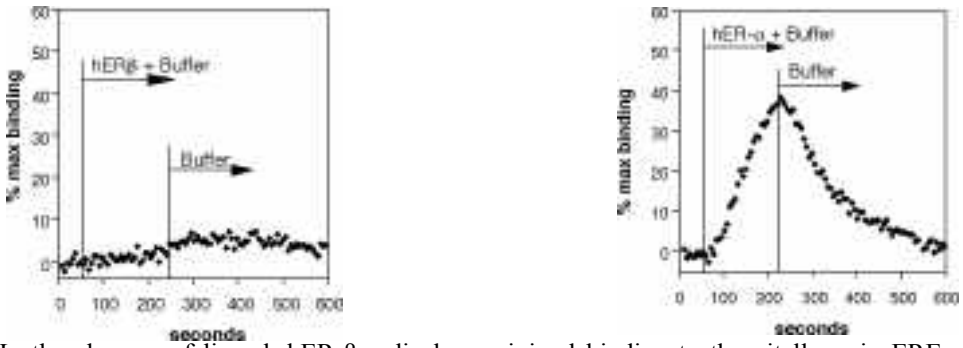

Figure 9: In the absence of ligand, hER-ß displays minimal binding to the vitellogenin ERE consensus sequence. On the other hand, hER-α displays rapid, unstable binding to the vitellogenin ERE consensus sequence. Binding between hER-α and the ERE in the absence of estrogen has often been noted in the literature.[49, 50]

Figure 10 demonstrates that for both ER types, 17β-estradiol has the effect of producing a more stable complex between ER and ERE.

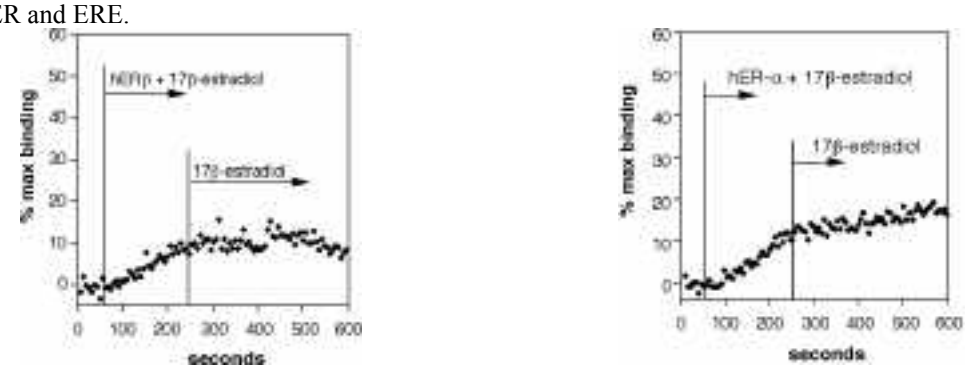

Figure 10: The hER-ß -17ß-estradiol complex associates more slowly and dissociates more rapidly with the ERE than does the hER-α-17ß-estradiol complex. The off rate began dominating the sensorgram by around 380 seconds for hER-ß, but had not clearly dominated the sensorgram at 500 seconds for hER-α. This is consistent with the radioligand binding experiments performed at HRL (E2-hER-α K_d =2-7x10^{-10} M; E2-hER-ß K_d= 1-5x10^{-9} M).

One of the most provocative results obtained was that hER-α and hER-β responded differently to the presence of different types of estrogenic compounds. Genestein was selected as a common phytoestrogen found in many vegetable products and diethylphthalate was selected as a fairly ubiquitous xenoestrogen. Sensorgrams shown in Figures 11 and 12 indicate that genestein more strongly activates binding between ER-β and ERE, while diethylphthalate activates binding of hER-α, but not hER-β.

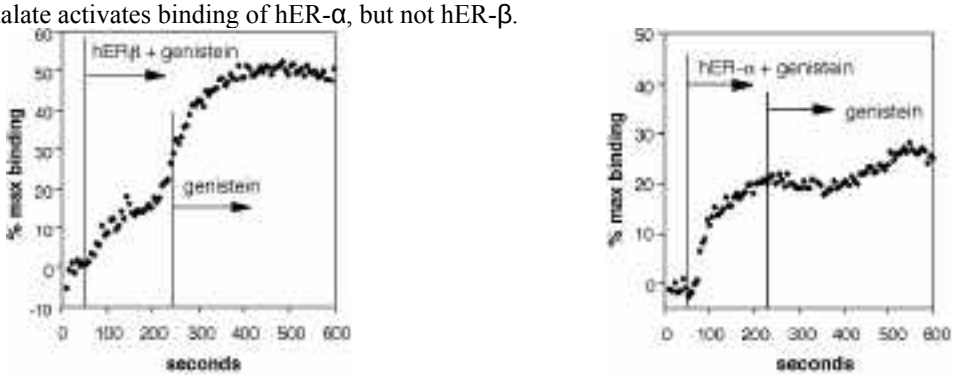

Figure 11: For both hER-β and hER-α, genistein produced the greatest increase in the rate of association between the receptor and the ERE. The effect on hER-β was greater than on hER-α. In both cases genistein produced an interaction sensorgram reflecting multiple on rates and off rates. For hER-ß, the most rapid increase in binding occurred around 200 seconds into the experiment, immediately after changing the solution flowing into the sensor from hER-β+ genistein to genistein alone.

Although these results are preliminary, they are consistent with the hypothesis that ER-α and ER-β act in a balanced manner on specific EREs under endogenous conditions. The phytoestrogen, genestein produces an imbalance in favor of ER-β, while the xenoestrogen diethylphthalate produces an imbalance in favor of ER-α.Genestein has been shown to be beneficial for bone and vascular health.[35] Phthalates have been linked to abnormal sexual development.[19] Whether this observation will generalize remains to be seen. In any case, the evanescent sensor proves to be a rapid way of comparing the effects of such compounds.

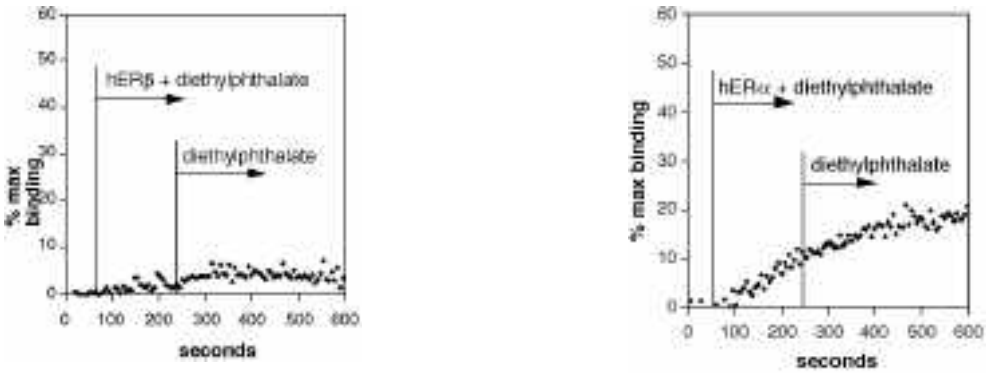

Figure 12: The xenoestrogen, diethylphthalate, had a minimal effect on hER-β, but produced a gradual persistent association between hER-α and ERE. This is consistent with literature indicating that phthalate esters have estrogenic activity and recent reports linking phthalate esters to abnormal sexual development in humans.

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Mineral Deficiency, Estrogenic Activity, and the Occurrence of Malformed Frogs in Minnesota

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Abstract

During the mid-to-late 1990s an increase in the incidence of malformed frogs was observed in 68 counties in Minnesota. The parasitic trematode *Ribeiroia* has been linked to the occurrence of malformed Pacific treefrogs (*Hyla regilla*) in Oregon and California [Johnson et al. 1999] and may explain the occurrence of malformed frogs at some of the sites in Minnesota. However, it does not explain the presence of malformed frogs at all the sites and the decrease in malformation rates during recent years at some sites.

During 1999, 2000, and 2001 surveys were conducted of wetlands in Minnesota and water samples were collected. The water samples were analyzed for the presence of various anthropogenic compounds (i.e.: pesticides and phthalates), estrogenic activity, and mineral content. In addition, the water samples were tested for the ability to disrupt *Xenopus laevis* embryo development and induce malformations.

Estrogenic activity was determined using an evanescent field biosensor in which activity is measured by the ability of a component in a sample to interact with the estrogen receptor and inhibit the binding to another estrogen. Estrogenic activity was detected in the water from several wetlands, particularly those that contained malformed frogs. The level of estrogenic activity at two of the sites, HIB and ROI2, was equivalent to several orders of magnitude more estradiol than could be explained by the amount of ubiquitous estradiol that was measured using commercially available ELISA kits.

Studies using *Xenopus laevis*, the South African clawed frog, have demonstrated that low potassium levels resulted in developmental retardation [Tietge et al. 2000]. Seasonal fluctuations in potassium levels at two sites, NEY and CWB, correlated with biological activity as determined using the Frog Embryo Teratogenesis Assay: *Xenopus* bioassay. In contrast, the low concentration of sodium in the water from ROI2 was found to induce *Xenopus* retardation despite the presence of potassium at levels sufficient to support normal development. Upon extension of the incubation time from 96 to 120 hours, embryos reared in ROI2 water displayed a high rate of gut malformations. Mineral supplementation of all water samples prevented the developmental retardation and resulted in normal embryos.

Classification of the ponds based on mineral content, the presence of estrogenic activity, and reports regarding the occurrence of *Ribeiroia* infected frogs indicated that malformed frogs were only observed at sites that contained at least two of these factors. Limited mineral content can potentially affect anuran development and the occurrence of malformations by numerous mechanisms. Retardation in the development of tadpoles may increase the window of susceptibility towards an infectious agent or the action of an endocrine disruptor. Likewise, growth in a restrictive media may compromise the embryos and make them more susceptible to infection or endocrine disruption.

Introduction

Amphibian populations around the world have been declining at an alarming rate for several decades [Houlahan et al. 2000]. In contrast, the incidence of malformed frogs in Minnesota was estimated at only 0.2% prior to the mid-1990s [Hoppe 2000]. Between 1995 and 1997, malformed frogs were reported in over 62% of the counties in Minnesota. Surveys conducted across the state of Minnesota since 1996 examined 13,763 northern leopard frogs (*Rana pipiens*) and found 900 malformed frogs, a malformation rate of 6.5% [Murzyn R & Canfield, J,

MPCA personal communication]. The types of malformations observed have been documented by Meteyer et al. 2000. The widespread occurrence of malformed frogs and the importance of fresh water to the food chain has increased public concern regarding food safety and public health.

Numerous hypotheses have been proposed to explain the appearance of malformed frogs in Minnesota. These include exposure to parasites, pesticides, predation, retinoids, increased ultraviolet B (UVB) radiation from thinning of the ozone layer, and aerial deposition of pollutants from neighboring metropolitan and industrial areas [Rosenberry 2001 and references cited within].

The ubiquitous nature of UVB has made it a plausible trigger for the occurrence of malformed frogs over a large geographical region, such as the state of Minnesota, during a short period of time. Research has demonstrated that UVB radiation can directly cause deformities in frogs [Ankley et al. 1998, Blaustein et al 1997]. However, it has not been established whether tadpoles in the various wetlands would be exposed to the necessary UVB doses and whether the incidence of frog malformations correlated with UVB penetrability at these sites. In addition, this direct UVB hypothesis does not explain the decreased percentage of malformed frogs in many wetlands during recent years. UVB as an indirect cause of frog malformations by photo-activation of biological agents [Klesecker et al. 2001] or chemical compounds is a possibility. A photo-activation process could display fluctuations consistent with the observed changes in malformation rates. It should therefore be possible to isolate the causative agent or its degradation products.

The premise that an aquatic agent is responsible for the occurrence of malformed frogs is supported by laboratory experiments, which have demonstrated that water collected from various wetlands that contained deformed frogs induced malformations in frog larvae [Burkhart et al. 1998]. The aquatic agent(s) may be either biological or chemical. Parasitic cysts of the trematode *Ribeiroia* have been implicated in the occurrence of malformed Pacific tree frogs (*Hyla regilla*) and were able to induce multiple limbs in the laboratory [Sessions et al. 1999, Johnson et al. 1999, Daszak et al. 1999]. However, parasites are probably not the sole cause of frog malformations in Minnesota. Although *Ribeiroia* has been found in frogs collected from several wetlands [Sutherland 2001, Johnson PTJ & Lunde KB, personal communication], no *Ribeiroia* has been found at several other sites that harbor malformed frogs. Furthermore, filtration of the water from sites that contained malformed frogs did not reduce the incidence of malformations in laboratory experiments with *Xenopus laevis* and C-18 solid phase extraction of water samples was able to concentrate the teratogenic activity [Fort et al. 1999].

In this study, we used the frog embryo teratogenesis assay: *Xenopus* (FETAX) bioassay to screen water samples collected from various wetlands and studied the role of minerals in the occurrence of malformed frogs in Minnesota. The presence of phthalates and the pesticide content of the water samples were examined by GC/MS. In addition, ubiquitous levels of estradiol were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits. These results, along with biosensor studies reported in the accompanying paper [Erb et al. 2001], are consistent with a model in which hydrological events during the mid 1990s may have contributed to the rate of frog malformations. A decrease in either sodium or potassium content to less than 2 ppm would have retarded tadpole development and could have altered the windows of susceptibility toward infection or the action of a biologically active compound (e.g., endocrine disruptors).

Materials and Methods

Chemicals and supplies

GC/MS analytical standards were purchased from Ultra Scientific (North Kingstown, RI, USA), all other chemicals used were of the best analytical grade available. Adult *Xenopus laevis* frogs were obtained from Xenopus I (Ann Arbor, MI, USA) and maintained at 22 ºC on a diet of Aquamax Grower 500 Fish Food (PM Nutrition International, Inc., Brentwood, MO, USA). The use of plastic was minimized and all glassware was rinsed twice with 1 M HCl, methanol, acetone, and deionized water, and baked dry prior to use.

Water Samples from Minnesota Wetlands

Water samples were collected from sites known to contain deformed frogs, either currently or in the past, or for which no malformed frogs were reported (control sites). The water samples were collected in Trace Clean HDPE jars (VWR, Chicago, IL, USA) and stored on ice during transport prior to freezing at -20 ºC. Whenever possible, water samples were collected to coincide with egg deposition (April), tadpole development (May/June), and metamorphosis (July/August).

Site Properties

Table 1 lists and details the wetlands from which water samples were collected during 1999 through 2001. Included are statements regarding the status of frogs at the sites, a description of the environment surrounding the wetland, the pH, and the conductivity of the water at the time of sampling. CWB, HIB, NEY, ROI, ROI2, and TRD are sites that historically harbored malformed frogs. The environments surrounding the sites differ considerably. CWB is a lake partially surrounded by agriculture and woods. Marsh and grassland surround HIB with no agriculture in the vicinity other than a small family vegetable garden to which neither pesticides nor chemical fertilizers were applied. NEY was totally surrounded by fields of soy beans and corn. ROI and ROI2 were two wetlands situated between Lakes Darwin (DAR) and Washington (WAS) and located less than 50 m apart. Lake Darwin is connected to Lake Washington via ground water. During wet seasons, as the water table

Table 1: Site Properties

Site	Environ.[a]	Frog Status	I [b]	pH [b]
			uS	
CWB	wood,ag,rec	1999: 66 of 110 *R.septentrionalis* malformed [c]	39 - 46	7.0 - 9.0
DAR	wood,rec	no reports of malformed frogs	319 - 414	7.7 - 9.0
HIB	marsh	2000: 77 of 113 *R.pipiens* malformed	241 - 259	7.5 - 8.9
LYN	ag	no reports of malformed frogs	207 - 370	6.9 - 8.0
NEY	ag	9% malformation rate [c]	318 - 380[c]	7.0 - 8.0
ROI	wood,rec	1999: 21 of 389 *R.pipiens* malformed [c,d]	151 - 330	6.2 - 6.6
R0I2	wood,rec	declining population, only *R.sylvatica* left	101 - 244	6.0 - 7.4
TRD	semi-rural	1999: 38%, 2000: 9% *R.pipiens* malformed [e]	321 - 330	6.6 - 7.2
WAS	rec	no reports of malformed frogs	343 - 386	6.9 - 8.5
Mineral Media			1640	7.6

[a] Environment surrounding the site: ag -agricultural; marsh-marshland; rec-recreational; semi-rural, wood-wooded.
[b] Conductivity and pH range observed during samplings conducted between 1999 - 2001.
[c] Canfield J. (MPCA), personal communication
[d] The close proximity between ROI and ROI2 made it difficult to ascribe any observations exclusively to one site.
[e] Hoppe DM, personal communication

rises, ROI and ROI2 were also interconnected via the ground water. However, during times of drought, as may have occurred during the late 1980s, the water table drops and ROI2 and ROI were effectively disconnected from the two lakes. There was no agricultural activity in the vicinity of either ROI or ROI2. ROI was adjacent to woods, while woods completely surrounded ROI2. Recreational housing was nearby and on the shore of Lake Washington. TRD was located in a park/wayside stop on the outskirts of a town and near agriculture. HIB, NEY, and TRD were manmade ponds. Frogs collected from CWB, HIB, and NEY have been shown to contain cysts from the trematode *Ribeiroia*, while no cysts were detected in frogs obtained from ROI/ROI2 or TRD [Sutherland 2001].

FETAX Analysis

The frog embryo teratogenesis assay-*Xenopus* (FETAX) bioassay was used to screen compounds for teratogenic activity. Phytochemicals, endocrine disruptors, retinoids, herbicides, and pesticides have been shown to induce malformations in the FETAX bioassay [Davis et al. 1981, Dumpert 1987, Fort and Bantle 1990, Mann and Bidwell 2000]. FETAX analyses were conducted according to American Society for Testing and Methods (ASTM) guidelines (E 1439-91) [ASTM 1991] with the following modifications. Three independent scorers examined all samples for malformations and the results were averaged. Typical variances between scorers were < 10%. In addition, the profile of each embryo was individually photographed for length measurements. Length measurements were made using SigmaScan Pro 5.0 (SPSS, Chicago, IL, USA) and entailed following the notochord to the base of the body and across to the mouth or cement gland. Length measurements were expressed as the mean in mm $\pm$ standard deviation (number of embryos /number of different clutches).

The term malformation in the FETAX bioassay refers to the presence of features that differ from those observed with embryos raised for 96 hours, to stage 46, on a mineral media also known as FETAX water [Bantle et al. 1999, Nieuwkoop and Faber 1994]. Embryos that lacked deformities but were slightly retarded and displayed features indicative of a stage earlier than 46 [i.e. the presence of a residual cement gland and a loosely coiled gut] would be classified as malformed. In order to clarify this ambiguity, a new sub-classification under the category of malformations, developmentally delayed, was introduced to indicate developmentally retarded embryos lacking structural deformities. The degree of developmental delay was classified as either severe or slight depending on whether both a cement gland and an uncoiled gut were present. When feasible, water samples that induced an inordinate amount of developmental delay were re-screened with an extended incubation period to determine if the embryos would reach stage 46 after 120 h of incubation. This procedure enabled the detection of subtle developmental antagonists that might otherwise be classified as teratogens.

The composition of the mineral media routinely employed was 10.7 mM NaCl / 1.14 mM $NaHCO_3$ / 0.403 mM KCl / 0.135 mM $CaCl_2$ / 0.349 mM $CaSO_4 \cdot 2H_2O$ / 0.623 mM $MgSO_4$. The role of sodium, potassium, magnesium, and calcium in *Xenopus* development were studied using supplemental media designated sABCD. In naming the supplemental media, sABCD the A and B refer to the sodium and potassium content in ppm while the C and D refer to the magnesium and calcium concentrations in 10 ppm increments. Hence, s2511 media contains 2 ppm sodium, 5 ppm potassium, 10 ppm magnesium, and 10 ppm calcium. A third type of media employed was Syn-ROI2 which contained 1.2 ppm Na/5.7 ppm K/4.1 ppm Mg/9.7 ppm Ca and was formulated to have the same mineral content as ROI2 water from 07-01-99. Syn ROI2-10X contained the same proportion of minerals as Syn ROI2 but at 10X concentration.

Pesticide and Phthalate Analysis

Pesticides in the water samples were determined using a modification of the procedure developed by Lehotay et al (1998). As modified, the procedure entailed passing 4 ml of the water sample supplemented with 100 ppb terbuthylazine, as an internal standard, over an IST Isolute ENV+ 200 mg 10 ml column (Jones Chromatography, USA) and washing with two column volumes of water. Bound material was eluted with 2 ml of a solution consisting of 2.5 acetonitrile:7.5 acetone:6 methylene chloride and dried under nitrogen. Samples were then suspended in 100 ul chloroform and analyzed by GC/MS using a Micromass Autospec MS fitted with a HP5890 GC. The samples were injected using a HP6890 autosampler using a cool on-column injection method and separated using a 30 m (0.25 mm I.D.) CP-Sil 5 CB Low Bleed/MS capillary column (Varian Inc.). The temperature program started at 80 °C (held 1 min) and increased at 10 °C /min to a maximum of 310 °C. Identification and quantification of the pesticides was based on the elution times listed in Table 2 and the m/z peak areas, respectively. The apparent limits of detection (LOD) for the various pesticides present in standard mixtures following ENV+ extraction are also presented in Table 1. Standard mixtures were analyzed at concentrations of 0.01, 0.05, 0.1, 0.5, 1.0, and 10 ppb. The lowest concentration at which a pesticide was detected with a maximum variance of 40% from the expected fragmentation ratio for the monitored ions was considered the apparent LOD. Further analyses of purified atrazine standards indicated a LOD of 0.025 ppb and a recovery for atrazine of 87%.

Table 2: Pesticides Monitored

Pesticide	Retention Time	m/z Monitored		LOD[a]
	(min:sec)			(ppb)
prometon	16:41	225.159	210.1355	0.05
atrazine	16:50	215.0938	217.0909	0.025
lindane	16:46[b]	216.9145	218.9116	0.05
alachlor	18:47	269.1183	271.1157	0.5
cyanazine	19:01	225.0655	227.0627	0.5
chlorpyrifos	19:32	313.9574	315.9545	0.5
metolachlor	19:37	238.0999	240.0973	0.05
chlordane	21:09[c]	372.826	374.8231	0.05
dieldrin	22:00	344.8989	346.896	0.5
endosulfan	22:27	338.8553	340.8523	0.5
endosulfan sulfate	23:09	386.84	388.8371	0.05
methoxychlor	23:56	227.1072		0.05

[a] Limit Of Detection (LOD) based on analysis of standards at 0.01, 0.05, 0.1, 0.5, 1.0, and 10 ppb. As per the site water samples, the standards contained terbuthylazine as an internal reference and were column extracted prior to analysis.
[b] additional peaks also used for quantification at 17:03, 17:27, and 17:35 with LOD values closer to 0.01 ppb.
[c] additional peak used for quantification at 21:28.

The presence of phthalates in the water samples were determined using GC/MS. Two to four liters of water were passed through a 5 g C-18 column and eluted with 20 ml of methanol or acetone. Aliquots of the methanol and acetone extracts were dried and suspended in chloroform for analysis using GC/MS. Alternatively, the presence of phthalates in the water samples was also determined by chloroform extraction of lyophilized water samples. The operation of the GC/MS was as previously described for the pesticides.

Estradiol Analysis

Estradiol in the water samples was determined using competitive ELISA kits from Cayman Chemical (Ann Arbor, MI) and Neogen Corporation (Lexington, KY).

Biosensor Analysis

A fiber optic evanescent biosensor, model HH01 Fiber Optic Biosensor Fluorometer manufactured by Threefold Sensors (Ann Arbor, MI) [Smith et al. 1999] was utilized to examine water samples for the presence of compounds capable of affecting estrogen mediated signal transduction as previously described [Erb et al 2001, Garber et al. 2001]. Briefly, the biosensor monitors in real-time the inhibition of Cy5-human estrogen receptor-α binding to estrone-3-glucuronide, a weak estrogen immobilized on the surface of an optical fiber by estrogenic compounds in the water samples.

Mineral and Complex Ion Analysis

Mineral and complex ion analysis of the water samples was done by the North Dakota State University Soil and Water Environmental Laboratory, Fargo, ND. K, Ca, Mg, Na, S, Cu, Fe, Zn, Hg, Pb, and As were determined by ICP-atomic absorption spectroscopy. Carbonate and bicarbonate were determined by titration with 0.0100 N sulfuric acid. Chloride was determined by titration with 0.005 N silver nitrate. Nitrate was determined colorimetrically by the nitration of salicylic acid. The limits of detection were: K, 0.05; Ca, 0.002; Mg, 0.001; Na, 0.01; S, 0.01; Cu, 0.003; Fe, 0.003; Zn, 0.002; Hg, 0.02; Pb, 0.03; As, 0.3; and all anions (carbonate, bicarbonate, chloride, and nitrate), 0.1 ppm.

Statistical Analyses

Statistical analyses of the FETAX data were performed using Dunnett's test. Data that yielded Dunnett q' test statistics of less than the $\alpha_T = 0.05$ q' critical value were considered insignificant. Length measurements were analyzed using n values representative of the sample size (number of embryos measured) and also recalculated using the number of different clutches to gauge experimental variability. Determinations of the correlation coefficients between embryo length and mineral composition were made using the Pearson product-moment correlation coefficient (r). The variance of whole populations were calculated using the equation $[n\Sigma x^2 - (\Sigma x)^2] / n^2$ and the VARP command in Excel (Microsoft).

RESULTS

Pesticide and Phthalate Content

Analysis of water collected at CWB (08-11-99), LYN (04-24-01 and 05-09-01), TRD (04-24-01), ROI2 (07-01-99, 08-17-00, and 04-25-01), and DAR (07-01-99) for alachlor, atrazine, chlordane, chlorpyrifos, cyanazine, dieldrin, endosulfan, endosulfan sulfate, lindane, methoxychlor, metolachlor, and prometon indicated only atrazine to be present at levels greater than the limit of detection. The levels of atrazine detected at the various sites are presented in Table 3; all were considerably less than the 3 ppb maximum contaminant level (MCL) [Nelson et al. 2001].

None of the samples showed elevated levels of phthalates relative to controls.

Table 3: Atrazine Content

Site	Date	Atrazine (ppb)
CWB	08/11/99	0.079 ± 0.006
DAR	07/01/99	0.168 ± 0.019
LYN	04/24/01	0.035 ± 0.003
LYN	05/09/01	0.045 ± 0.006
ROI2	07/01/99	0.081 ± 0.002
ROI2	08/17/00	< 0.025 [a]
ROI2	04/25/01	0.050 ± 0.013
TRD	04/23/01	0.040 ± 0.007

[a] LOD estimated at 0.025 ppb

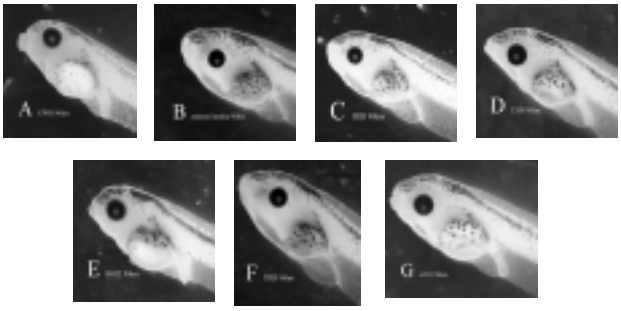

Figure 1: *Xenopus laevis* embryos at 96 hours following completion of the FETAX bioassay. The embryos were incubated on A) CWB 08-11-99; B) mineral media; C) HIB 08-21-00; D) LYN 05-09-01; E) ROI2 07-01-99; F) TRD 04-24-01, and G) s2211 media.

Biological Activity

The FETAX results obtained with water from the various wetlands are presented in Figure 1 and Table 4. Though the mortality rate for most sites was ≤ 5%, the slightly higher rates observed with water from DAR, NEY, and ROI2 may not be significant due to a higher variability between egg clutches as evidenced by a 12% standard deviation with the water from ROI2. Of particular interest is the high incidence of developmentally delayed embryos obtained with water from CWB, ROI2, and LYN as evidenced by the presence of cement glands and uncoiled guts (Figure 1A, D, and E). The severity of the developmental delay, the amount of residual cement gland and lack of gut coiling, correlated with embryo length. The embryos reared in LYN water (Figure 1D) were the least delayed. Embryos reared in s2211, a medium that contained only 2 ppm sodium, 2 ppm potassium, 10 ppm magnesium, and 10 ppm calcium, also displayed residual cement glands and lack of complete coiling (Figure 1G). In contrast, no such characteristics were observed with embryos reared in mineral media (Figure 1B).

Table 4: FETAX Analysis of Water Samples

		96 hour FETAX			
Site	Date	Mortality	Malformed	Dev.Delay	Length [b]
		(%)[a]	Rate (%)[a]	(%)[a]	(mm)
CWB	08/11/99	4	77	72	7.1 ± 0.5 (225/8)
DAR	07/01/99	10	13	3	8.9 ± 0.5 (175/7)
HIB	08/21/00	5	15	1	9.3 ± 0.4 (150/4)
LYN	05/09/01	4	94	91	8.5 ± 0.4 (25/1)
NEY	10/05/99	9	13	3	8.9 ± 0.4 (100/3)
ROI	07/01/99	5	9	5	9.0 ± 0.4 (50/2)
R0I2	07/01/99	15	76	69	7.5 ± 0.5 (275/11)
TRD	04/24/01	0	11	0	9.2 ± 0.3 (25/1)
WAS	07/01/99	3	9	2	8.7 ± 0.5 (75/3)
Mineral Media		4	6	1	9.3 ± 0.5 (250/7)

[a] percentage of the initial 25 embryos in the assay.
[b] lengths are expressed in mm ± 1 Std. Dev (number of eggs tested / number of different clutches).

Role of Minerals

Water from CWB and ROI2 supplemented with minerals equivalent to the concentration present in the mineral media have been shown to yield normal stage 46 *Xenopus* embryos in the FETAX bioassay [Garber et al. 2001]. Likewise, the suspension of lyophilized material in the mineral media also resulted in normal stage 46 embryos, while suspension in deionized water resulted in severely delayed embryos, identical to those observed with both the untreated and filtered water [Garber et al 2001]. These results are consistent with the work of other researchers [Burkhart et al 1998] and support the conclusion that neither a volatile compound nor a biological agent that can be removed by filtration was the cause of the developmental delay observed in the bioassay. Rather, the mineral composition of the water may have an effect on embryonic development in the FETAX bioassay.

Table 5: Variation of Embryo Length with Mineral Content and Sampling Date

ROI2						
date	07/01/99	07/01/99-well	04/13/00	06/13/00	08/17/00	04/25/01
length	7.5 ± 0.5 (275/11)	8.5 ± 0.4 (100/3)	8.1 ± 0.5 (175/5)	7.7 ± 0.4 (150/5)	7.5 ± 0.3 (50/2)	7.3 ± 0.5 (25/1)
sodium	1.197 [a]	2.6	1.7	1.4	0.8	0.5
potassium	5.7	7.9	13.4	16.2	7.7	9.2
magnesium	4.1	16.3	4.0	4.6	4.9	3.1
calcium	17.8	6.7	16.6	15.1	26.7	11.4
chloride	0.8	3.9	2.5	1.4	0.4	0.7
carbonates	53	88	75	98	26	31
CWB						
date	08/11/99	03/01/00	05/03/00	06/01/00	08/11/99-se-well	
length	7.1 ± 0.5 (225/8)	7.0 ± 0.5 (175/4)	7.2 ± 0.5 (125/4)	7.0 ± 0.4 (100/4)	7.0 ± 0.6 (75/3)	
sodium	1.8	1.0	1.4	1.1	1.7	
potassium	1.2	0.7	1.0	1.5	1.9	
magnesium	1.8	1.0	1.6	1.4	1.3	
calcium	4.2	4.9	4.7	3.9	5.6	
chloride	1.1	1.4	1.2	0.4	0.7	
carbonates	18	22	22	21	38	
NEY						
date	10/05/99	04/25/00	06/06/00			
length	8.9 ± 0.4 (100/3)	8.4 ± 0.3 (75/2)	7.1 ± 0.6 (100/3)			
sodium	4.9	3.7	3.2			
potassium	4.6	3.4	1.0			
magnesium	21.2	18.0	10.2			
calcium	24.7	11.1	8.0			
chloride	13.3	11.7	9.0			
carbonates	100	81	52			

[a] Na, K, Mg, Ca, Cl, and carbonates (CO_3^{2-} + HCO_3^-) are espressed in ppm

A survey of lakes in Minnesota has identified low potassium as a concern, with CWB being especially low in potassium [Burkhart et al. 2000]. Research on the effects of potassium on *Xenopus* development during the first 96 hours following egg fertilization indicated that low levels of potassium, comparable to that present at CWB, retarded *Xenopus* development [Tietge et al. 2000].

Table 5 compares the seasonal fluctuations in the mineral content at ROI2, CWB, and NEY with *Xenopus* embryo length following 96 hours of incubation in water from these sites (FETAX analysis). Also listed are the analyses of the ground water collected from a well on the southeast side of CWB (designated CWB-SE-well) and of a well adjacent to ROI2. Fluctuations in the sodium and chloride content of ROI2 correlated with embryo length with correlation coefficients of r = 0.969 and 0.976, respectively. In contrast, no correlations were observed between the length of *Xenopus* embryos reared in CWB water and either the sodium or potassium content of the water (r = 0.000 and -0.738, respectively). This result with CWB reared embryos was probably a consequence of the small variance (0.006 mm) in average length (range 7.0 - 7.2 mm) and the interplay between insufficient sodium and insufficient potassium levels in the water. The observation of a

correlation coefficient of 0.785 between the chloride content of CWB water and embryo length in the FETAX bioassay is currently being examined. Inasmuch as NEY was only sampled three times, the correlation coefficient between embryo length and potassium (r = 0.998) was of limited significance.

The addition of 5 ppm sodium to ROI2 (07-01-99) water resulted in a 16% increase in embryo length to 8.7 mm. Though still substantially smaller than embryos raised on mineral media, the features were indicative of embryos only slightly developmentally delayed. In order to determine whether the effects of ROI2 water on *Xenopus* embryo development were entirely due to an insufficient sodium content, the effects of various media were compared following 96 hours of incubation (FETAX bioassay) and 120 hours (extended FETAX). The results of these experiments are presented in Table 6.

Table 6: Effects of Mineral Media Composition

	96 Hours				120 Hours			
	Mo.[a]	M[b]	D[c]	length (mm)	Mo.	M	D	length (mm)
Mineral Media	4	6	1	9.3 ± 0.5 (250/7)	2	6	0	10.1 ± 0.6 (225/7)
S2211	6	80	76	8.6 ± 0.6 (150/6)	3	37	31	9.4 ± 0.5 (150/6)
S2511	8	66	61	9.1 ± 0.3 (50/2)	4	19	18	9.7 ± 0.3 (50/2)
S5211	10	10	1	9.1 ± 0.4 (50/2)	4	11	0	10.1 ± 0.4 (50/2)
S5511	3	26	19	9.2 ± 0.4 (75/3)	3	13	11	10.1 ± 0.4 (75/3)
Syn ROI2	7	93	82	8.2 ± 0.4 (75/2)	7	93	89	8.7 ± 0.5 (75/2)
Syn ROI2-10X	2	13	12	9.5 ± 0.4 (50/2)	14	20	0	10.4 ± 0.4 (50/2)

[a] Mo. (Mortality rate): percentage of embryos that did not survive to the completion of the experiment
[b] M (malformation rate): percentage of embryos displaying features not indicative of stage 46 or later.
[c] D (delayed): percentage of embryos displaying features of a stage earlier than 46 with no other deformities.

None of the synthetic media resulted in a significant increase in embryo mortality (P>0.05) in the FETAX bioassay. However, developmentally delayed *Xenopus* embryos were observed after 96 h of incubation in synthetic media that contained less than 2 ppm sodium in the following order of decreasing retardation of development: Syn ROI2 > S2211 > S2511 (all P<0.01). Embryos reared on mineral media, Syn ROI2-10X, and S5211 displayed insignificant levels of developmental delay (P>0.05). S5511 resulted in normal sized embryos with normal gut coiling but with a slight residual cement gland.

Extended Incubation

After 120 h of incubation in the various media (Table 6), all embryos displayed an increase in length. Embryos reared on mineral media, Syn ROI2-10X, and S5211 were still indistinguishable P>0.05). A slight residual cement gland was still detected on the embryos reared on S5511 even though their length and other properties were indistinguishable from the embryos reared on mineral media. The majority of embryos reared on S2211 and S2511 were at stage 46 or later with only slight residual cement glands detected in a minority of the embryos. In contrast, the majority of the embryos reared on Syn ROI2 were still not at stage 46 (P<0.01). These results indicated that a decrease in sodium content to 2 ppm was sufficient to delay tadpole development. In addition, at lower concentrations of sodium, such as 1.2 ppm in the presence of reduced magnesium (Syn ROI2), the retardation of development was more severe and not overcome by an additional 24 h of incubation (P<0.01).

Embryos were also reared on LYN and ROI2 water for an additional 24 h (120 h total) to determine whether the developmental delay observed at 96 h was symptomatic of a teratogen or just a slowing of development. Embryos reared on LYN water grew to 9.2 $\pm$ 0.3 (25/1) and displayed features of normal stage 46 or later embryos with only a slight residual cement gland present. In contrast, the embryos reared on ROI2 water displayed gut malformations with a 0.7 mm increase in size.

Figure 2 depicts *Xenopus* embryos reared on ROI2, Syn ROI2, and mineral media for 96 and 120 h. The guts of the embryos reared in ROI2 and Syn ROI2 water for 96 h were not coiled and were indicative of stage 43-44 embryos. After 120 h, significant differences were observed between embryos reared on ROI2 and Syn ROI2 water. The guts of the Syn ROI2 embryos had started to coil while the guts of the ROI2 embryos displayed invaginations (Figure 2E,F). This was observed in 44 out of 100 embryos after 120 h of incubation on water collected at ROI2 on 7-01-99, 8-17-00, and 4-25-01. Reexamination of the comparable embryos exposed for 96 h to ROI2 water indicated that possibly 20 out of the 100 had indications of gut malformations, though the details were not obvious.

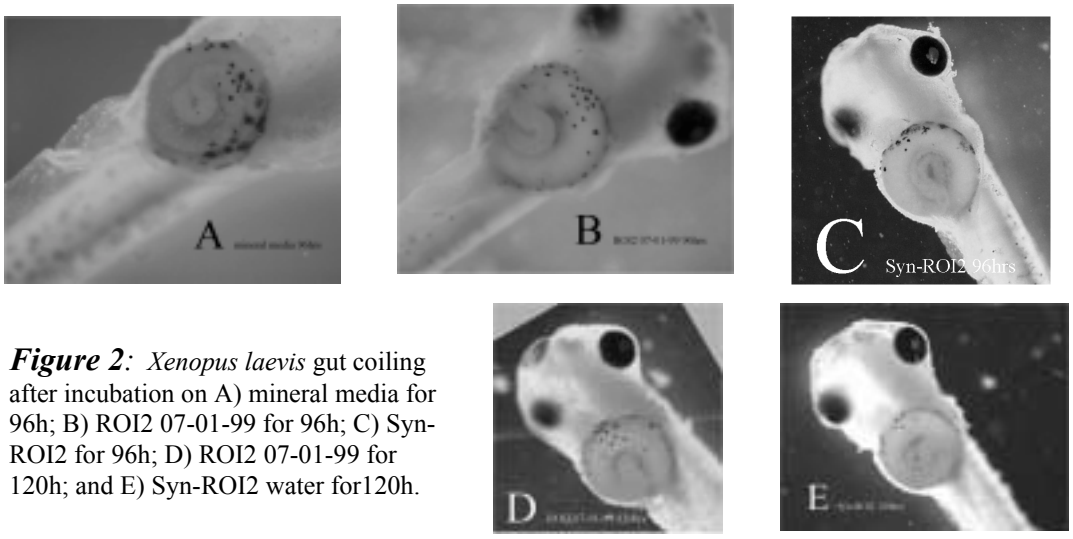

***Figure 2**: Xenopus laevis* gut coiling after incubation on A) mineral media for 96h; B) ROI2 07-01-99 for 96h; C) Syn-ROI2 for 96h; D) ROI2 07-01-99 for 120h; and E) Syn-ROI2 water for120h.

Estrogenic Activity

An examination of the various water samples for the presence of compounds capable of interacting with recombinant human estrogen receptor-α were presented in the accompanying paper [Erb et al 2001] and in preliminary form [Garber et al 2001]. ROI2, HIB, and ROI displayed levels of estrogenic activity in the biosensor equivalent to concentrations of estradiol greater than 10^{-6} M. ELISA analyses were conducted to determine whether such high levels of ubiquitous estradiol were present in the water samples. Figure 3 depicts the results obtained using ELISA kits manufactured by Neogen Corporation and Cayman Chemical. Both products gave comparable results with tap water indicative of a threshold of 20 ppt. However, with environmental samples the Neogen ELISA kit indicated a background of approximately 40 ppt while the Cayman product was approximately 20 ppt. Overall, the results obtained with 14 different samples representing 11 sites indicated a correlation of 0.764 between the ELISA kits (not all shown). The differences between the two ELISA kits may be representative of different cross-reactivities with other components in the samples. According to the manufacturers, the Neogen ELISA had a cross-reactivity with estriol and estrone of 0.41% and 0.10%, while the Cayman product was 0.57% and 4%, respectively. Analyses of 18 water samples soon after collection in 1999 indicated higher levels of estradiol [Garber et al. 2001]. Prolonged storage may therefore affect the concentration or stability of freely soluble estradiol. In either case, the trend between the sites was comparable. ROI2 contained the greatest level of estradiol with the following order of decreasing estradiol content: ROI2 samples collected on 06-13-00 > 07-01-99 ~ 08-17-00 >> well water. Assuming that the ELISA measurements did not include any cross-reacting compounds present, the maximum estradiol content of the ROI2 water was 1nM, several orders of magnitude less than the amount predicted by the biosensor. It is

therefore very likely that the biosensor detected an estrogenic compound in water samples that was not ubiquitous estradiol.

Comparison of the estradiol levels for the various ROI2 samples with the lengths of the embryos generated in the FETAX bioassay resulted in a negative correlation coefficient of approximately -0.3. This is consistent with retardation of *Xenopus* development being primarily due to the mineral content of the water and not due to ubiquitous estradiol. However, the malformations observed upon rearing *Xenopus* in ROI2 water may be partially due to the ubiquitous estrogenic activity inasmuch as the Syn ROI2 reared embryos did not display these features.

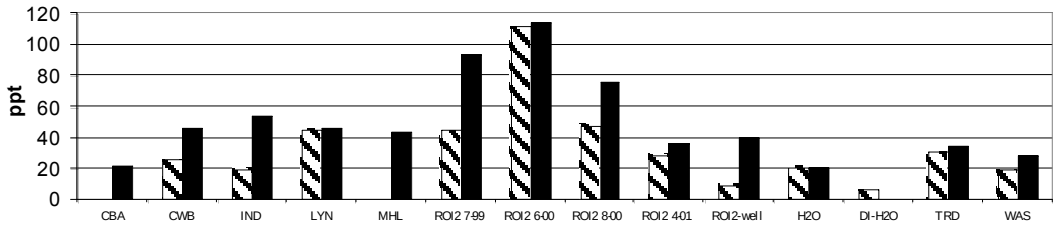

Figure 3: ELISA analysis of estradiol content of water samples. Solid bars - ELISA kit manufactured by Neogen Corporation, hatched bars - ELISA kit manufactured by Cayman Chemical. Note: CBA and MHL were not analyzed using the Cayman Chemicals ELISA kit and deionized water was not analyzed using the Neogen ELISA kit.

Discussion

Pesticides

Though the data indicate that levels of contamination for various pesticides were less than the established MCL values, it is not possible to definitively conclude that pesticides did not play a role in the occurrence of malformed frogs at these sites. This is because MCL values have not been established [Ruhl et al. 2000] for half the pesticides examined (i.e. chlorpyrifos, cyanazine, dieldrin, endosulfan, and prometon) and a considerable difference exists between MCL values and the aquatic life criteria. An example is methoxychlor, which has an aquatic life criterion 33,000-fold less than the official MCL value of 100 ppb. Furthermore, the aquatic life criteria for dieldrin, chlorpyrifos, and methoxychlor are 0.056, 0.041, and 0.03 ppb, respectively [Gilliom 2001, EPA 1999], which are less than the limits of detection of the assay employed.

It is not likely that the atrazine detected at the various sites was the sole cause of the frog malformations. Research on female largemouth bass has demonstrated that exposure to 50 ppb atrazine increased serum estradiol levels while 25 ppb had no effect [Grady et al 1998, Schmitt & Dethloff 1999]. Other research has demonstrated that 20 ppb atrazine had no effect on gray tree frog (*Hyla versicolor*) larval development though the next higher concentration examined, 200ppb, did [Diana et al. 2001]. The concentrations of atrazine detected in this study at the various sites were less than 0.1 ppb. In order for any of the sites to have contained 50 ppb atrazine it would require that all the atrazine was deposited 1.5 or 5.7 years prior assuming either a 60 day half-life [Wauchope 1992] or a 230 day half-life [Klaassen 1979]. The possibility that a synergism between the atrazine and another effector caused the malformations can not be ruled out, especially because very little is known about the effects of mixtures [Gilliom et al. 1999].

Role of Minerals

Supplementation studies and statistical analysis of the bioassay data confirm the importance of potassium and sodium for proper *Xenopus* development. Furthermore, the data indicate that a threshold concentration of approximately 5ppm sodium and potassium were necessary to avoid developmental retardation, with the most dramatic effects observed below 2 ppm. Statistical analysis of the bioassay data and mineral content of 30

water samples collected from 18 different wetlands (data not shown) yielded only weak correlation coefficients for sodium (r=0.363), potassium (r=0.299), and chloride (r=0.357). This probably was indicative of the substantial differences in the nutrient contents of the sites and variations in the effects a particular mineral may elicit. In addition, the use of data obtained with samples that contained more than the threshold concentration of the mineral under analysis would also reduce the r value.

The observation that the mineral content of several sites that contained malformed frogs was insufficient to support the normal rate of *Xenopus laevis* and *Rana pipiens* tadpole (data not shown) development may explain the sudden widespread occurrence of malformed frogs throughout the state during the mid- to late 1990s. Hydrological events diluting the mineral content of the wetlands could have delayed tadpole development, which would have altered the windows of susceptibility towards either infection by biological agents (i.e. *Ribeiroia*) or the action of hormonally active compounds. A shift in the rate of development may have increased the likelihood of the tadpole being present at the same time as the teratogen and also increased the duration of its exposure. Consistent with this hypothesis is the Palmer Hydrologic Drought Index (PHDI) for central Minnesota which showed that the wettest period of record (105 years) occurred from the mid 1980's to 1996. This period also included a severe drought from 1988 to 1990, followed by an above average rate of recharge with spring peaks of stream flow exceeding the 75 percentile during 1995 [Stoner et al. 1998]. This elevation of the water table could have induced new hydrological pathways that mobilized both chemical and biological agents into the wetlands. The appearance of malformed frogs at HIB in 2000 may be an example of this process. Prior to heavy rainfall in 2000 and the subsequent flow of water from the west to the east across the site, no malformed frogs were observed.

Estrogenic Activity and Synergism

Whether estrogenic compounds can directly induce deformities at environmentally relevant concentrations under realistic conditions that moderately stress the organism is not known and is currently being explored. In amphibians the estrogen, thyroid, and retinoic acid systems are interconnected. Estradiol inhibits the interconversion of T4 to T3 and can induce involution of the thyroid gland [Hayes 2000]. The thyroid receptor functions as a heterodimer with the retinoid X receptor (RXR) [Reginato et al 1996]. Prolactin, a presumed thyroid hormone inhibitor [Tata 1996], can inhibit retinoic acid-dependent limb development [Maden and Corcoran 1996] and is itself regulated by T4 via the hypothalamus [Denver 1996]. Thus, it is not surprising that the administration of 1.5 mg/l (ppm) estradiol induced limb deformities in frogs [Takahashi 1958] and the administration of 10^{-5} M (2.7 ppm) estradiol inhibits development and affects metamorphosis [Nishimura et al. 1997]. Though the morphology of these deformities was different than those observed in the wild, these results indicate the interconnectivity between steroidal hormones and metamorphic development.

A mechanism by which the mineral content of the wetlands may contribute to the occurrence of malformed frogs is through synergism with a second effector. The mechanism of this synergism is purely speculative. It is conceivable that the two components function at the molecular level *in-vivo* and overwhelm the ability of the organism to develop normally. As such, the low sodium content at ROI2 may have stressed the developing tadpole and made it more susceptible to disruption by the estrogenic activity detected in the water. Similarly, the high level of estrogenic activity at HIB may have contributed to the tadpoles being susceptible to infection by *Ribeiroia*.

Conclusion

It is currently not possible to ascribe the occurrence of frog malformations in Minnesota to a single causative agent. Though an association was reported between the occurrence of malformed frogs in the St. Lawrence River Valley of Quebec, Canada and agricultural activity [Ouellet et al 1997], no obvious correlation exists between agricultural activity and the sites in Minnesota. Malformed frogs were observed both in man-made and naturally occurring ponds, near agriculture, urban areas, and relatively pristine areas consisting of woods and marshland. Dilution of critical minerals in the wetlands due to hydrological events could retard tadpole development and alter windows of susceptibility towards infectious agents and endocrine disruptors which have been detected in the various wetlands associated with malformed frogs.

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Effects of Exposure to Low Levels of Water-Borne 17β-Estradiol on Nest Holding Ability and Sperm Quality in Fathead Minnows

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Abstract

This study assessed the effects of exposure to low levels of estradiol (E2), as they have been found in some treated sewage effluents, on nest holding ability and sperm quality, two measures likely related to reproductive success, in fathead minnows (*Pimephales promelas*), a species with paternal nest care. Male fathead minnows were exposed for 21 days to either 50 ng/L (E2) in 25 µl/L ethanol, an E2 concentration found in the effluent of some British and Canadian sewage treatment plants (STP), or to a 25 µl/L ethanol control (aqueous flow-through exposure). After exposure, groups of six males from one treatment were placed into an observation aquarium with females and a limited number of spawning sites and allowed to spawn for four days. Plasma vitellogenin (VTG) concentrations, the ability to acquire and defend a nest site, and sperm quality was compared between E2 exposed and control males. E2 exposure resulted in a significant increase in VTG concentrations in E2 exposed males, but did not affect nest holding ability or sperm quality. These preliminary results indicate little effect of 21 day exposure to levels of E2 that match the highest reported E2 loads in STP effluent, and parallel our findings from a previous study using goldfish. However, caution is warranted in extrapolating these results to populations of wild fish, as other factors such as the overall estrogenic potency of effluent, the duration and timing of exposure to estrogenic compounds, and the competition between fish for spawning opportunities need to be estimated.

Keywords: Endocrine Disruption; Estradiol; Fathead Minnow; Behavior; Sperm Production

Introduction

The steroid 17ß-estradiol (E2) is an important hormone in endocrine pathways of fishes (Arcand-Hoy and Benson 1998) and occurs frequently in effluent discharge of municipal sewage treatment plants (STP) (Desbrow *et al.* 1998; Ternes *et al.* 1999, Rodgers-Gray *et al.* 2000) where it can cause endocrine disruption in fishes (Purdom *et al.* 1994; Harries *et al.* 1997; Routledge *et al.* 1998). Numerous studies have assessed the effects of E2 and associated xenoestrogens on wild fish (Folmar *et al.* 1996; Jobling *et al.* 1998; Lee *et al.* 2000; Folmar *et al.* 2001), fish that were caged (Purdom *et al.* 1994; Harries *et al.* 1996; Harries *et al.* 1997; Routledge *et al.* 1998; Rodgers-Gray *et al.* 2001), and on fish in the laboratory (Kramer *et al.* 1998; Panter *et al.* 1998; Miles-Richardson *et al.* 1999; Korte *et al.* 2000; Bjerselius *et al.* 2001). All of these studies found induction of the yolk protein vitellogenin (VTG) in exposed male fish, as well as other effects such as gonadal abnormalities (Gimeno *et al.* 1998; Jobling *et al.* 1998; Miles-Richardson *et al.* 1999; Rodgers-Gray *et al.* 2001), changes in behavior (Bayley *et al.* 1999; Bjerselius *et al.* 2001), and sperm production (Gimeno *et al.* 1998; Bjerselius *et al.* 2001). However, little is known about the reproductive effects of exposure to E2 at these low concentrations. In a previous study (Schoenfuss *et al.* in press), we found that 50 ng/L E2 exposure resulted in minor reductions in spawning behavior and sperm production in male goldfish (*Carassius auratus*) which have no parental care. Based on these results, we designed a new experiment to determine whether exposure to similar E2 concentrations would be more detrimental to fish with greater paternal involvement in reproduction. E2 exposure concentration were chosen to match the highest E2 concentrations reported in sewage effluent in Great Britain (Desbrow *et al.* 1998; Rodgers-Gray *et al.* 2000) and Canada (Ternes *et al.* 1999). In this study we exposed male fathead minnows (*Pimephales promelas*), a species with paternal nest care, to low levels of E2 and assessed the effects of exposure on the fishes ability to hold nest sites and to produce viable sperm.

The fathead minnow is widely used in toxicological studies (Ankley *et al.* 1998) and reproduces in the laboratory. Fathead minnows breed year round with the male guarding a cavernous nest site until eggs hatch.

The ability to defend a nest is determined by the individual's social status in which dominant males, identifiable by their tubercles, dorsal pad, and bright banding pattern, establish successful breeding territories (Cole and Smith 1987). Smith (1974) suggested that these morphological characteristics are associated with elevated androgen levels. The male defends the nest site continuously until hatching as eggs are quickly eaten by other fathead minnows in the absence of the nest guarding male (Sargent 1989). Estrogen exposure could increase circulating E2 levels in male fish and suppress androgen levels, by altering neuroendocrine feedback loops (Trudeau *et al.* 1993; Jobling *et al.* 1996; Ankley *et al.* 1998).

We expect nest holding ability to be a sensitive indicator of reproductive success as the opportunity to spawn is conditional upon possession of a nest site (Unger 1983; Huntingford and Torricelli 1993). We also expect sperm production to serve as a sensitive and relevant measure of the effects of estrogen exposure as sperm production is important in externally fertilizing fish (Petersen and Warner 1998). A reduction in sperm quantity (which normally increases in association with interactions with ovulating females (Stacey and Sorensen 1986; Dulka *et al.* 1987; DeFraipont and Sorensen 1993) or quality can lead to reduced fertilization rates (Nakatsuru and Kramer 1982; Zheng *et al.* 1997; Suquet *et al.* 1998; Cosson *et al.* 2000). By exposing fathead minnows to E2 we addressed two questions. (1) Is sperm quality in fathead minnows compromised by E2 exposure? (2) Are E2 exposed male fathead minnows able to maintain nest sites?

Materials & Methods

Experimental animals

Fish for this study were bred and reared at the University of Minnesota. Fish were held in 28°C water (flow-through, 300 ml/min) from an in-house well, at a constant photoperiod (16 h:8 h light:dark, 0800 lights on), and were fed frozen brine shrimp (San Francisco Bay Company, CA) twice daily *ad libitum*.

Experimental design

An experiment was conducted to assess the effects of E2 exposure on nest holding ability and sperm quality in male fathead minnows. In four trials males were exposed for 21 days to either E2 or an ethanol control. After exposure, groups of males were placed with females and allowed to spawn. Male behavior was observed, after which plasma VTG concentrations, and sperm quality were quantified.

Exposure

For each trial, 16 spermiating fathead minnows were netted from a stock aquarium and distributed evenly among four 20 L aquaria. Aquaria were aerated, shielded on three sides, and fish were maintained otherwise as in the stock aquaria. Males were held for 21 days in either (i) 50 ng/L E2 dissolved in 25 µl/L ethanol, or in (ii) a 25 µl/L ethanol control. Experiments with guppies (*Poecilia reticulata*) and goldfish (Bayley *et al.* 1999; Schoenfuss *et al.* in press) found no effects of ethanol exposure on spawning behavior and sperm production. Both treatments were administered via continuous flow-through delivery (280 ml/min). A peristaltic pump continuously supplied two aquaria with an E2 stock solution, while the other two aquaria received ethanol at concentrations similar to the one used to prepare the E2 stock solution. E2 was extracted weekly from E2 exposure aquaria and bi-weekly from the control aquaria. Water samples were concentrated using C18 SepPaks (Waters Corporation, MA), and than measured with an E2 ELISA test kit (Cayman Chemicals, MI). Aquarium concentrations of E2 were approximately 50% of the 50 ng/L nominal concentration at 24 ± 3.6 ng/L E2 (mean ± standard error). E2 concentrations in the ethanol control aquaria were below detection limit (9 ng/L).

Spawning assay

After 21 days, groups of six male fish from the same treatment were moved from the exposure aquaria into 160 L observation aquaria (day one). Each observation aquarium contained six mature female fathead minnows and three nest sites (3" PVC pipe sections) to ensure that only nest sites are limiting reproductive success. In preliminary studies performed with unexposed males in a similar setting, males routinely occupied all nest sites within 24 hours. Males were marked using a combination of the same three latex colors that were injected

subcutaneously. A pre-experiment found no effects of latex injection on the reproductive ability of male fathead minnows.

Male fathead minnows were observed twice each morning (days two through five) for five min intervals. During the observation period the nest holding male in each of the three nest cavities was identified. Each nest defended by a male fathead minnow was scored as a "nest holding event" for the treatment. Data from all four replicates were pooled resulting in a total of 48 'nest holding events' per aquarium and treatment (three nests x four observation days x four replicates).

On the fifth day, fish were anesthetized with 0.1% phenoxyethanol (Sigma, St. Louis, MO), placed upside-down into a grooved sponge, and milt (sperm and seminal fluid) was drawn into a capillary tube by applying light abdominal pressure following established protocols (Stacey and Sorensen 1986). Care was taken not to contaminate the sample with feces or urine. For storage, 1 µl of milt was transferred into 100 µL of isoosmotic sperm extender following published protocols (Chao *et al.* 1987). For the sperm analysis, 10 µl of sperm extender solution was suspended in 100 µl distilled water (1:2000 final dilution) and vortexed. The drop in osmotic pressure activated the sperm sample (Billard *et al.* 1995) which was then analyzed using a Hamilton-Thorn motility analyzer (Danvers, MA). Mean Progressive Velocity (MPrV), a measure of sperm velocity along its path was used to characterize sperm quality (Kime *et al.* 1996). Each sperm sample was analyzed three times and values were averaged to determine the MPrV for each fish. MPrV was calculated only for those fish with expressible milt.

VTG assay

After completion of sperm analysis, blood was collected from the caudal vasculature, stored on ice, and centrifuged at 10,000 rpm for 1 min. Plasma was transferred into aprotinine coated microcentrifuge tubes and stored at –20°C for later analysis. Plasma VTG concentrations were determined using a fathead minnow-specific ELISA following published protocols (Parks *et al.* 1999). For the statistical analysis VTG concentrations below detection limit were reported as zero.

Statistical analysis

Sperm quality and plasma vitellogenin concentrations were analyzed using a non-parametric Mann-Whitney U test as some of the data were not normally distributed. The proportional nest holding data were analyzed using a Fisher's Exact test. All calculations were performed with the Prism 2.0 statistical package for the Macintosh (Graphpad Software Inc., CA).

Results

Table 1. Plasma vitellogenin (VTG) concentrations, sperm quality, and nest holding ability in male fathead minnows exposed to E2 or a control.

	Control		**E2**		
	N	Mean ± Stand. Err.	N	Mean ± Stand. Err.	
VTG (mg/ml)	23	0.01±0.006	24	**29.12 ± 0.62	p<0.01
MPrV (µm/sec)	11	45.45 ± 2.12	12	45.41 ± 0.95	ns
Nest Holding (days)		44		48	ns
(%)		92%		100%	ns

Plasma VTG concentrations in E2 exposed male fathead minnows were significantly higher than in control males ($p<0.01$, Mann-Whitney U test; Table 1). Despite VTG induction in exposed males, sperm quality did not differ between E2 exposed and control males and the MPrV was virtually identical ($p>0.05$, Mann Whitney U test, Table 1). The ability to hold nest sites also did not differ between E2 exposed and control fish. The number of nest holding events was similar for E2 exposed and control male fathead minnows ($p>0.05$, Fisher's Exact test; Table 1). E2 exposed male fathead minnows were holding nest sites during all 48 observations, while control males held nests during 44 out of 48 observations. As males were color-coded it was also

possible to identify changes in the identity of the nest holder from day to day. However, very few incidents in which the nest holding male changed from one day to the next were observed in either treatment.

Discussion

Our preliminary results indicate that three week exposure to 50 ng/L E2, a concentration exceeding E2 loads in the effluent of at least two of the few STP effluents that have been analyzed for E2 loads, consistently induced VTG production in mature male fathead minnows. The reproductive consequences associated with this physiological effect appear minor under the experimental conditions presented in this study. These results match our earlier findings in goldfish, for which 10 week exposure to E2 at similar concentrations results in VTG induction but had relatively minor effects on spawning behavior and sperm production (Schoenfuss *et al.* in press). Ours is the first study to evaluate the effects of aqueous exposure to E2 at concentrations that simulate E2 loads in some STP effluents on nest holding ability and sperm quality in fathead minnows.

The E2 exposure protocol used in this study is notable because the absolute exposure concentration in this study (24 ng/L) is below the highest E2 loads reported for STP effluent. Although the E2 concentration in this study was higher than effluent E2 concentration reported for US STP effluents, some studies have reported E2 concentrations in STP effluent as high as 88 ng/L in Great Britain (Desbrow *et al.* 1998, Rodgers-Gray *et al.* 2000) and 64 ng/L in Canada (Ternes *et al.* 1999). Thus, our exposure protocol represents a high, but still realistic exposure scenario. Furthermore, E2 is but one of many estrogenic compounds that have been found in STP effluent and the overall estrogenic potency of the effluent could be much greater than simulated in this experiment.

The plasma VTG concentrations found in E2 exposed male fathead minnows in this study match those reported by Parks *et al.* (1999) in fathead minnows exposed to E2 via injection. Despite VTG induction, the fish appeared healthy and active and in each replicate spawning behavior could be observed within 24 h in both treatments. Nest holding ability of E2 exposed fathead minnows was also not affected by E2 exposure, with E2 exposed males holding all nest sites in all replicates of the experiment. The ability to defend a nest site is crucial for the reproductive success of male fathead minnows as eggs in any unguarded nest are quickly eaten by other fathead minnows (Sargent 1989). Our results are similar to those reported in two studies on guppies, that did not find reproductive impairment at 30 ng/L E2 exposure (Toft and Baatrup 2001), and saw no behavioral impairment at 100 ng/L E2 exposure (Bayley *et al.* 1999). In contrast, a previous study conducted in our laboratory (Schoenfuss *et al.* in press) found a significant decline in reproductive behavior in male goldfish exposed to 50 ng/L E2 for 10 weeks. Bjerselius *et al.* (2001) also found behavioral impairment at E2 exposure concentrations exceeding 1 μg/L E2, but that E2 exposure level is far above any recorded for STP effluents.

MPrV, a measure of sperm quality did not vary between E2 exposed and control fish. Sperm quality has been suggested previously (Kime *et al.* 1996; Kime 1998; Kime and Nash 1999) as a tool to analyze the effects of EDCs on the reproductive fitness of fish. However, despite the strong physiological response (VTG induction) in male fish exposed to E2, sperm quality appeared unaffected. In a previous study using goldfish (Schoenfuss *et al.* in press), the absolute number of motile sperm per fish, a product of sperm quality and sperm quantity, declined with 50 ng/L E2 exposure after 10 weeks. In contrast, Toft and Baatrup (2001) saw an increase in sperm numbers in guppies exposed to 30 ng/L E2. In this experiment we were unable to measure sperm quantity as the small size of the fathead minnow renders it impossible to strip consistently all milt while avoiding contamination by urine.

Although we did not find any impairment of nest holding ability and sperm quality in male fathead minnows exposed to E2, we remain cautious about extrapolating these laboratory results to populations of wild fish. Indeed, ongoing studies in our laboratory suggest that intra-species interactions might be an important component in evaluating the effects of E2 exposure. Furthermore, STP effluent consists of complex mixtures of chemicals, many of which have been shown to be estrogenic to fish and their combined action may exceed our exposure concentrations. Our understanding of the effects of STP effluent on fish populations is still rudimentary and further studies will be necessary to determine the effects of estrogen and xenoestrogen exposure on all life stages of fish. Answering these questions will allow us to determine the effects of effluent exposure on wild fish populations.

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Removal of Pharmaceutical Residues and Other Persistent Organics From Municipal Sewage and Surface Waters Applying Membrane Filtration

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Abstract

In out of area missions, it may be necessary to produce drinking water even from highly contaminated surface waters. These waters may contain a large number of organic, inorganic, and microbiological contaminants such as PhACs, pesticides, flame retardants, and heavy metals. The mobile drinking water purification units must be able to remove such contaminants as much as possible to meet the requirements of the maximum tolerance levels for drinking water set by the German and also by the European legislation. In the course of a research project, we investigate two drinking water purification units using the membrane filtration technique. Presently, these units undergo extensive long-term trials. If the testing of the new devices is positive regarding their functionality and their ability to remove all possible contaminants (also including radioactive compounds) they shall substitute the conventional devices which use large amounts of chemicals and charcoal filtration for water purification. The new membrane purification units are much lower in costs and the generated drinking water has much higher acceptance regarding it's organoleptic quality. In 2000, we tested a mobile drinking water purification unit, commercially available since 1999. This unit is able to generate up to 1600 liters of drinking water per hour. In a field study at the Teltowkanal, a canal carrying a high burden of municipal sewage effluents, the functionality and the efficiency of this device was tested to remove high amounts of algae, microbes, and organic and inorganic pollutants. The results from this fatigue test proved the ability of the water purification unit to reduce all contaminants to meet the maximum tolerance levels set by the drinking water regulation. Residues of PhACs have almost totally been removed from the surface water where these contaminants are found at individual concentrations up to the µg/L level. The pre-filtration device was very effective to remove algae and solid particles to protect the membranes from clogging and to enable an almost maintenance-free operation. Some results from this study will be presented in this paper.

1. Introduction

1.1 General considerations

In recent years, the occurrence and fate of pharmaceutically active compounds (PhACs) in the aquatic environment was recognized as one of the emerging issues in environmental chemistry and a matter of public concern. Residues of PhACs have been found as contaminants in several investigations of sewage, surface and groundwater samples (Stan and Heberer 1997; Halling-Sørensen et al. 1998; Heberer and Stan 1998; Ternes 1998; Daughton and Ternes 1999; Möhle et al. 1999; Wilken et al. 2000; Heberer in press). Due to the high amounts of pharmaceuticals prescribed in human medical care considerable amounts of persistent drug residues are passed through the municipal sewage plants which therefore act as point sources for the neighboring waters. Thus, drug residues are found at concentrations up to the µg/l-level in surface waters contaminated by municipal sewage effluents. Wherever contaminated surface water is used for ground water recharge in drinking water production, the polar drug contaminants can also cause problems to drinking water supplies by leaching into the ground water aquifers of the drinking water plants (Heberer et al., 1997 & 2001; Heberer in press).

In general, there is a need to focus on reducing or, if possible, eliminating the release of organic compounds into surface water. This may be achieved by restricting their use or by using modern technologies such as new, highly effective sewage treatment technologies. The use of some organic contaminants may be prohibited to protect the aquatic environment but the main purpose of pharmaceuticals is given by their medical indication. Thus, the use of a particular compound can and should not be generally banned for environmental reasons. Nevertheless, restrictions for their use may be desirable and achievable in the future. This may be especially important with regard to possible effects such as resistances of bacteria against antibiotics. As far as the medical indication allows, drugs showing a negative environmental behavior in environmental risk assessment studies could and should be replaced by other compounds.

Many of the above mentioned aspects have already been discussed and will possibly be put into future legislation. In the meantime, it is necessary to assess the present situation. PhACs occur at concentrations up to the µg/L-level in surface water and in a few cases, at very low concentrations also in ground- and drinking water. These low concentrations may, from a toxicological point of view, not be harmful to humans but their occurrence in ground- or drinking water is also not desirable from a hygienic point of view (Heberer and Stan, 1998; UBA, 2000) or with regard to the precautionary principle. Thus, there is a need to develop and study new drinking water treatment technologies to remove such organic contaminants from drinking water

1.1 Aims of the research project funded by the German Ministry of Defense

In 1999, the German Ministry of Defense initiated a research project on this issue. This project has three defined objectives. The first one is the investigation of PhAC residues in ground water wells used for drinking water production by the German Army. Secondly, the occurrence and fate of PhAC residues in the effluents from an Army hospital in Berlin is currently investigated. This is done both by calculations of the pharmaceutical loads deriving from prescription amounts and knowledge on pharmacokinetics and by confirmatory measurements of target compounds in the individual and combined hospital effluents. The fate of these residues will be investigated down to the sewage treatment plants and the receiving surface waters. A final goal is to provide an environmental risk assessment for the compounds discharged from this particular hospital and to compare and apply these results to other military and civil hospitals. In the third part of this project we investigate the effectiveness of new, mobile drinking water treatment techniques for the removal of PhACs, some other contaminants, and microbes.

1.1 Use of mobile drinking water purification units in out of area missions

In the course of out of area missions, a reliable and save drinking water supply for the soldiers and the other military personnel is one of the most important logistics and has to be guaranteed. At such locations, public water supply is often not available (not sufficient, damaged or destroyed) or safe. Thus, it may be necessary to produce sufficient and clean drinking water even from highly contaminated surface water. Such waters may contain various organic, inorganic, and microbial pollutants such as PhACs, pesticides, industrial chemicals, heavy metals or pathogen bacteria. Independent of the origin of the surface water, the mobile drinking water purification units have to reduce the concentrations of any contaminant as much as possible to supply hazardous-free drinking water to the consumers and to meet the requirements of the European drinking water regulations (EEC, 1980; 1998) or additional standards (e.g. STANAG 2136). The requirements for drinking water purification units designed for military use are even stronger than those for civil use as they also have to guarantee the removal of any kind of nuclear, biological, and chemical contamination (NBC).

Conventional mobile purification units need much personnel for maintenance and large amounts of chemicals and active charcoal. Drinking water produced by conventional techniques meets all regulations but often lacks in acceptance among the consumers due to the way it is produced by using chemicals and charcoal powder and due to the resulting turbidity problems. With regard to their growing international duties, the German Ministry of Defense has decided to replace the conventional purification units by modern ones using membrane filtration for purification. Before these units are used in out of area missions, they need to be tested over a long time-scale under extreme and “worst-case” conditions such as large amounts of various contaminants (including NBC), cold and hot ambient temperatures, and high ambient humidity.

In this paper, the first results from our field investigations on the performance of a mobile drinking water purification unit, at an urban canal highly polluted by municipal sewage effluents, are presented. This system which uses bag filter pre-filtration, reverse osmosis, UV-radiation (optional), and depot-chlorination (optional) for water treatment is also commercially available.

1. Description of the mobile purification unit, the field site and the analytical methods

1.1 Description of the mobile water purification unit

The field study at the Teltowkanal was carried out with the mobile drinking water purification unit WTC 1600 GT (figure 1), commercially available from Alfred Kärcher GmbH & Co., Winnenden, Germany. It has been designed to generate drinking water from surface, river, sea, and brackish water. The capacity of the unit has been calculated to produce a minimum of 1600 liters of drinking water per hour (only in single pass mode) meeting the requirements of the European and the German drinking water regulation (TVO).

Figure 1: Photography of the mobile drinking water purification unit Kärcher TWC 1600 GT at the field site near the Teltowkanal in September 2000.

The tested unit consisted of the following components: A power supply generator, a raw water supply pump (including a connection hose and a device for floating use), a pre-filtration unit (duplex bag filters with a particle separation <0.5 µm abs.), two water-cooled high pressure pumps and a reverse osmosis unit applying cross-flow technique (figure 2), a UV-disinfection unit, a post depot-chlorination unit, a heating unit (to operate the system at very low temperatures), automatic cleaning device, measurement technology (e.g. for conductivity measurements), a microprocessor-operated control device (SPS) to operate the unit automatically, and a one axis trailer.

Specifications:

Ambient operation temperatures (air): -32°C to +49°C, below 0°C the heating system is required

Storage temperatures:	-40°C to +72°C
Raw water temperature:	0 to +49°C
Ambient humidity:	5 to 100%
Sea level:	0 to 3000 meters above sea level
Turbidity (NTU):	< 150
pH :	5-9
Mechanical resistance :	3.5 g
Raw water exploitation :	40-50%
Membrane elements :	Eight Filmtec SW 30 4040
Desalination rate :	< 99.7 % nominal
Raw water needed for operation :	about 4000L/h
Drinking water output (min.):	1600 liters per hour in single-pass mode (figure 3) 600 liters per hour in double-pass mode (figure 4)
Max. salt content:	55,000 ppm
Operating pressure :	max. 69 bar
Raw water pressure:	1.5-3 bar
Noise (generator):	max. 53 dB(A)
Sizes:	length: 2.8 meters; width: 1.6 meters; height: 1.05 meters
Weight (without trailer):	1550 kg

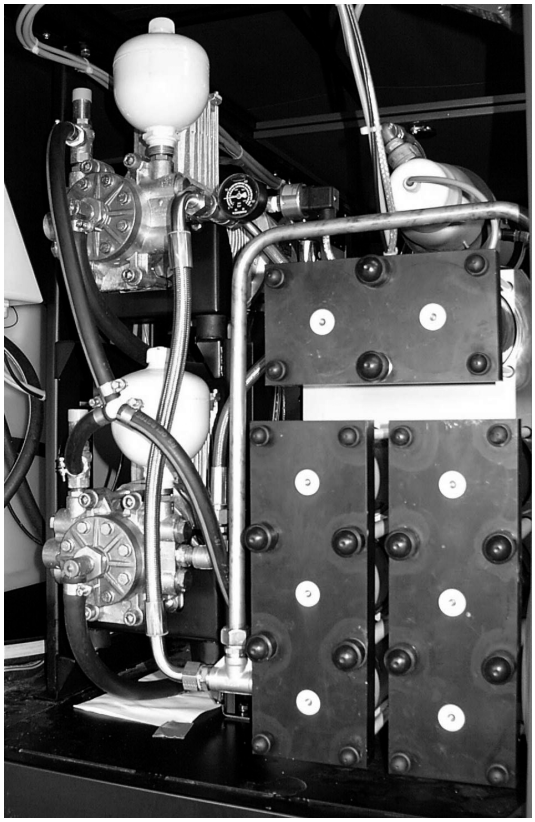

Figure 2: Photography of the reverse osmosis module of the mobile drinking water purification unit Kärcher TWC 1600 GT. High pressure pumps on the left and pressure pipes (eight 40'' membrane modules) of the reverse osmosis (right).

As shown in figure 3, the unit is ususally operated only in single-pass mode. The concentrate obtained from the first six membrane modules is again applied to the last two modules to increase the raw water extration yields. In case of nuclear cotaminations, the unit has to be run in double-pass mode, shown in figure 4.

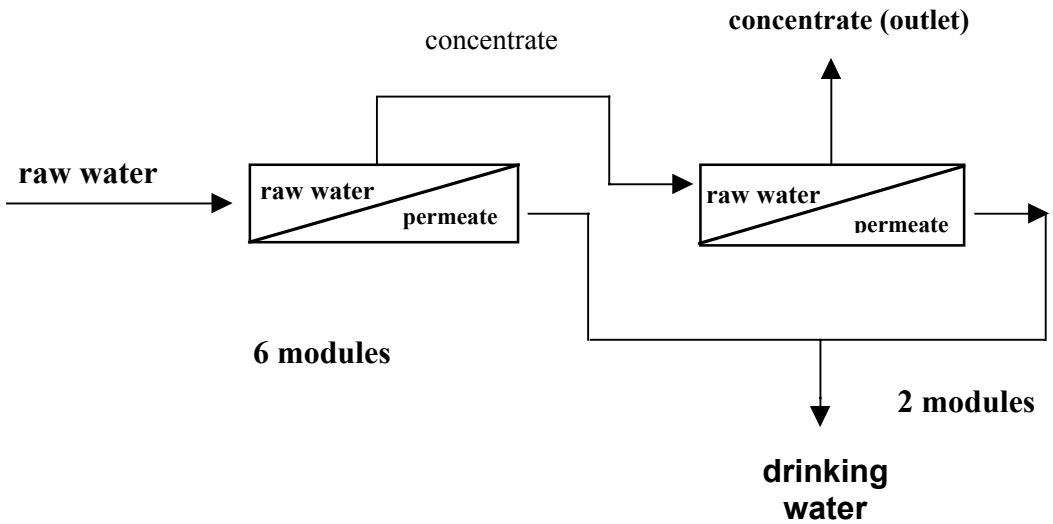

Figure 3: Flowscheme of the reverse osmosis unit operated in single pass-mode (generation of up to 1600 liters of drinking water per hour).

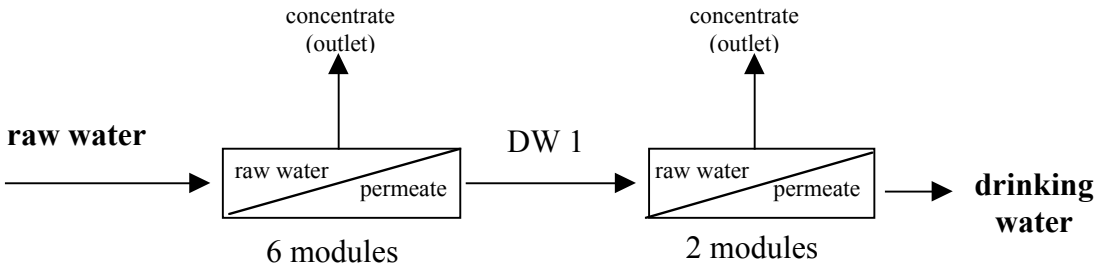

Figure 4: Flowscheme of the reverse osmosis unit operated in double pass-mode (generation of up to 600 liters of drinking water per hour).

1.1. Description of the Field site where the study was carried out in September 2000

The performance of the drinking water purification unit was tested for more than one year at different (extreme) environmental conditions using all possible kinds of contaminants (including NBC). The purpose of our field trial carried out in September 2000 was to check the performance of the mobile drinking water purification unit under realistic but also under "worst-case" conditions. We wanted to test various contaminants but our main target compounds were the PhACs that have never been tested before.

What was needed for the field trial, was a "natural" surface water containing municipal sewage discharges, contaminants from other sources, and many algae and particulates to test the practical performance including blocking of the pre-filtration unit. Thus, the Teltowkanal, a shallow canal located in the southern districts of Berlin (figure 5) was selected for the field study because it carries the highest loads of sewage discharges of all Berlin water ways. Additionally, it also contains many algae and much particulate organic matter that is distributed in the surface water by the shipping traffic. The Teltowkanal was build between 1901 and 1906 (SenStadtUm, 1987) and used as drainage for rainwater and industrial waste water from districts formerly located outside of Berlin. It was also used as a shipping canal for industrial supply and as a short cut for the shipping routes between the Oder and Elbe river. The Teltowkanal has a total length of approximately 35 kilometers and connects the Dahme and Havel river (SenStadtUm, 1987). Today, it is characterized by high proportions of sewage effluents being discharged into the canal by the municipal sewage treatment plants (STPs) in Stahnsdorf and by Berlin's two largest STPs in Waßmannsdorf and Ruhleben (only from April to October). In several sections of the canal, the municipal sewage effluents account for up to 40% of the average surface water flow (SENSUT, 2000), but under extreme conditions (dry periods with low surface water flows) the proportions of municipal sewage may also reach up to 84% (SENSUT, 2000). As far as contaminations from municipal sewage discharges are concerned, the surface water of the Teltowkanal represents some kind of "worst-case scenario". As shown in figure 5 , the field study was carried out downstream from the sewers of the STPs in Waßmannsdorf and Ruhleben to guarantee a high degree of contamination by municipal sewage effluents in the raw water.

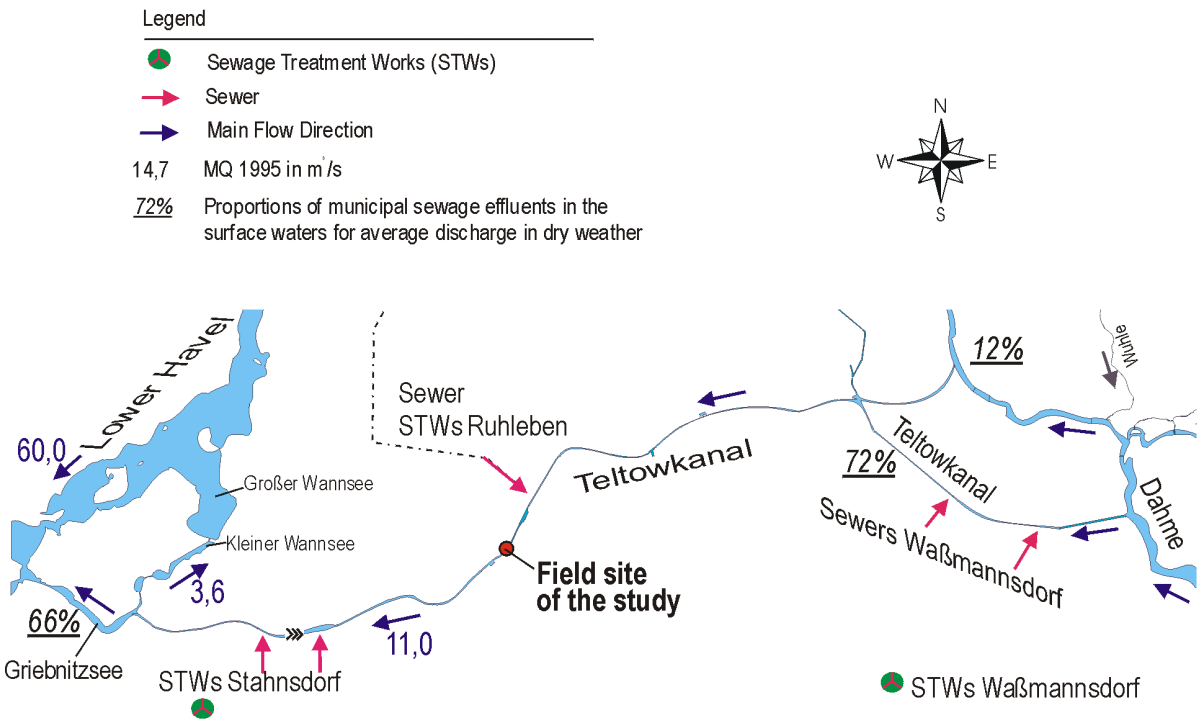

Figure 5: Map showing the field site at the Teltowkanal in Berlin, Germany, were the study was carried out in September 2000.

1.1 Parameters and Analytical Methods

All samples were analyzed for various organic contaminants including pesticides, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and volatile compounds (chlorinated VOCs and BTX) using standardized test methods (DIN, German industrial standard methods). Standard methods were also applied for the analysis of some heavy metals, kations, anions, and microbes. Additionally, the samples were

analyzed for 23 environmentally important organic contaminants including some PhACs and two flame retardants. Until now, no standard methods are available for these contaminants. Thus, we used a multi-method described below.

Analytical method for the determination of some PhACs, flame retardants, and other polar contaminants: All samples were filtered using 0.45 µm cellulose nitrate membrane filters and stored at 4°C prior to analysis. The pharmaceutical residues were analyzed using a multi-method described by Heberer et al. (1998) and Reddersen & Heberer (in prep.). For trace analysis, the samples were concentrated from (up to) one liter down to a final sample volume of 0.1 µl (concentration factor: up to 10,000). The method applies solid-phase extraction (SPE) using non-endcapped reversed-phase octadecyl adsorbent for the extraction of the analytes from the water samples. The extracted residues were then derivatized using pentafluorobenzyl bromide as derivatization reagent. The remaining residues were dissolved and analyzed applying gas-chromatography-mass spectrometry (GC-MS) using selected ion monitoring (SIM). The recoveries of the analytes were between 70 and 110%. For quality control, a suitable surrogate standard (2-(4-chorophenoxy)butyric acid) was added to the samples before sample preparation. The detection limits for the pharmaceuticals are between 1 and 10 ng/L, depending on the individual compounds, the sample volume and the sample matrix (Reddersen & Heberer, in prep.).

2. Results of the field trial

2.1 Introduction

The WTC 1600 GT water purification unit was tested on September 5th and 6th, 2000 at the Teltowkanal in Berlin, Germany. It was run in single-pass (09/05/00) and double-pass mode (09/06/00) without additional UV disinfection or chlorination. In terms of this field trial a total of more than 5000 individual values was obtained and evaluated. Although the surface water of the Teltowkanal contained much organic matter such as algae and solid particles, the pre-filtration and the whole system was working with high reliability. The operational life of the bag filters used for pre-filtration varied between 1.5 and 2.5 hours depending on the shipping traffic in the canal. Whenever a bag filter was blocked, the system automatically switched (by pressure control) to the second filter without interrupting the purification process. In the following sections, selected results showing the performance of the purification unit in single-pass mode are presented.

2.1 Organic parameters including PhACs, pesticides and some polar organics

Several PhACs, pesticides, flame retardants, and other polar contaminants were detected in the surface water of the Teltowkanal at maximum individual concentrations up to 2.1 µg/L (detected for tris-(chloroisopropyl)-phosphate). As shown for some PhACs in figure 6, temporal fluctuations of the concentrations were observed for several compounds. Due to the high proportions of municipal sewage effluents, the fluctuating loads are still reflected by their varying surface water concentrations of the individual PhACs. Other compounds such as clofibric acid (metabolite of blood-lipid regulating compounds) were detected over the whole sampling period (from 8 a.m. to 6 p.m.) at almost constant concentrations (between 140 and 160 ng/L, figure 6). This effect may be explained by the individual application practice and by differences in excretion from the human body.

In the permeate of the drinking water purification unit, none of the investigated PhACs or any other organic contaminant was detected at significant concentrations, neither when the system was operated in the single-pass mode nor when it was operated in the double-pass mode. The concentrations of all contaminants detected in the surface water of the Teltowkanal were reduced to concentrations below the analytical limits of detection (table 1). Figures 7 and 8 show the varying concentrations of two PhACs in the raw water from the Teltowkanal and the efficacy of their removal in single-pass mode by the tested drinking water purification unit. The removal rates which were calculated from the average raw water concentrations and the average permeate concentrations are also shown in table 1. In those cases, where the analytes were not detected in the permeate samples, the minimum removal rate was calculated from the individual limit of detection.

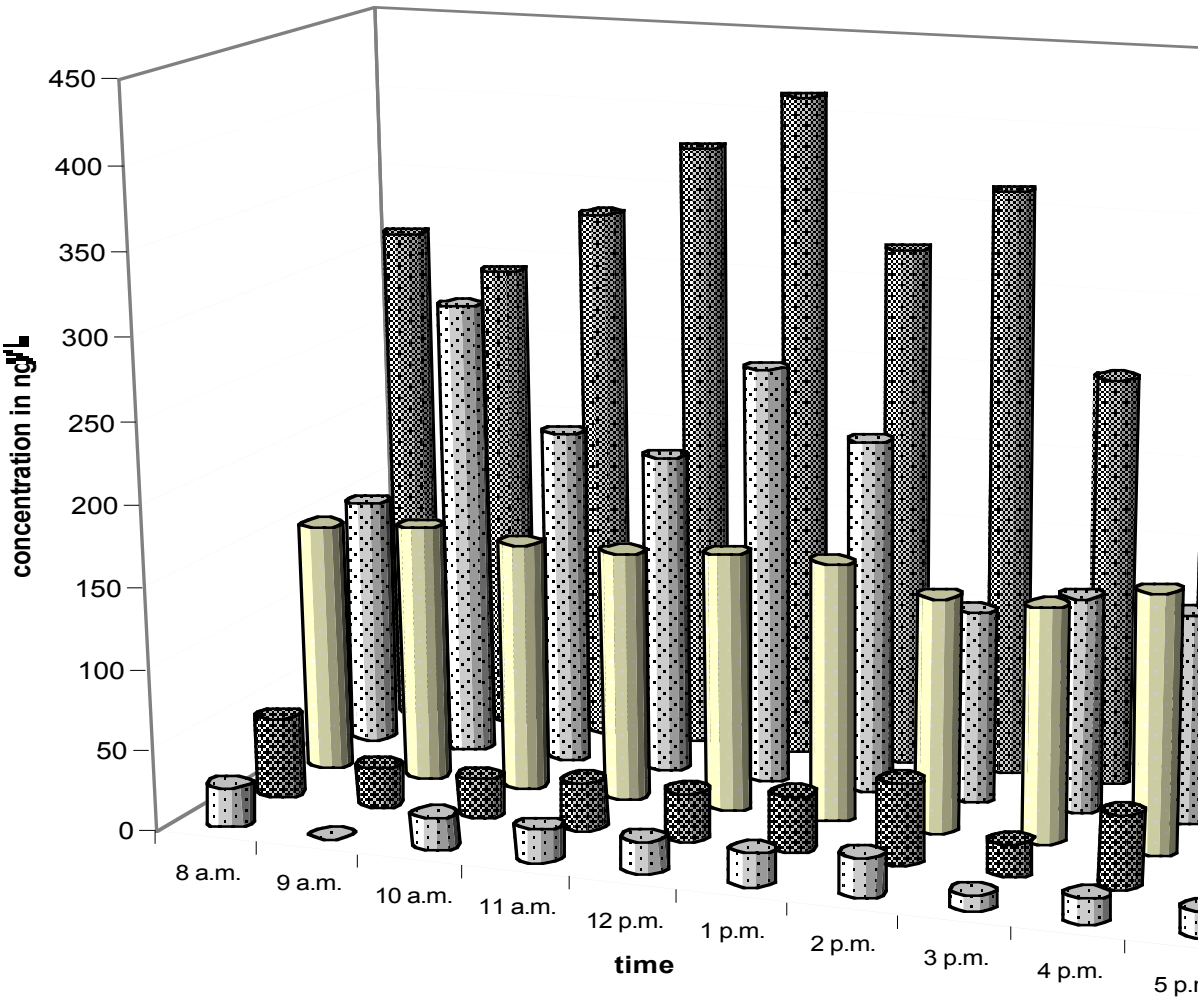

Figure 6: Temporal fluctuations of the concentrations measured for different drug residues at the selected location in the Teltowkanal. Repeated sampling on September 5th, 2000.

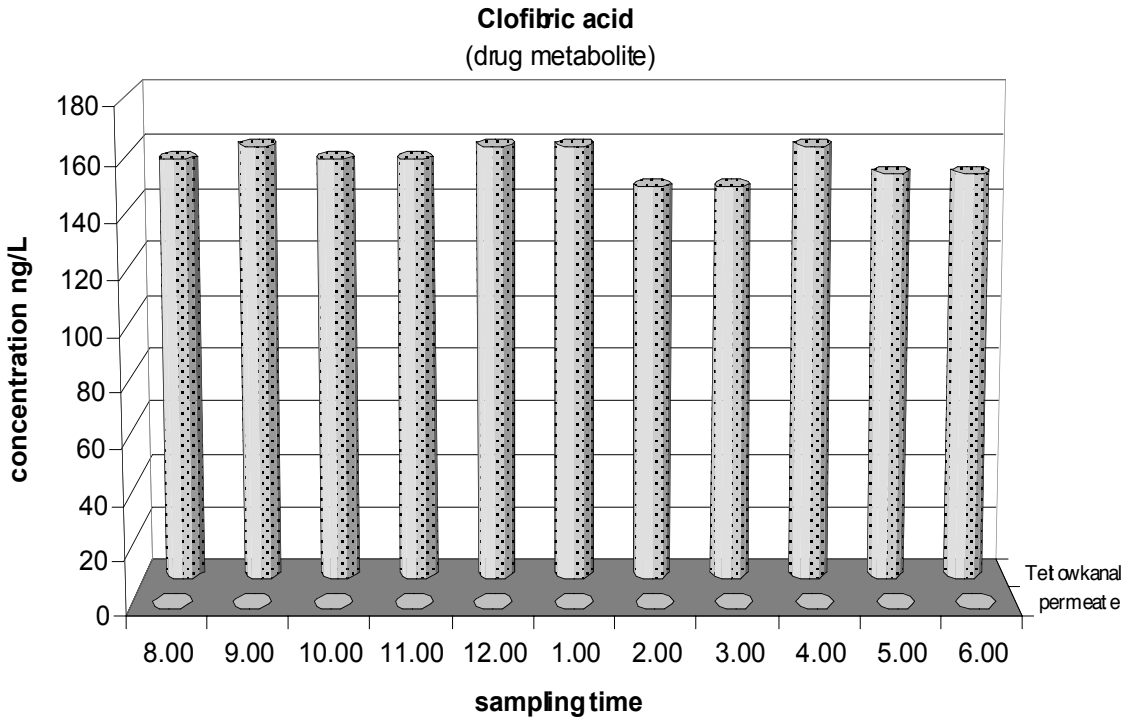

Figure 7: Concentrations of clofibric acid in the raw water from the Teltowkanal and in the permeate from the drinking water purification unit. Results from 09/05/00, when the system was operated in single-pass mode.

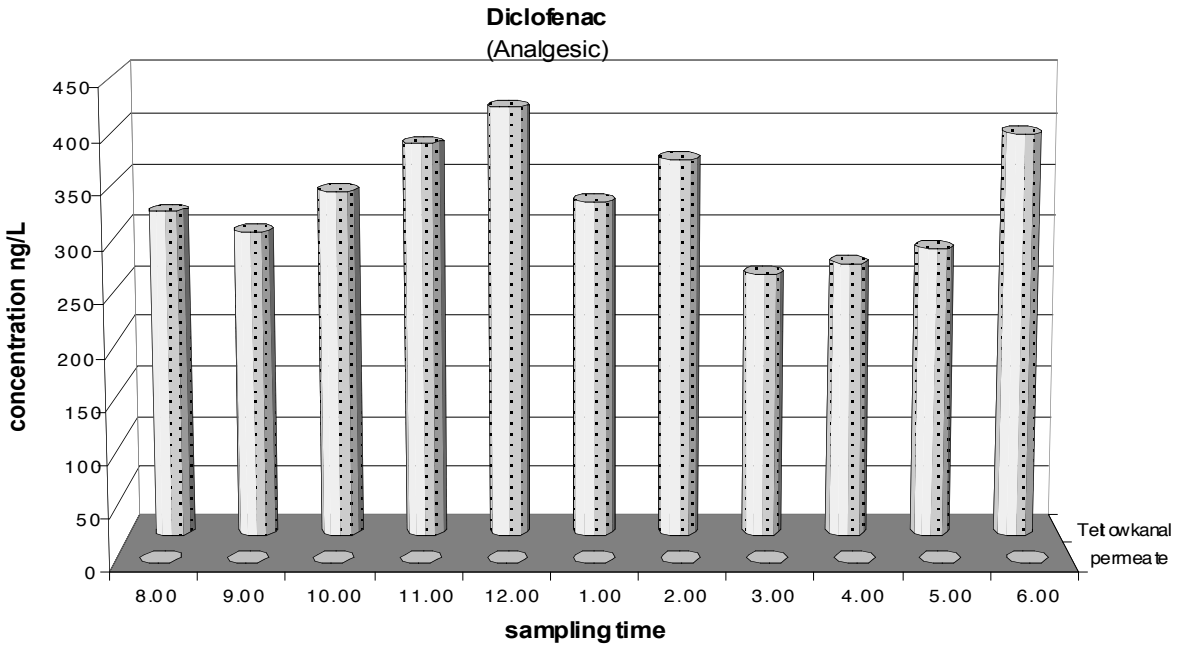

Figure 8: Concentrations of diclofenac in the raw water from the Teltowkanal and in the permeate from the drinking water purification unit. Results from 09/05/00, when the system was operated in single-pass mode.

Table 1: Average concentrations of selected contaminants in the raw water from the Teltowkanal and in the permeate after water purification using single-pass mode (09/05/00). Removal rates were calculated from the average raw water concentrations and the average permeate concentrations and, when analytes were not detected in the permeate samples, from the individual limit of detection.
[n.d.: not detected (below the limit of detection).]

Parameter	Average raw water concentration	Average permeate concentration	Removal rate in %
Anonymous*	290 ng/L	n.d.	> 99.7
Caffeine	430 ng/L	n.d.	> 99.8
Carbamazepine	330 ng/L	n.d.	> 99.7
Clofibric acid	155 ng/L	n.d.	> 99.4
Diclofenac	329ng/L	n.d.	> 99.7
Naproxen	38 ng/L	n.d.	> 95.0
N-(phenylsulfonyl)sarcosine	1143 ng/L	n.d.	> 99.9
Propyphenazone	170 ng/L	n.d.	> 99.4
Diurone	100 ng/L	n.d.	> 99.8
Mecoprop	93 ng/L	n.d.	> 98.9
Tris-(chloroethyl)phosphate	360 ng/L	< 10 ng/L	> 97.2
Tris-(2-chloroisopropyl)phosphate	945 ng/L	< 10 ng/L	> 98.9

* This compound has been identified as a metabolite of a pharmaceutical substance (Reddersen et al., in prep.). The identity of this compound cannot be publicized because of potential legal ramifications (Reddersen et al., in prep.).

2.1 Microbiology

As shown in table 2, large amounts of bacteria including several species of faecal bacteria were detected in the surface water of the Teltowkanal (raw water). In the permeate the number of bacteria was almost totally reduced by the drinking water purification unit. Cross-contaminations during sampling or transport may be possible at a low extent. This may explain the low number of positive counts in the permeate samples.

Table 2: Selected results from the microbial investigations

single pass mode		**8.00 a.m.**	**10.00 a.m.**	**12.00 p.m.**	**2.00 p.m.**	**4.00 p.m.**	**6.00 p.m**
colony count 20 +/. 2°C	canal	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
colony count 20 +/- 2°C	permeate	3	2	3	0	4	2
colony count 36 +/. 1°C	canal	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
colony count 36 +/. 1°C	permeate	3	3	6	0	4	1
E.coli / specific Enterobacteriaceae	canal	E.coli/ coliforms	E.coli/ coliforms	E.coli/ coliforms	E.coli/ coliforms	E.coli/ coliforms	E.coli/ coliforms
E.coli / specific Enterobacteriaceae	permeate	negative	negative	negative	negative	negative	negative
Streptococcus faecalis / S. faecium	canal	positive	positive	positive	positive	positive	positive
Streptococcus faecalis / S. faecium	permeate	negative	negative	negative	negative	negative	negative
sulfite-reducing anaerobic growing bacteria	canal	positive	positive	positive	positive	positive	positive
sulfite-reducing anaerobic growing bacteria	permeate	negative	negative	negative	negative	negative	negative

2.1 Inorganic parameters

As already shown for the organic contaminants, only small amounts of heavy metals, cations or anions were detected in the permeate from the drinking water purification unit, regardless from operating the system in single- or double-pass mode. The concentrations of all contaminants detected in the surface water of the Teltowkanal were reduced significantly often to concentrations below the analytical limits of detection (table 3). The removal rates which were calculated from the average raw water concentrations and the average permeate concentrations are also shown in table 3. In those cases, where of the analytes were not detected in the permeate samples, the minimum removal rate was calculated from the individual limit of detection.

Table 3: Average concentrations of selected inorganic parameters in the raw water from the Teltowkanal and in the permeate after water purification using single-pass mode (09/05/00). Removal rates were calculated from the average raw water concentrations and the average permeate concentrations and, when analytes were not detected in the permeate samples, from the individual limit of detection.

Parameter	Average raw water concentration	Average permeate concentration	Removal rate in %
Aluminium	0.39 µg/L	< 0.04 µg/L	> 87.2
Ammonia	0.34 mg/L	< 0.05 mg/L	> 85.3
Borate	0.24 mg/L	< 0.05 mg/L	> 79.5
Iron	0.77 µg/L	< 0.05 µg/L	> 93.5
Nitrate	18.84 mg/L	< 1.0 mg/L	> 94.7
Nitrite	0.39 mg/L	< 0.01 mg/L	> 97.4
Phosphate	0.96 mg/L	< 0.2 mg/L	> 79.2

Conclusions

The field trial of the mobile drinking water purification unit WTC 1600 GT showed the high efficiency and reliability of modern purification units applying membrane filtration. All contaminants detected in the surface water of the heavily polluted Teltowkanal such as PhACs, pesticides, flame retardants, heavy metals, anions, and cations were effectively removed by the system. For the investigated spectrum of compounds, the application of the double-pass mode was not found to be necessary. Double-pass mode operation is, however, inevitable to remove nuclear contamination. Although the surface water of the Teltowkanal contained much organic matter such as algae and solid particles, the pre-filtration and the whole system worked with high reliability. The operational life of the bag filters used for pre-filtration varied between 1.5 and 2.5 hours depending on the shipping traffic in the canal. The generated drinking water meets all requirements set by the German and European drinking water regulations and by some other regulations such as NATO STANAG 2136.

Outlook

This year in September, the performance of a prototype of a purification unit will be tested under “worst-case” conditions in another field trial. This newly designed system also applies membrane filtration (ultrafiltration and reverse osmosis) and is specified to produce as much as 5 m^3 per hour of drinking water in single-pass mode and 3.5 m^3 per hour in double-pass mode. In our investigations we will place this unit directly near a sewer of the municipal STPs in Ruhleben (Berlin, Germany).

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Natural Attenuation of Pharmaceuticals

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Abstract

Occurrences of pharmaceuticals in surface water and sewage water have widely been reported. Investigations show the presence of several classes of medicaments such as antirheumatics (e.g. Diclofenac), analgesics (e.g. Propyphenazone), and blood lipid regulators (Clofibric acid) even in groundwater. Compared with the occurrences in surface waters, the reported incidence of drugs in groundwater is much rarer. This may be due to the input function but also to natural attenuation. Laboratory results show that clofibric acid exhibits almost no degradation and only low sorption. Diclofenac exhibits a photochemical degradation, whereas Propyphenazone is sorbed to the aquifer material to a certain extend. These results from the laboratory were proved in the field where groundwater at the bank infiltration site Lake Tegel, Berlin (Germany) was sampled. Only Clofibric acid was found in the groundwater in concentrations of up to 290 ng/L whereas the other pharmaceuticals were measured, if any, in much lower concentrations.

Introduction

In the 1970s Garrison et al. (1976) and Hignite and Azarnoff (1977) detected clofibric acid in sewage effluent. Clofibric acid is the active metabolite of blood lipid regulators like clofibrate, etofibrate, and etofyllin clofibrate. When applying pharmaceuticals to humans, many of these compounds are excreted with only slight transformations or even unchanged and often conjugated to polar molecules (e.g. as glucoronides). Due to an incomplete elimination of a number of pharmaceuticals used in human medical care in sewage treatment plants several classes of drugs are found in sewage effluent. Among these classes are antirheumatics (e.g. Diclofenac), analgesics (e.g. Propyphenazone) as well as the above mentioned blood lipid regulators (e.g. Clofibric acid) (Fig 1). High concentrations of sewage contaminants may be expected in the receiving surface waters with regard to the high contribution of sewage treatment work's effluents, especially in areas with a high population density.

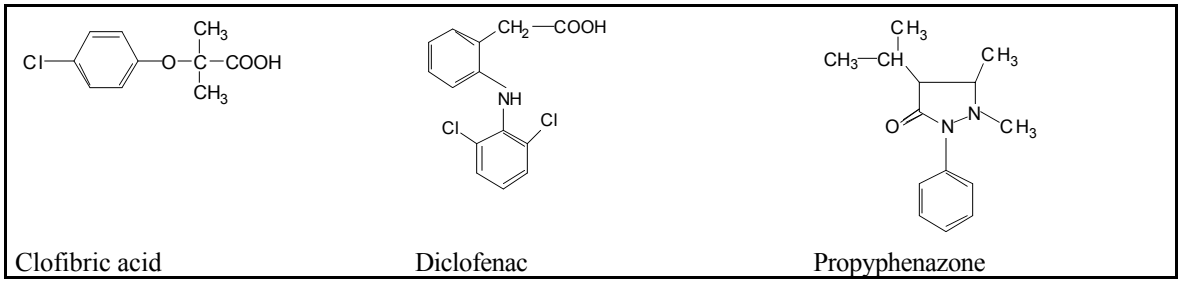

Fig. 1 Chemical structure of compounds studied

If surface water is contaminated by pharmaceuticals, these substances may reach groundwater in case of influent conditions (losing stream) and by bank filtration or artificial recharge of groundwater. Furthermore, groundwater contamination through sewage water may be due to leaky sewage systems. Table 1 gives a summary of occurrences of the above mentioned pharmaceuticals in sewage and surface water from Europe. The groundwater samples are collected from 17 wells in the catchment area of a Berlin drinking water works. Compared with occurrences in sewage effluent and surface waters, the reported incidence of drugs in groundwater is much rarer. Therefore, the processes of degradation regardless whether biological or chemical and sorption processes were studied in laboratory experiments. These results are compared to the concentrations along a transect at the bank filtration site Lake Tegel, Berlin, Germany.

Table 1 Highest reported concentrations published in the open literature of selected pharmaceuticals in the aquatic environment.

	Sewage Water (ng/L)	Surface Water (ng/L)	Groundwater (ng/L)
Clofibric acid	2540 - 9740[a]	< 1 - 1750[d]	n.d. - 7300[e]
Diclofenac	up to 6.220[b]	< 1 - 960[c]	n.d. - 380[e]
Propyphenazone	up to 1900[c]	< 5 - 350[c]	n.d. - 1465[e]

[a] Hignite and Azarnoff (1977) [b] Möhle *et al.* (1999)
[c] Heberer *et al.* (1998) [d] Stan *et al.* (1994).
[e] Heberer *et al.* (1997) n.d. not detected; < below limit of quantification.

Laboratory Experiments

Setup

A laboratory column (16 cm diameter and 35 cm length) was packed with Pleistocene sediments from well drilling for Berlin water works. The sediment was taken from a depth of approximately 60 m below ground level and consisted of medium grained sand. The column was first equilibrated with groundwater from the same location and depth for about 5 days. Afterwards lithium (LiCl) as tracer and the pharmaceuticals Clofibric acid, Propyphenazone, and Diclofenac were added to the same groundwater and the column was flushed for about 10 days. Afterwards, another 5 days the column was flushed with groundwater without lithium and the pharmaceuticals. The experiment took place at room temperatures of about 20°C. The groundwater can be characterised as hydrogen carbonate type water from the anaerobic (nitrate-reducing) zone of the aquifer. Total organic carbon in the sediment was 2.5 mg/kg and dissolved organic carbon in groundwater was 2.4 mg/L.

There was a steady upward flow through the column with a flow velocity of 0.3 m/d and constant supply of the tracer lithium (10 mg/L LiCl) and pharmaceuticals (10 µg/L). Physico-chemical parameters (E_H, pH, temperature, oxygen saturation, specific electrical conductivity) were continuously measured with a data logger. Water samples were collected hourly and the main anions and cations were analysed out of selected samples. Further investigations included laboratory degradation experiments with groundwater but without sediment at different light and temperature conditions.

Results

Concentrations of anions and cations as well physico-chemical parameters remained the same during the experiment except for chloride and specific conductance which increased when adding LiCl as tracer together with the pharmaceutical substances. The studies show that the pharmaceuticals exhibit different transport behaviour.

Clofibric acid (Fig. 2) is transported with tracer velocity (Scheytt *et al.*, 1998) and degradation experiments prove the persistence of this substance under different light and temperature conditions (Scheytt *et al.*, 1999). Further experiments revealed that Clofibric acid exhibits only low sorption tendency with Kd values (Henry Isotherme) around 0.25 ml/g (Scheytt, 2001).

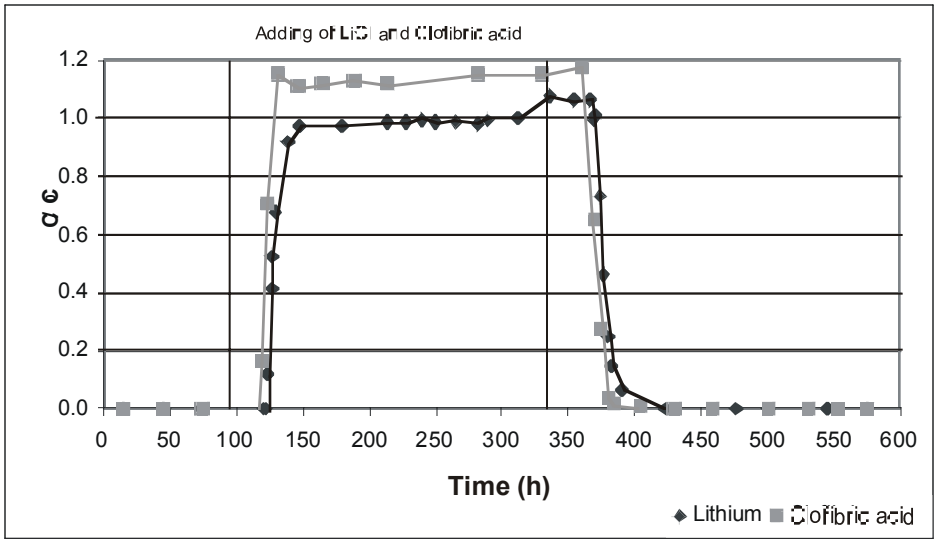

Fig. 2 Concentration of lithium (tracer) and Clofibric acid at the outflow of the laboratory soil column.

Propyphenazone exhibits quite high sorption with a retardation factor R_d of 2.7. Mass balance of input and output reveals that about 70% of the substance is found in the outflow of the column. Fig. 3 shows the concentration of Propyphenazone during the experiment. Due to sorption the increase in concentration after adding this pharmaceutical is retarded compared to the tracer and the ratio of measured concentration at the outflow C to the initial concentration of the inflow (C_0) is only about 0.5. Interestingly, C/C_0 increases remarkably to about 1.2 at the time, when adding of Propyphenazone ended. The reason for this peak could be that Propyphenazone is preferably sorbed on colloids. These colloid are mobilised at that time due to the change of the flow regime in the column. This change could have been induced by the change of the water tank. Additionally, the high amount of Propyphenazone found at the outflow of the column shows that sorption of Propyphenazone is at least partly reversible.

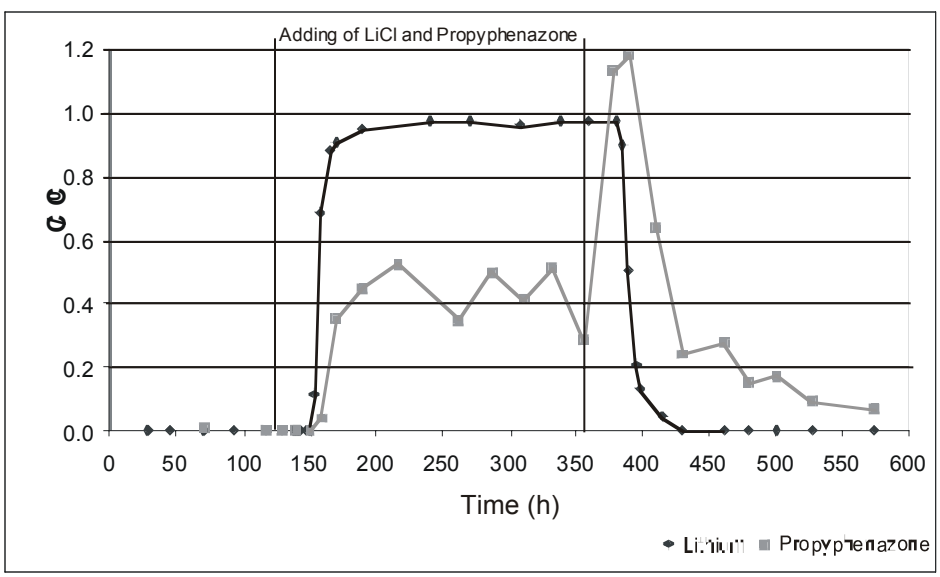

Fig. 3 Concentration of lithium (tracer) and Propyphenazone at the outflow of the laboratory soil column.

Diclofenac concentrations (Fig. 4) showed some similarities to Propyphenazone. Like Propyphenazone, concentrations increased after the end of adding the tracer and Diclofenac to a C/C_0 value of 0.8. Significant differences where a lower retardation factor (3.5) but also lower values for C/C_0 (0.3). Mass balance revealed that only 52% of the substance was found at the outflow of the column.

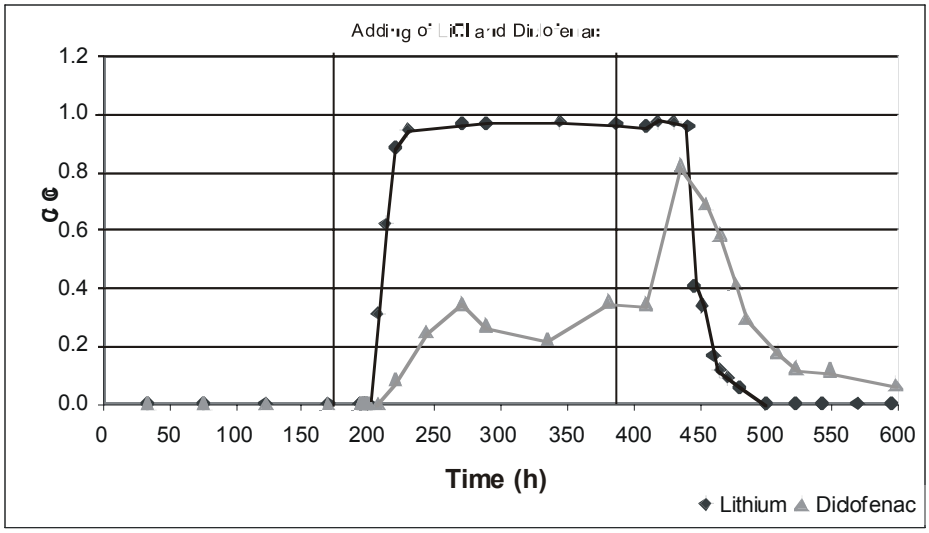

Fig. 4 Concentration of lithium (tracer) and Diclofenac at the outflow of the laboratory soil column.

Bank Filtration Site Tegel

The fate of pharmaceuticals was investigated at the bank filtration site Lake Tegel (Fig 5) in Berlin, Germany, within the framework of a bank filtration research project of Free University Berlin. This site is located on the east bank of Lake Tegel and belongs to a running water work of Berlin water supply (Berliner Wasserbetriebe) with infiltration of water from Lake Tegel.

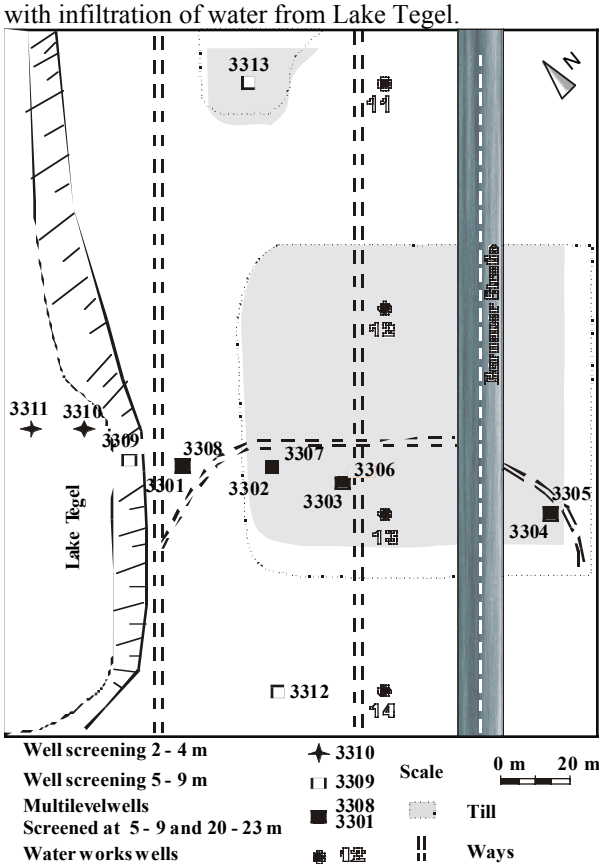

Fig. 5 Location of transect at Lake Tegel

The aquifer at the bank filtration site has a thickness of about 50 m and consists of quaternary (Wechselian) sand and gravel with hydraulic conductivities between 10^{-4} m s^{-1} and 10^{-3} m s^{-1}. Groundwater samples for this study originate from 13 monitoring wells and 3 drinking water wells (e.g. B 13, Fig. 5) at 5 distances (deeper wells, Fig. 6) from Lake Tegel. The transect was established along a line of about 150 m. The deeper wells (3301 to 3304) are screened between 20 and 23 m below surface (Fig. 6), the shallow wells between 5 and 9 m below surface. The analytical programme included among other parameters the measurement of physico-chemical parameters, the concentrations of main anions and cations as well as the concentrations of Clofibric acid, Propyphenazone, and Diclofenac.

The most likely source of the pharmaceuticals in Lake Tegel is an input through treated sewage water from the sewage treatment plant Schönerlinde in the north of Lake Tegel. The concentrations of the pharmaceuticals were measured in Lake Tegel at the same time when the groundwater samples were collected. Clofibric acid in Lake Tegel was found in concentrations of 120 ng/L (May 1998), 140 ng/L (June 1998) and 190 ng/L (November 1998). Concentrations of Propyphenazone in Lake Tegel were 30 ng/L (May 1998) and 60 ng/L (June 1998), Diclofenac was both times below limit of detection.

The aquifer is divided by a till with aerobic groundwater conditions above the till and anaerobic conditions beneath the till. Lake Tegel is underlain by silts and clays that are rich in organic material, leading to an relatively impermeable layer. Hence, the most important flow path is through the thin layer at the bank of Lake Tegel (Fig. 6). The recharge of groundwater through the silt and clay layer and groundwater flow beneath Lake Tegel is of minor importance. The portion of landward water in the water works wells accounts for 15 % to 25 % of the totally withdrawn water.

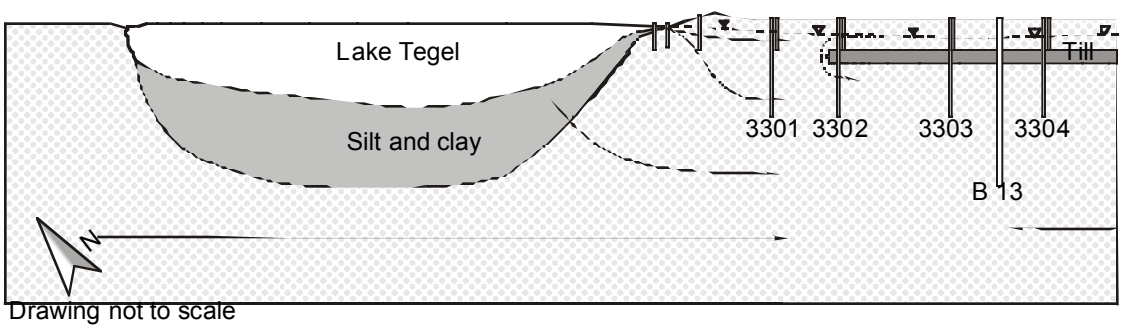

Fig. 6 Schematic geologic section of bank filtration site Lake Tegel with location of deeper wells and water works well (numbered) and shallow wells.

The quantitative results for the three pharmaceuticals are shown in Table 2. All of the concentrations were within the limits presented in Table 1. Clofibric acid appeared in concentrations up to 290 ng/L, Propyphenazone up to 105 ng/L and Diclofenac up to 50 ng/L. A distinct decrease in concentration of Propyphenazone and Diclofenac could be observed between wells 3301 and 3302. In the water works wells Clofibric acid as well as Propyphenazone were analysed, although Propyphenazone was not found in the wells upgradient of the water works wells.

Interestingly, Clofibric acid was not only found in groundwater originating from bank filtration but also in groundwater from well 3304 that samples landward water. The concentration of clofibric acid is at certain times even higher in landward water than in bank filtration water, indicating a potential further input beside bank filtration. This further input path could not be identified until now.

Table 2 Distribution of pharmaceuticals in groundwater in deep wells of transect Lake Tegel.

Compound	**Sampling Date**	Seaward wells			Water works wells			Landward well
		3301	3302	3303	B 12	B	B 14	3304

					13			
Clofibric acid	May 1998	15	n.d.	n.d.	45	30	50	225
	June 1998	20	< 10	n.d.	50	35	40	< 5
	November 1998	20	240	290	70	90	10	55
Propyphenazone	May 1998	40	n.d.	n.d.	35	30	90	n.d.
	June 1998	55	n.d.	n.d.	45	40	105	n.d.
Diclofenac	May 1998	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	June 1998	50	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Concentrations in ng/L
n.d. not detected; < below determination limit

Attenuation processes

The disappearance of the pharmaceutical compounds may be caused by dilution, sorption, or degradation. The reduction in concentrations within a distance of some 10 meters (wells 3301 to 3302) is for Propyphenazone and Diclofenac so high that it cannot be explained by dilution alone, when compared to chloride acting as a conservative tracer in this part of the aquifer. All the pharmaceuticals shown in Table 2 are water-soluble with low to moderately-high octanol/water distribution coefficients, indicating that they will be mobile and probably will migrate in the aquifer. Among the three pharmaceuticals, Diclofenac has the highest octanol/water distribution coefficient (log K_{OW} = 4,51, Avdeef *et al.*, 1998), followed by Clofibric acid (log K_{OW} = 2,84, Henschel *et al.*, 1997) and Propyphenazone (log K_{OW} = 2,32, Holm *et al.*, 1995), which means that Diclofenac is expected to show the strongest retardation in the aquifer. However, in the laboratory studies Propyphenazone exhibited the highest retardation factor (R_d = 2.7), the retardation factor of Diclofenac (R_d = 3.5) was significantly lower and Clofibric acid was not retarded at all.

Furthermore, the concentrations of Diclofenac in Lake Tegel should be much higher if generally observed concentrations in sewage effluent are considered. Data from degradation studies suggest that Diclofenac is degraded significantly by photolytic degradation. This observation is in good accordance with the results of Buser *et al.* (1998) who found Diclofenac being rapidly photodegraded in water of Swiss lakes. Furthermore, Buser *et al.* (1998) found Diclofenac being degraded in natural water but not in desalted water, indicating that this degradation is microbiologically catalysed. Most likely the concentration of Diclofenac decreases in surface water on the way from the sewage treatment plant to Lake Tegel, but not completely as Diclofenac was found in one sample of well 3301. The remaining substance in groundwater is either sorbed to the subsurface material as photolytic degradation is unlikely in groundwater or degraded. But sorption is more likely as Zwiener *et al.* (2000) reported very low degradation under anaerobic conditions compared to aerobic oxygen conditions.

Concentration distribution of Propyphenazone is more difficult to explain, as there are occurrences of this compound in well 3301 and in the water works wells but no further analyses along the flow path (Table 2). In the column experiment Propyphenazone was retarded with a factor of 2.7 thus explaining the decrease in concentration along the flow path between well 3301 to 3302. The water works wells are pumped in alternating intervals. Due to the high amount of water pumped, the flow regime is strongly influenced at the beginning of a pump interval. This may lead like in the column experiment to a mobilisation of colloids and to a mobilisation of Propyphenazone that is sorbed on those colloids . In the case of bank filtration site Tegel sorption seems to be the main process for attenuation of Propyphenazone. Holm *et al.* (1995) found indications that degradation is much more important than sorption in groundwater downgradient of the landfill Grindsted (Denmark). The attenuation in landfill leachate from Grindsted took place under strongly reducing conditions whereas the redox conditions along the transect of Lake Tegel are anaerobic but not methanogenic.

Clofibric acid did not show any attenuation which is in good accordance with the laboratory results that prove the high persistence and high mobility of this compound.

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Role of Subsurface Wetland Treatment in Fate of Endocrine Disruptors in Wastewater[1]

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Abstract

There is widespread concern over environmental and human health effects arising from exposure to endocrine disrupting chemicals (EDCs) and other pharmaceutically active compounds in water. Many such compounds are present at trace quantities in wastewater effluent and persist in water bodies influenced by the disposal of treated wastewater. We examined the fate of EDCs during subsurface wetland treatment of secondary effluent and groundwater (control) flows in parallel, research-scale, subsurface-flow wetland raceways at the Constructed Ecosystems Research Facility (CERF) located in Tucson, Arizona. The CERF facility enabled us to distinguish between 1) persistent effluent-derived EDCs and 2) phytoestrogens of wetland origin as sources of estrogenic activity in wetland effluent. Estrogenicity was analyzed using a competitive binding assay involving the human estrogen receptor. The assay is based on a presumed relationship between chemical affinity for the estrogen receptor and disruption of endocrine system activity. Results of this work are relevant to water managers in arid regions that may incorporate wetlands into sequential wastewater treatments leading to potable reuse of reclaimed water.

Keywords

Endocrine disruptors, estrogen receptor, 17β-estradiol, binding assay, wastewater effluent, constructed wetlands, phytoestrogens

Introduction

There is considerable concern over environmental and human health effects arising from exposure to endocrine-disrupting chemicals in water (Ashby *et al.*, 1997; Colburn *et al.*, 1996; Jobling *et al.*, 1995; Kavlock, 1999; Sumpter *et al.*, 1998; Tyler *et al.*, 1998). Many such compounds are present at trace quantities in wastewater effluent and persist in water bodies influenced by the disposal of treated wastewater. Contaminant-related endocrine system anomalies include aberrant animal behavior and disruption of reproductive patterns. Gulls from Lake Ontario and southern California; alligators in Lake Apopka, FL; dolphins in the Mediterranean Sea; and fresh-water fish are among the documented examples of waste-related endocrine system abnormalities

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(Colburn *et al.*, 1996; Roefer *et al.*, 2000; Tyler *et al.*, 1998). In the United Kingdom, elevated levels of vitellogenin, an egg precursor protein, were detected in male trout and other fish species taken immediately downstream from wastewater effluent discharge points (Jobling *et al.*, 1998; Purdom *et al.*, 1994; Tyler *et al.*, 1998). There have been similar observations in the United Sates. Male carp from the Las Vegas Wash and Las Vegas Bay, areas impacted by wastewater discharge, also exhibited elevated levels of vitellogenin (Roefer *et al.*, 2000).

Endocrine disruptors in municipal wastewater may include a combination of pharmaceutical products and industrial chemicals (nM to μM concentration range). Classes of pharmaceuticals that have been detected in receiving waters include antibiotics, antidepressants, anticonvulsants, analgesics, anesthetics, cholesterol-reducing medicines, and synthetic hormones from birth control pills and replacement therapies. Industrial chemicals with known estrogenic effects include pesticides, polychlorinated biphenyls, phthalates, alkylphenols, bisphenol A, and polycyclic aromatic hydrocarbons. An added threat to aquatic life from the presence of these chemicals in receiving waters is their potential magnification through the food chain.

Since chemicals can interfere with endocrine activity through numerous mechanisms, it may be necessary to apply a combination of bioassays to adequately characterize endocrine system disruption. Available methods for quantifying estrogen disruption *in vitro* include receptor binding, reporter gene, and cell proliferation assays. These assays differ considerably in terms of effort and demand for technical skill, and their sensitivities to aqueous phase 17β-estradiol vary by orders of magnitude (Table 1).

Receptor binding assays are relatively fast and comparatively straightforward. They generally involve only a single receptor (e.g. ER-α or ER-β) and thus cannot provide a universal indication of endocrine disruption. Furthermore, although both agonist and antagonist compounds can be detected, binding assays do not generally distinguish between them. For these reasons (sensitivity, agonist/antagonist recognition), receptor binding assays (and *in vitro* assays in general) cannot replace *in vivo* tests based on lifespan, behavior, or measurement of biomarkers like vitellogenin that more directly indicate endocrine system disruption (Harries *et al.*, 2000; Jobling *et al.*, 1996, 1998; Tyler *et al.*, 1998).

Table 1. Comparison of endocrine disruptor assay sensitivities and effort. Sensitivity is represented by the lowest concentration at which estrogenic effects due to 17 β-estradiol can be readily detected.

Assay	Representative Sensitivity (nM)	Time Required (days)	Reference
Receptor Binding	~2.0	1	Bolger *et al.*, 1998
Gene Expression	0.5	1-3	Routledge and Sumpter, 1996
			Coldham *et al.*, 1997
			Graumann *et al.*, 1999
Cellular Proliferation	0.01	5-7	Korner *et al.*, 1998
			Soto *et al.*, 1995
In Vivo (vitellogenin)	1.8	10-30	Sherry *et al.*, 1999

Due to growing use of reclaimed water and interest in the use of constructed wetlands for treatment of municipal wastewater, the fate of endocrine disrupting compounds during wetland treatment has become increasingly important. The primary objective of this research was to evaluate the efficacy of constructed wetland treatment in a hot arid desert environment for attenuation of estrogenic compounds in municipal wastewater. A secondary objective was to determine whether there is measurable estrogenic activity in wetland-derived organics. Here, the displacement of an intrinsically fluorescent, non-steroid estrogen analog (ES2) from ER-β was measured via a fluorescence polarization technique (Bolger *et al.*, 1998). Plane-polarized light excites ES2, which emits primarily polarized light when bound to ER-β and non-polarized light when free

in solution. The assay was used to look for estrogen-system disruption by wastewater effluent before and after treatment in constructed wetlands.

Methods

Research was conducted at the CERF facility, a pilot-scale research site operated by the University of Arizona's Office of Arid Lands Studies for the Pima County Department of Wastewater Management. The facility began operation in 1989 and consists of five raceways (200 ft long by 27 ft wide by 4.6 ft deep) and one additional, larger raceway (212 ft by 36 ft by 8.5 ft) (Figure 1). All raceways are underlain by 30-mil 3-ply Hyperlon (heavy plastic sheeting) to prevent seepage losses. Raceways #2-5 are subsurface horizontal-flow wetland cells containing 2-3 ft of gravel and various herbaceous shrub and tree species. During the period of investigation, water depth was maintained at 1-2 inches below the gravel surface. Conservative tracer tests using bromide indicated that the average water detention time in these raceways was 5-6 days. Raceway #6 is an aquatic pond maintained at a depth of 3 ft and covered with duckweed (*Lemna* spp.). Raceways received either unchlorinated secondary effluent from the Roger Road Wastewater Treatment Plant or potable water (ground water) as influent. Raceway #6 provided pretreatment (suspended solids removal) for effluent that was sourcewater for raceways #3 and #5. Previous research at CERF is described in Karpiscak *et al.* (1996) and Karpiscak *et al.* (in press).

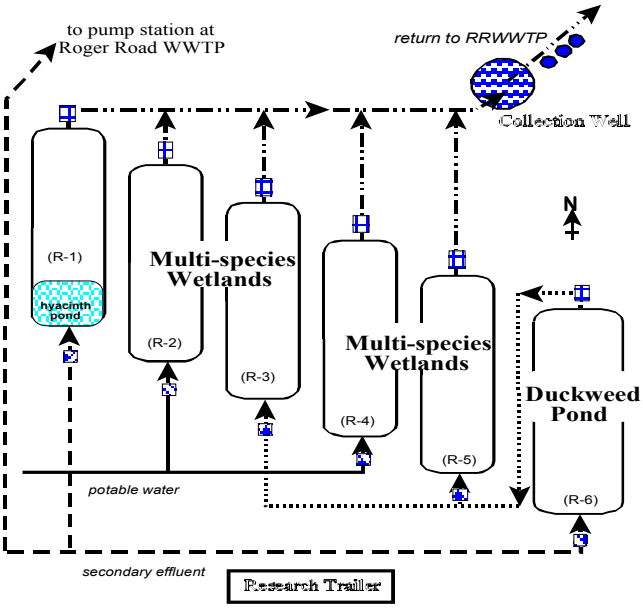

Figure 1. Physical layout of CERF wetland facility. Graphic courtesy of Office of Arid Lands Studies.

Samples were collected in 1-L amber glass bottles that had been acid washed and muffled (550°C). Organics were extracted from 500-1000 mL samples on C18 extraction disks (Empore, 3M) and eluted twice with 10 mL of reagent-grade methanol. Eluates were combined and evaporated to dryness. Residual organics were resuspended in 0.15 M NaCl to achieve volume concentration factors of 200. Compounds that are efficiently separated from water on C18 disks include polychlorinated biphenyls, organochlorine pesticides, phenoxyacid herbicides, phthalates and tirazines—in short, most of the wastewater contaminants that have been implicated in endocrine disrupting activities (manufacturer's data). Previous work in our laboratory demonstrated that constituents in secondary effluent responsible for disruption of ES2/ER-β binding are mostly removed by passage through the C18 disk, indicating that endocrine-disrupting compounds are effectively retained during this step (Turney *et al.*, submitted).

Binding assays followed procedures established for the Estrogen Receptor-β Competitor Assay (PanVera). Samples and controls were analyzed in mixtures consisting of sample (variable volume based on serial dilution), the reagent/buffer solution containing ER-β (10nM), ES2 (1nM) and additional (reagent-free) assay buffer to bring the reaction volume to 100 μL. The reagent/buffer solution comprised ≥50% of the final volume. Concentrates and control samples provided from 50% to 0.01% of the total assay volume. To minimize ionic strength effects in buffered mixtures containing relatively large (fractional) volumes of the samples, NaCl (Mallinckrodt) was added to samples and concentrates to yield 0.15M solutions, as in the assay buffer. To establish upper and lower assay limits, assays were carried out using the reagent/buffer solution (ES2, 1nM; ER-β, 10nM) and 1nM ES2 in buffer (receptor-free, no bound ES2). At least 2 hours were provided for the displacement reactions to reach equilibrium at room temperature in the dark. The assay buffer, ER-β, and ES2 were purchased from PanVera.

Fluorescence polarization was measured with a Beacon 2000 variable temperature fluorescence polarization system (PanVera, Model P2300) using an excitation wavelength of 360nm and a fluorescence detection wavelength of 530nm at 25°C. The instrument provides a numerical indication of the degree of light polarization at the fluorescent wavelength. The fluorescence polarization method is fully described in Turney *et al.* (submitted). The IC_{50} for 17β-estradiol varied between 8.3×19^{-9} – 1.1×10^{-8} M (2.26 – 2.99 μg/L). The "IC_{50} sample" was derived experimentally (the volume fraction that produced 50% ES2 displacement), and the concentration factor (200x) resulted from the C18 extraction procedure.

The possibility that procedural artifacts contributed to estrogenic response in the binding assay was evaluated by extracting 1-L of Milli-Q water and eluting retained material with methanol. Results from C18 disc extracts were indistinguishable from unextracted Milli-Q water (results not shown), indicating that organic bleed originating from the C18 disks did not contribute to estrogenic activity. There was a modest dissociation (<20%) of ES2 from ER-β in the 50% Milli-Q water sample (negative control). At each corresponding dilution, negative control data was used to adjust ES2 dissociation results obtained in actual samples.

Dissolved organic carbon (DOC) was measured using a Shimadzu Model TOC5000-Total Organic Carbon Analyzer, after passage of the sample through a pre-rinsed 0.45 μm cellulose acetate filter (Millipore). The lower level of detection of the TOC analyzer was 0.5 mg/L.

Results and Discussion

Positive control experiments showed that 17β-estradiol was detected at a concentration of approximately 2 nM (0.54 μg/L) (Figure 2). At a concentration of 100 nM (27.2 μg/L) 17β-estradiol almost completely displaced ES2 (the fluorescent estrogen) from ER-β, a human estrogen receptor (Figure 2). IC_{50}, the concentration at which half the bound ES2 ligand was displaced, was 9 nM (2.45 μg/L). It is evident that the competitive binding assay can detect known endocrine-disrupting compounds in the assay buffer at or near concentrations that are physiologically relevant to fish (i.e. nM).

Competitive binding assays detect all compounds that interact with the estrogen receptor, causing displacement of the labeled estrogen ligand, not just 17β-estradiol. Other compounds typically present in wastewater that may contribute to ligand displacement include 17α-ethinyl-estradiol, estrone, alkylphenol polyethoxylates (nonylphenol, octylphenol, and breakdown products), and many other chemicals such as PCBs or pesticides. The effluent used in this study was derived from a domestic wastewater; it seems likely that estrogens and ionic surfactant compounds (or their metabolites) are primarily responsible for the endocrine disruption observed.

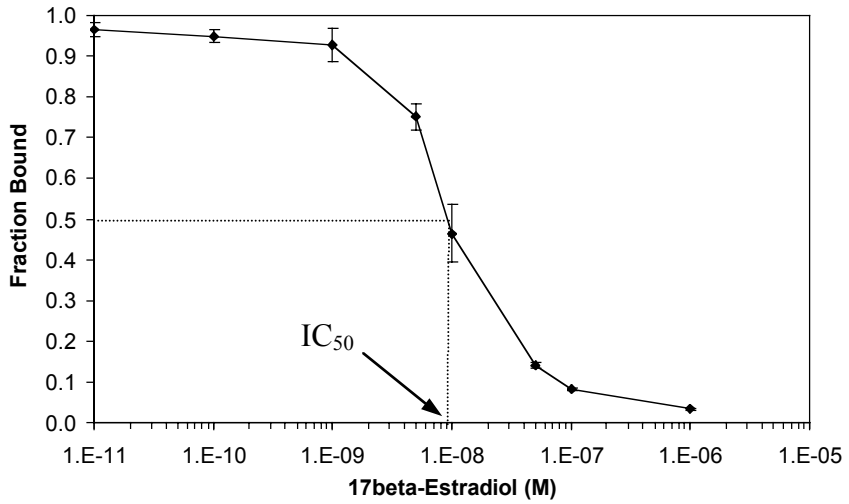

Figure 2. Competition binding assay response for 17β-estradiol. The IC_{50} was 9 nM (dashed line)

Figure 3 shows binding assay data for waters taken before and after treatment in raceway #6 (duckweed pond) and after raceway #5 (subsurface flow wetland). The estrogenic response of wetland-derived organics is also shown. Concentration factors for all eluates were 200x. Significant estrogenic activity was present in all raceway effluent samples. Changes in estrogenic activity of municipal wastewater due to passage through raceways #6 and #5 were modest or insignificant, IC_{50} values for all were between 0.59 and 1.4% for the 200x eluate samples (Table 2). Measurable estrogenic activity was also consistently detected in effluent from raceway #4, suggesting that the wetland-derived natural organic matter (NOM) produced in this raceway is mildly estrogenic. The IC_{50} of wetland-derived NOM (raceway #4 effluent) ranged from 6.1 - 37% of the 200x eluate samples. There was no measurable estrogenic activity in the 200x eluate of raceway #4 influent (municipal tapwater) (data not shown).

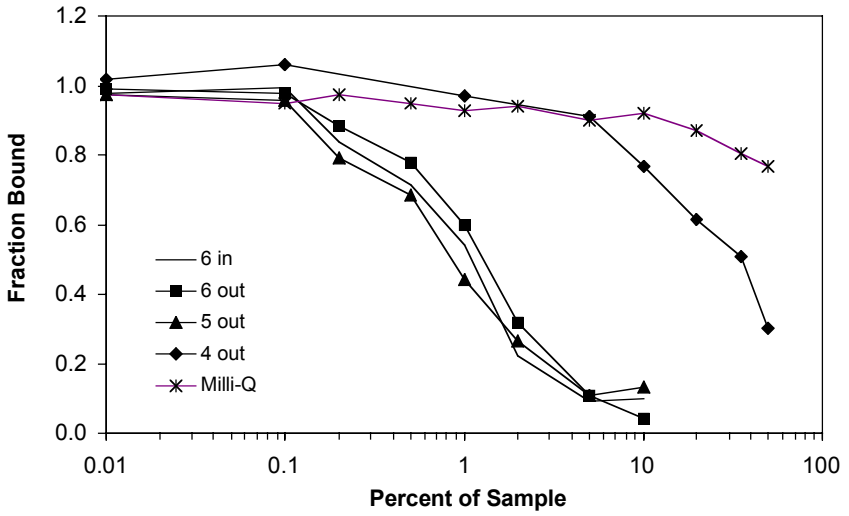

Figure 3. Competition binding assay results for samples collected from CERF wetland raceways on June 5, 2001. Total volume reduction after C18 extraction, elution, and resuspension into 0.15 M NaCl was 200x for all eluates.

To normalize the data and facilitate comparisons, IC_{50} values were converted to equivalent 17β-estradiol concentrations (Table 2). These values were also normalized to DOC concentration to provide an indicator for

the relative estrogenic activity per mg of DOC. In all three sampling episodes, total estrogenic activity (IC_{50}) varied little across a free-water-surface raceway (#6) or during subsequent treatment in a subsurface flow raceway (#5) (Table 2). When normalized to DOC however, there was a significant increase in the DOC-specific estrogenic response after the combined treatments in raceways #6 and #5. Reductions of DOC in raceway #5 averaged 40%; a corresponding reduction of estrogenic activity was observed in only one sampling episode (June 12). These results suggest that wetlands do not efficiently remove estrogenic compounds. In addition, the estrogenic activity of wetland-derived NOM (raceway #4, Figure 2) suggests that wetlands produce phytoestrogenic compounds, although the amount of estrogenic activity per mg DOC was less in wetland-derived NOM than in effluent organic matter (Table 2). Previous work (Quanrud *et al.*, in press) demonstrated that evapotranspiration water losses (20-50%) in these raceways occur during summer months and may have accounted for the maintenance of estrogenic activity during passage through raceway #5. Thus, removal efficiency of estrogenic compounds during wetland treatment in a hot, arid desert environment may be season dependent and inversely related to temperature.

Table 2. Summary of competition binding assay results for three sample collection episodes from CERF wetland raceway.

	IC_{50} (% of sample)	Equivalent β-Estradiol (μg/L)	DOC (mg/L)	β-Estradiol equivalent per DOC (ng/mg)
May 29, 2001				
#6 in	0.59	2.54	15.09	168
#6 out / #5 in	0.85	1.76	14.20	124
#5 out	0.61	2.45	8.00	307
#4 out	37.0	0.04	1.49	27
June 5, 2001				
#6 in	1.2	0.94	17.64	53
#6 out / #5 in	1.4	0.81	16.40	49
#5 out	0.85	1.33	8.47	157
#4 out	36.0	0.03	1.68	19
June 12, 2001				
#6 in	0.60	2.08	15.07	138
#6 out / #5 in	0.32	3.91	14.99	261
#5 out	0.60	2.08	7.70	271
#4 out	6.1	0.20	1.23	164

Note 1: concentration factor for all samples was 200x
Note 2: a decrease in IC_{50} (% of sample) indicates an increase in estrogenic response
Note 3: inlet DOC for raceway #4 averaged 0.65 mg/L

Establishment of correlations between binding assay data and the results of more physiologically relevant bioassays will strengthen interpretation of these data. This work is underway. Until such correlations are available, it must be recognized that compounds that interfere with ES2/ER-β binding would not be relevant from the perspective of whole-organism toxicity if they cannot penetrate cell membranes. Future work will include cell proliferation and reporter gene assays for the same classes of samples.

Conclusions

The following conclusions are supported by experimental results:

1. There is measurable estrogenic activity in municipal secondary effluent.

2. Subsurface flow wetland treatment did not attenuate estrogenic activity in municipal wastewater effluent.
3. Wetland-derived NOM exhibited measurable estrogenic activity.
4. The amount of estrogenic activity per mg of DOC increased significantly after wetland treatment, suggesting that responsible compounds are not removed or that removal of wastewater-derived estrogenic activity is counterbalanced by concentration effects (via evapotranspiration) and addition of wetland-derived estrogenic compounds.

Acknowledgment

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Disclaimer

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Removal of Pharmaceuticals During Conventional Wastewater Treatment, Advanced Membrane Treatment and Soil-Aquifer Treatment

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Abstract

The release of pharmaceuticals and personal care products (PPCPs) through domestic effluents into the environment is potentially associated with a human health risk where water is subsequently used to augment a drinking water supply. There is evidence that substances of pharmaceutical origin are not completely eliminated during wastewater treatment or biodegraded in the environment. A lack of knowledge still persists regarding the effectiveness of conventional wastewater treatment, advanced membrane treatment and soil-aquifer treatment (SAT) with respect to remove PPCPs. The scope of this study was directed to examine different wastewater treatment technologies (activated sludge, trickling filter, nanofiltration, reverse osmosis) at full-scale facilities in Arizona and California and their capability to remove PPCPs. Additionally, the fate of selected PPCPs was studied during SAT at sites where secondary and tertiary treated effluents are used for subsequent groundwater recharge. The groundwater recharge sites selected differ in the geohydrological settings of their recharge facilities. Composite samples were collected and analyzed for pharmaceuticals using GC/MS analysis. The selected pharmaceuticals represent lipid regulators, analgesics/anti-inflammatories, and antiepileptics.

The study revealed that drugs commonly found in domestic effluents and surface waters in Middle Europe were also present in U.S. effluents in similar concentration ranges. Analgesics/anti-inflammatory drugs, such as ibuprofen and naproxen, were determined in the investigated effluents at concentrations varying between detection limit and 6,280 ng/L. This large range indicates a high potential of removal during conventional wastewater treatment. Facilities employing longer detention times during treatment (nitrifying and denitrifying plants) showed significant lower effluent concentration for analgesic drugs as compared to trickling filter or activated sludge facilities applying shorter detention times. A similar trend was observed for the lipid regulator gemfibrozil, which was significantly removed in denitrified effluents, whereas a trickling filter treated effluent exhibited concentration of 1,235 ng/L. Antiepileptic drugs, such as carbamazepine and primidone, showed no dependency on the wastewater treatment applied. Primidone concentration in all investigated effluents varied between 100 and 220 ng/L and no impact of applied treatment was obvious. Carbamazepine was not detected in effluents where high concentrations of lipid regulating or analgesic drugs were detected indicating local variations in the consumption of this antiepileptic drug in different service areas. None of the investigated drugs was detected in tertiary treated effluents after nanofiltration or reverse osmosis. After soil-aquifer treatment, analgesic/anti-inflammatory drugs were efficiently removed after retention times of less than 6 months and remaining concentrations were near or below the detection limit of the analytical method. A similar behavior of ibuprofen, ketoprofen, naproxen, and fenoprofen during SAT confirmed a high potential for biodegradation of anti-inflammatory drugs in groundwater recharge systems. The antiepileptics carbamazepine and primidone represented the most dominant of all investigated drugs in well treated domestic effluents (nitrifying/denitrifying plants). During SAT, carbamazepine and primidone were not removed under aerobic unsaturated flow conditions. Removal of carbamazepine and primidone also did not seem to occur during travel times of more than 6 years in the subsurface characterized by saturated, anoxic flow conditions.

These findings indicate that most drugs, commonly found in other studies, were also present in secondary and tertiary treated effluents of facilities in the Southwestern U.S. The type of wastewater treatment employed affects the remaining concentration especially of anti-inflammatory drugs. Antiepileptic drugs represented the most dominant drugs in well treated wastewater and tended to persist during subsequent SAT. Employing nanofiltration or reverse osmosis treatment could guarantee the rejection of all drugs investigated.

Introduction

The release of pharmaceuticals and personal care products (PPCPs) through domestic effluents into the environment is potentially associated with a human health risk where water is subsequently used to augment a drinking water supply. The occurrence of PPCPs in domestic effluents is reported in studies since the late 1980s (Richardson and Bowron, 1985; Stan et al., 1994; Halling-Sørensen et al., 1998; Stan and Heberer, 1997). Meanwhile, more than 50 pharmaceuticals from various prescription classes such as analgesics, antibiotics, anti-epileptics, anti-rheumatics, beta blockers, chemotherapeutics, steroid hormones, and X-ray contrast media have been detected in the aquatic environment (Stan & Heberer, 1997; Ternes, 1998; Halling-Sørensen et al., 1998; Daughton & Ternes, 1999; Heberer et al., in press; Drewes et al., in press). Table 1 summarizes categories of drugs which were frequently detected in wastewater affected streams considering studies in the U.S., Europe, Canada, and Japan. Since most pharmaceuticals are hydrophilic and some are rather resistant to biotransformation, these compounds represent a potential source of contamination for drinking water sources. The major concern associated with the presence of PPCPs in surface or groundwater which serves as source for drinking is not necessarily acute effects on human health, but rather the manifestation of imperceptible effects that can accumulate over time to ultimately yield truly profound changes (Daughton & Ternes, 1999). Since effects might occur over long periods of time in certain non-target populations, changes would not be distinguishable from natural events or ecologic succession. Current comprehensive environmental risk assessments and epidemiologic studies lack to consider this type of long-term effects.

Table 1 Partial list of pharmaceuticals observed in the aquatic environment

Area of Application	Compounds
Antibiotics (Sulfonamides)	Sulfamethoxazole, sulfachlorpyridazine Sulfamerazine, sulfamethazine Sulfathiazole, sulfadimethoxine sulfamethiazole
Analgesics/anti-inflammatory drugs	Diclofenac; Ibuprofen; Ketoprofen; Naproxen Indometacine; Fenoprofen; Phenazone Acetaminophen; Acetylsalicylic acid Demethylaminophenazone Meclofenamic acid; Tolfenamic acid
Lipid regulators	Bezafibrate; Gemfibrozil; Clofibric acid Fenofibric acid
Antiepileptics	Carbamazepine; Primidone
Beta-blockers (antihypertentives)	Metoprolol; Propranolol; Nadolol; Carazolol Timolol; Betaxolol; Bisoprolol
Diagnostic agents	Iopromide; Diatrizoate
Others	Metformin (antidiabetic agent) Fluoxetine (antidepressant)

Sources: Stan & Heberer, 1997; Halling-Sørensen et al., 1998; Ternes, 1998; Buser et al., 1999; Daughton & Ternes, 1999; Buxton, 2000

There are a number of routes how pharmaceuticals can affect surface and groundwater, but primarily the sources are both untreated and treated sewage. There is evidence that substances of pharmaceutical origin are not completely eliminated during conventional wastewater treatment or biodegraded in the environment (Ternes, 1998; Daughton & Ternes, 1999; Heberer, in press, Drewes et al., in press). However, removal rates reported in these studies vary significantly and a lack of knowledge still persists regarding driving mechanisms of pharmaceutical removal during wastewater treatment. The objective of this study was to investigate occurrence of emerging contaminants in secondary and tertiary treated wastewater, their behavior during advanced membrane treatment, and their fate during subsequent soil-aquifer treatment (SAT) leading to groundwater recharge at wastewater facilities in Arizona and California. Pharmaceutical compounds for this study were selected based on their production, per-capita-consumption, and occurrence and were commonly found in domestic effluents and surface waters in the U.S. and Middle Europe. In the category of analgesic/anti-inflammatory drugs diclofenac, ibuprofen, ketoprofen, naproxen, fenoprofen, propyphenazone, meclofenamic

acid, and tolfenamic acid were identified. Gemfibrozil, clofibric acid, and fenofibrate were selected to represent lipid regulators. Carbamazepine and primidone were identified as antiepileptic drugs. Wastewater samples were collected and analyzed for selected target compounds using GC-MS analysis.
Diclofenac is a popular drug in human medical care as an analgesic, antiarthritic and antirheumatic compound. Buser et al. (1998) reported for tributaries of a lake in Switzerland, which received effluent discharge, diclofenac concentrations between 11 and 370 ng/L whereas in the lake effluent concentrations of <1-12 ng/L were observed pointing to a removal of > 90 percent. Based on additional controlled laboratory studies, the author concluded that photolysis was the predominant degradation process for diclofenac. Stumpf et al. (1996) determined the occurrence of pharmaceuticals in different surface waters in Germany. Along the River Rhine, diclofenac was the most prominent drug detected at concentrations of up to 300 ng/L. Ibuprofen is a non-steriodal anti-inflammatory, analgesic and antipyretic drug, widely used in the treatment of rheumatic disorders, pain and fever. It is an important non-prescription drug and has a relatively high therapeutic dose (600 to 1200 mg/d). Buser et al. (1999) conducted activated sludge biodegradation studies and showed extensive removal of ibuprofen. Based on these experiments, the authors concluded that for a complete degradation of ibuprofen, a hydraulic retention time (HRT) of the wastewater in the activated sludge system in excess of 6 h is required (Buser et al. 1999). Ibuprofen exhibited concentrations along the River Rhine varying between 6 to 41 ng/L (Stumpf et al., 1996). The lipid regulators clofibric acid and bezafibrate were also detected along the River Rhine at concentrations between 12 to 51 ng/L and 106 to 295 ng/L, respectively. No degradation of clofibric acid was observed in incubation experiments conducted by Buser et al. (1998). This apparent persistence could explain why clofibric acid was detected in the North Sea at concentrations of 1.2 to 2.4 ng/L. Zwiener & Frimmel (2000) conducted experiments using advanced oxidation processes (AOP). Findings of this study revealed 98 percent degradability of clofibric acid by OH-radical mediated reactions during AOP. Other frequently prescribed analgesic drugs, such as acetylsalicylic acid (Aspirin), fenoprofen, and ketoprofen, and the lipid regulator gemfibrozil were not detected along the River Rhine (Stumpf et al., 1996). However, gemfibrozil was readily detected in other German streams and rivers during this study at concentrations varying between 24 and 190 ng/L. Betablockers are also commonly used as therapeutic drugs. Metoprolol and propranolol are the most important betablockers with therapeutic doses of 100 to 200 mg/d and 40 to 80 mg/d, respectively (Hirsch et al., 1996). Based on a survey of different surface waters in Germany, Hirsch et al. (1996) reported the highest concentration of betablockers for metoprolol with a median concentration of 31 ng/L followed by propranolol exhibiting a median concentration of 7 ng/L. No betablockers were detected in finished water. Carbamazepine and primidone are representing antiepileptic drugs which are applied at high daily therapeutic doses of 600 to 1200 mg. Carbamazepine is used as anticonvulsant (anti-epileptic), relieves pain associated with trigeminal neuralgia, and has been found useful in the treatment of acute mania. Sacher et al. (1998) estimated the amount of carbamazepine prescribed per year in Germany to be 60 to 120 t, which would be similar to the yearly amount of acetylsalicylic acid prescribed and higher than the amounts of ibuprofen, bezafibrate and diclofenac used per year. Acetylsalicyclic acid and ibuprofen are sold as over the counter drugs. Thus, in Germany, the total amounts of acetylsalicylic acid sum up to a total amount of approximately 1000 tons per year. Several studies in Europe indicate that carbamazepine and primidone are barely removed during wastewater treatment, bank filtration and groundwater recharge making them highly relevant for drinking water treatment (Ternes, 1998; Kuehn & Mueller, 2000; Brauch et al., 2000; Heberer, in press; Heberer et al., 2001; Scheytt et al., 2001).

Methodology

Field sites

Results reported here were determined at full-scale wastewater treatment facilities in Arizona and Southern California. The study focused on the City of Mesa (Arizona) Northwest Water Reclamation Plant (NWWRP), the Sweetwater Recharge and Storage Facility (SUSRF) in Tucson, the Scottsdale Water Campus (Arizona), and the San Jose Creek East Water Reclamation Plant (WRP) and Whittier Narrows WRP operated by the County Sanitation Districts of Los Angeles County (CSDLAC), California. The treatment characteristics of each facility are summarized in Table 2. Treatment at the Mesa Northwest WRP and the Scottsdale Water Campus consists of activated sludge treatment including nitrification/denitrification with disinfection and tertiary filtration. Wastewater treatment at the Tucson site consists of trickling filter treatment where no nitrification or denitrification is established. The San Jose Creek East WRP employs secondary treatment with

partial nitrification, tertiary filtration and disinfection. Treatment at the Whittier Narrows WRP consists of nitrification and denitrification, tertiary filtration and disinfection. Advanced membrane treatment was investigated at full-scale at the Scottsdale Water Campus, where tertiary treated effluent is treated by microfiltration (MF) (U.S. Filter-Memcor 90M10C) followed by reverse osmosis (RO) (TFC-HR 8832 Magnum, Koch Membrane Systems). In addition, pilot-scale testing of a nanofiltration membrane (DOW/FilmTec NF-90) and a reverse osmosis membrane (TFC-HR, Koch Membrane Systems) was performed using tertiary effluent from the NWWRP Mesa. Figure 1 illustrates the flowscheme of the membrane pilot-scale plant used in this study. Operational details of the membrane pilot study are described in Drewes et al. (2001).

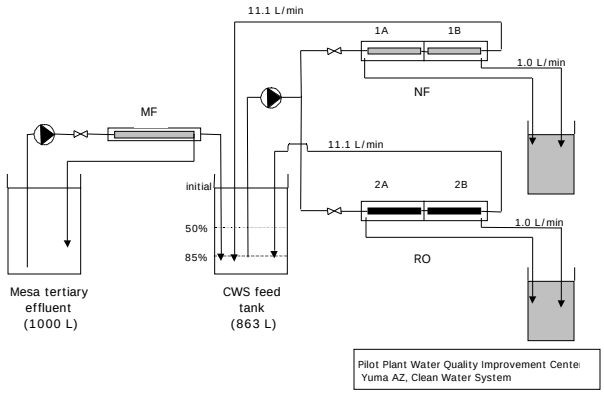

Figure 1 Flowscheme of the pilot-plant membrane testing facility (Drewes et al., 2001)

Table 2. Wastewater treatment technologies employed at different facilities in Arizona and California.

Facility	**Wastewater treatment**
Northwest Water Reclamation Plant Mesa, AZ	activated sludge treatment (nitrifying/denitrifying); disinfection; tertiary filtration
Tucson Sweetwater Underground Storage and Recharge Facility, AZ	activated sludge treatment (no nitrification); disinfection
Scottsdale Water Campus, AZ	activated sludge treatment (nitrifying/denitrifying); disinfection; tertiary filtration, microfiltration, reverse osmosis
San Jose Creek East Water Reclamation Plant, Whittier CA	activated sludge treatment (partly nitrifying); tertiary filtration; disinfection
Whittier Narrows Water Reclamation Plant, Whittier CA	activated sludge treatment (nitrifying/denitrifying); tertiary filtration; disinfection

Two groundwater recharge sites in Arizona, the Tucson SUSRF and the NWWRP in Mesa, were selected to study the fate of selected target drugs during subsequent groundwater recharge. The recharge facilities differ both in the type of above-ground treatment and the geohydrological settings. At SUSRF, treated effluent is fed to eight recharge basins. Flow at the Tucson site is characterized by unsaturated conditions and a vadose zone of approximately 40 m. At the Mesa NWWRP the tertiary treated effluent is applied to four recharge basins. The majority of flow occurs under saturated conditions as a consequence of the local hydrogeological conditions. The two settings represent significantly different redox conditions (aerobic versus anoxic). The majority of recharged water at the Mesa site has not been recovered and a plume of infiltrated wastewater extends greater than 2,000 meters downgradient from the recharge site. Five downgradient groundwater monitoring wells within the upper alluvial unit were sampled at the Mesa site during this study to characterize water quality changes in the wastewater plume.

Sampling location

At the Mesa site, two-hour composite samples of tertiary treated effluent were collected twice. A two-hour composite sample of the Tucson secondary effluent was collected at the inlet of recharge basin 1 (RB-1). At San Jose Creek East and Whittier Narrows, two-hour composites of tertiary treated effluent were collected once and grab samples of tertiary treated effluent and RO permeate were collected at the Scottsdale Water Campus. At the Tucson site, a hydraulically corresponding sample was collected from a deep well (WR-199) located in basin 1 (RB-1) representing regional groundwater. Additionally, a groundwater sample from monitoring wells WR-205 adjacent to recharge basin RB-8 were collected. The monitoring wells represent groundwater from the upper alluvial unit. Groundwater flow at this site is directed to the northwest. Figure 2 presents a map indicating the location of recharge basins and monitoring wells.

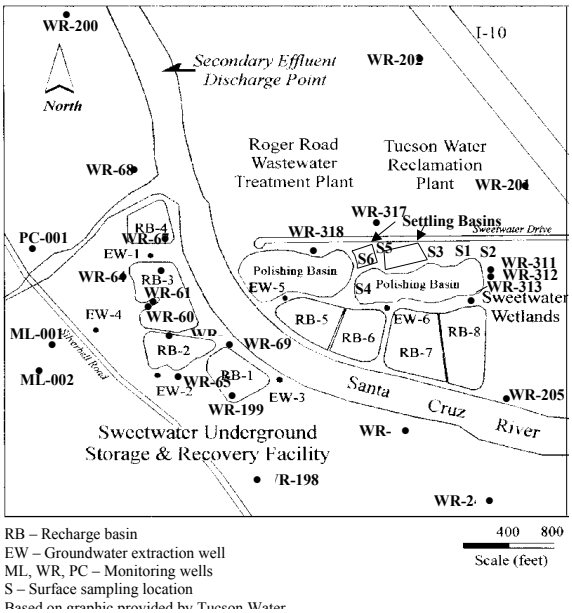

Figure 2. Location of recharge basins and monitoring wells at Tucson Sweetwater Underground Storage and Recovery Facility (adopted from Fox et al., 2001)

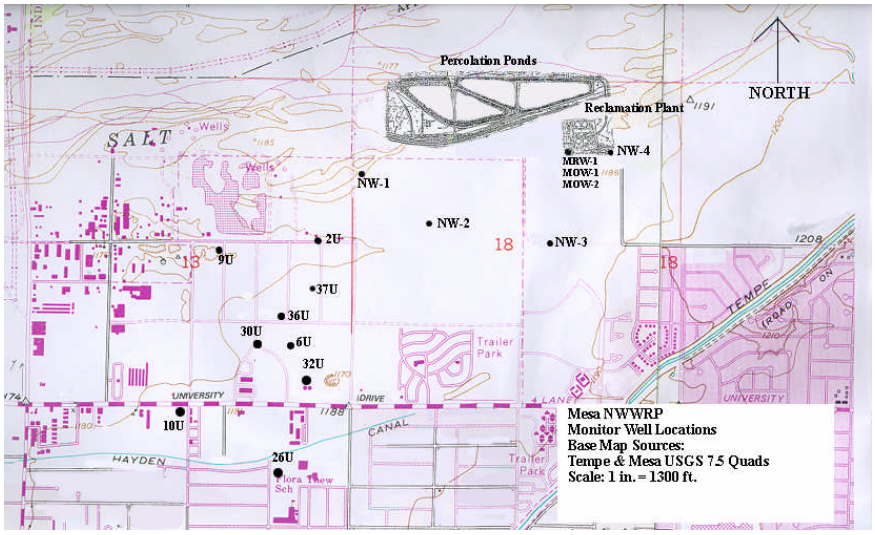

Figure 3 Location of recharge basins and groundwater monitoring wells at the Mesa Northwest Water Reclamation Plant (adopted from Fox et al., 2001).

At the Mesa site, groundwater samples were collected at monitoring wells located downgradient of the recharge basins. The monitoring well samples represent travel times of 6-18 months (OW2-90', NW-4,

NW-2), 2 years (2U), and more than 6 years (6U), respectively. Groundwater flow at this site is directed to the southwest. Additionally, local drinking water based on surface water supply was included in the study to represent background water quality. The location of monitoring wells at the Mesa site is indicated in Figure 3.

Analytical methods

All samples were filtered using 0.45 µm cellulose nitrate membrane filters and stored at 4 ^{0}C prior to analysis. The pharmaceutical residues were analyzed using two multi-methods described by Heberer et al. (1998) and Reddersen & Heberer (in prep.). For trace analysis, the samples were concentrated from (up to) one liter down to a final sample volume of 0.1 µl (concentration factor: up to 10,000). Both methods apply solid-phase extraction (SPE) using non-endcapped reversed-phase octadecyl adsorbents for the extraction of the analytes from the water samples. The extracted residues were then derivatized using pentafluorobenzyl bromide and N-(tert.-butyldimethylsilyl)-N-methyl-trifluoroacetamide (MTBSTFA) as derivatization reagents, respectively. The remaining residues were dissolved and analyzed applying gas-chromatography-mass spectrometry (GC-MS) using selected ion monitoring (SIM). The recoveries of the analytes were between 70 and 110 percent. For quality control, two suitable surrogate standards (2-(4-chorophenoxy)butyric acid and dihydrocarbamazepine) were added to the samples before sample preparation. The detection limits for the pharmaceuticals are between 1 and 10 ng/L, depending on the individual compounds, the sample volume and the sample matrix (Reddersen & Heberer, in prep.).

Results and Discussion

Occurrence in secondary and tertiary treated effluents

The study revealed that analgesic and anti-inflammatory drugs like diclofenac, ibuprofen, fenoprofen, ketoprofen, and naproxen were present both in secondary and tertiary treated effluents. Of all anti-inflammatory drugs analyzed, only two were detected in Mesa and Whittier Narrows tertiary treated effluents. Meclofenamic acid and tolfenamic acid were not detected in effluent samples. Propyphenazone was only detected at low concentration in Tucson's secondary treated effluent. Table 3 summarizes results of pharmaceuticals analyzed in the investigated effluents. None of the pharmaceutical drugs was detected in Mesa drinking water.
In general, effluent samples of facilities employing nitrification and denitrification exhibited significantly lower concentrations of ibuprofen and naproxen. Ibuprofen varied between 5 and 40 ng/L in nitrified and denitrified effluents, whereas ibuprofen was detected at concentrations varying between 2550 and 3380 ng/L in not or partial nitrified effluents. A similar trend was observed for naproxen, which concentrations varied between below detection limit and 110 ng/L in effluents with low hydraulic retention time, whereas elevated concentrations of 710 ng/L and 6280 ng/L occurred in San Jose Creek East's and Tucson's effluent samples, respectively. Based on a survey of 49 wastewater treatment plants in Germany conducted by Ternes (1998), ibuprofen and naproxen were also regularly found in German secondary treated effluents with maximum concentrations of 3400 ng/L and 520 ng/L, respectively. However, the 90-percentile of ibuprofen and naproxen in this study was only 1200 ng/L and 420 ng/L, respectively, indicating significantly lower concentrations in the German effluents as compared to Tucson secondary effluent. The most prominent acidic drug detected in German effluents was diclofenac which might indicate a different application practice between Germany and the U.S.

Of three lipid-regulating substances investigated, only gemfibrozil and clofibric acid were detected in effluent samples (Table 3). The Tucson secondary effluent with the shortest hydraulic retention time exhibited the highest concentration of gemfribozil (1235 ng/L) followed by San Jose Creek East effluent (145 ng/L) and Scottsdale's tertiary effluent (65 ng/L). Clofibric acid was detected only at sites in California, exhibiting concentrations of 5 and 25 ng/L. None of the three lipid regulators was detected in Mesa tertiary effluent. According to the survey conducted by Ternes (1998) for German secondary effluents, gemfibrozil exhibited a maximum concentration of 1500 ng/L with a 90-percentile of 840 ng/L, which reflects a similar concentration range as compared to the Tucson secondary effluent.
The antiepileptics carbamazepine and primidone were the most dominant of all investigated drugs in Mesa tertiary effluent (Table 3). Mesa effluent concentrations of these drugs varied between 155 and 220 ng/L.

Carbamazepine in Scottsdale's tertiary effluent was slightly higher (445 ng/L). A similar concentration of primidone was observed in all other effluent samples varying between 100 and 120 ng/L. Carbamazepine was not detected in Tuscon, San Jose Creek East and Whittier Narrows effluent samples. The concentration range of carbamazepine observed in the effluents was also reported for surface water under the influence of effluent discharge in Germany (Sacher et al., 1998; Heberer, in press). Based on the survey conducted by Ternes (1998), carbamazepine was present in secondary effluents of all 30 facilities investigated and the effluents exhibited significant higher concentrations with a maximum concentration of 6300 ng/L and a 90-percentile of 3700 ng/L.

Table 3. Pharmaceuticals detected in secondary and tertiary treated wastewater in ng/L

Use/origin	**Compound**	**Mesa tertiary effluent (ng/L)**	**Tucson secondary effluent (ng/L)**	**Scottsdale tertiary effluent (ng/L)**	**San Jose Creek East tert. effluent (ng/L)**	**Whittier Narrows tert. effluent (ng/L)**
Lipid regulator	Gemfibrozil	n.d.	1235	65	145	n.d.
	Clofibric acid	n.d.	n.d.	n.d.	5	25
	Fenofibrate	n.d	n.d.	n.d.	n.d.	n.d.
Antiepileptic	Carbamazepine	155 195	n.d.	445	n.d.	n.d.
	Primidone	185 220	110	100	120	105
Analgesic/anti-inflammatory	Diclofenac	n.d.	80	15	20	n.d.
	Ibuprofen	16 16	3380	40	2550	5
	Ketoprofen	n.d.	45	35	35	20
	Naproxen	8 8	6280	115	710	n.d.
	Fenoprofen	n.d.	35	n.d.	15	n.d.
	Propyphenazone	n.d.	20	n.d.	n.d.	n.d.
	Meclofenamic acid	n.d.	n.d.	n.d.	n.d.	n.d.
	Tolfenamic acid	n.d	n.d.	n.d.	n.d.	n.d.

n.d. – not detected

Considering the fact that the per-capita water demand in Arizona and California is three to four times higher as compared to Germany resulting in a more diluted wastewater in the Southwestern U.S. might also explain differences observed between the U.S. and German effluents. Since the service area of all investigated U.S. wastewater treatment plants receive sewage of similar composition, the significant difference in the presence of pharmaceuticals might point to the important role of wastewater treatment applied. Wastewater treatment at Tucson and San Jose Creek East is characterized by short hydraulic retention times (HRT < 10 hrs.) resulting in high concentrations of ammonia (above 20 mg/L NH_4^+-N) and total organic carbon (TOC) which are varying between 10 and 25 mg/L (Fox et al., 2001). The Mesa, Scottsdale, and Whittier Narrows nitrified and denitrified effluent, where long HRT are employed (> 10 hrs.), exhibits total nitrogen concentrations below 10 mg/L and TOC concentrations of less than 7 mg/L (Fox et al., 2001). With the exception of antiepileptic drugs, facilities employing long HRT exhibited significant lower concentration of analgesic and lipid regulator concentrations as compared to sites applying shorter hydraulic retention times.

Fate during Advanced Membrane Treatment

Advanced membrane treatment using nanofiltration or reverse osmosis was studied at the pilot-scale using tertiary treated effluent from the Mesa NWWRP. Two NF and RO membranes were chosen for this study according to total organic carbon (TOC) rejection and their applicability in full-scale water reclamation facilities. The tertiary treated effluent was additionally treated using microfiltration (Osmonics/Desal EW4040F) in order to avoid fouling of the NF and RO membranes. Two elements of each NF and RO membrane were installed in one 2.5'' diameter pressure vessel with a length of 80''. The vessels were operated in a constant flux with variable pressure mode. A permeate flux of ~1.0 Liter/min was implemented for both

membranes considering an average flux of approximately 14 gfd (34.2 m/h) as applied in full-scale facilities. Both vessels were arranged in a recycle pass mode in order to achieve a rate of recovery between 80 to 90 percent.

The nanofiltration membrane NF-90 showed a salt rejection of 92.4 percent and the TOC rejection varied between 92 and 94.5 percent (Drewes et al., 2001). The molecular weight cut-off (MWCO) of the NF-90 was reported to be 200 Dalton. None of the investigated pharmaceuticals was detected in the NF permeate (Table 4). The reverse osmosis membrane TFC-HR exhibited a salt rejection of 97.6 percent and a TOC rejection of 96 percent (Drewes et al., 2001). The MWCO was reported to be 100 Dalton and no pharmaceutical compound was identified in the RO permeate (Table 4). Findings from the pilot-scale are confirmed by samples collected after full-scale RO treatment (employing the same type of RO membrane, TFC-HR) at the Scottsdale Water Campus, where no drugs were identified in the RO permeate sample (Table 5).

Table 4. Concentration of pharmaceuticals in Mesa tertiary effluent and corresponding NF and RO permeates in ng/L (n = number of samples; n.d. = not detected)

Compound	**Mesa tertiary effluent, n = 2**	**NF-90 n = 2**	**RO TFC-HR n = 2**
Lipid regulator			
Gemfibrozil	n.d.	n.d.	n.d.
Clofibric acid	n.d.	n.d.	n.d.
Fenofibrate	n.d	n.d.	n.d.
Antiepileptic			
Carbamazepine	155	n.d.	n.d.
	195	n.d.	n.d.
Primidone	185	n.d.	n.d.
	220	n.d.	n.d.
Analgesic/anti-Inflammatory			
Diclofenac	n.d.	n.d.	n.d.
Ibuprofen	16	n.d.	n.d.
	16		
Ketoprofen	n.d.	n.d.	n.d.
Naproxen	8	n.d.	n.d.
	8		
Fenoprofen	n.d.	n.d.	n.d.
Propyphenazone	n.d.	n.d.	n.d.
Meclofenamic acid	n.d.	n.d.	n.d.
Tolfenamic acid	n.d	n.d.	n.d.

Table 5. Concentration of pharmaceuticals in Scottsdale tertiary effluent and corresponding RO permeate in ng/L (n.d. = not detected)

Compound	Scottsdale tertiary effluent, n = 2	RO TFC-HR n = 2
Lipid regulator		
Gemfibrozil	65	n.d.
Clofibric acid	n.d.	n.d.
Fenofibrate	n.d.	n.d.
Antiepileptic		
Carbamazepine	445	n.d.
Primidone	100	<10
Analgesic/anti-Inflammatory		
Diclofenac	15	n.d.
Ibuprofen	40	n.d.
Ketoprofen	35	n.d.
Naproxen	115	n.d.
Fenoprofen	n.d.	n.d.
Propyphenazone	n.d.	n.d.
Meclofenamic acid	n.d.	n.d.
Tolfenamic acid	n.d.	n.d.

Fate during Groundwater Recharge

The fate of pharmaceuticals present in wastewater during subsequent soil-aquifer treatment (SAT) was studied at the Mesa NWWRP and the Tucson Sweetwater recharge facility. Table 6 presents pharmaceuticals detected in Mesa monitoring wells. With the exception of monitoring well NW-2, neither ibuprofen nor naproxen or any other acidic drug were detected in groundwater wells downgradient of the Mesa recharge basins. However, antiepileptic drugs were detected in all monitoring wells downgradient of the recharge basins.

Table 6. Pharmaceuticals detected in Mesa tertiary effluent and monitoring well samples

Use/origin	Compound	Mesa tert. effluent (ng/L)	OW2-90' (ng/L)	NW-4 (ng/L)	NW-2 (ng/L)	2U (ng/L)	6U (ng/L)
Lipid regulator	Gemfibrozil	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Clofibric acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Fenofibrate	n.d	n.d.	n.d.	n.d.	n.d.	n.d.
Antiepileptic	Carbamazepine	155 195	115	235	125	145	85
	Primidone	185 220	140	120	160	90	100
Analgesic/anti-Inflammatory	Diclofenac	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Ibuprofen	16 16	n.d.	n.d.	16	n.d.	n.d.
	Ketoprofen	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Naproxen	8 8	n.d.	n.d.	n.d.	<10	<10
	Fenoprofen	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Propyphenazone	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Meclofenamic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Tolfenamic acid	n.d	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. – not detected N/A – not available

Since Mesa tertiary effluent concentrations of carbamazepine and primidone varied in a similar range as the monitoring wells samples, a significant removal of antiepileptic drugs during groundwater recharge was not obvious although the sampled groundwater represented travel times in the subsurface of more than 6 years. This finding is consistent with studies conducted at bankfiltration systems in Germany. Kuehn and Mueller (2000) and Sacher et al. (1998) reported that carbamazepine persisted during bank filtration at the River Rhine. Concentrations of the bank filtrate in these studies varied between 20 and 550 ng/L and were similar to concentrations observed in the river water.
Comprehensive studies previously conducted at this site proved the substantial removal of wastewater constituents during groundwater recharge resulting in TOC concentrations of approximately 1 mg/L and a significant removal of 85 percent of ethylendiaminetetraacidic acid (EDTA) and more than 95 percent of alkylphenolpolycarboxylates (APECs) (Fox et al., 2001). However, antiepileptic drugs seems to persist during groundwater recharge.

At the Tucson Sweetwater recharge facility, lipid regulators were not detected in monitoring wells underneath the recharge basins. Table 7 summarizes results of pharmaceuticals analyzed in Tucson groundwater monitoring wells. Although, high concentrations of naproxen and ibuprofen were detected beside other acidic drugs in Tucson secondary effluent, only naproxen in concentrations varying between < 10 ng/L and 20 ng/L and propyphenazone at a concentration of 15 ng/L were detected in monitoring wells WR-199 and WR-205. These findings indicate a high potential of biodegradation of acidic drugs during groundwater recharge. Consistent with observations at the Mesa site, antiepileptic drugs exhibited the most dominant concentrations in Tucson groundwater monitoring wells. Primidone concentration in both monitoring wells was 155 ng/L and 115 ng/L, respectively, representing a similar concentration range as observed in the secondary effluent. Although, carbamazepine was not detected in Tucson secondary effluent, concentrations of 610 ng/L and 455 ng/L were detected in monitoring wells WR-199 and WR-205, respectively. This inconsistency might be due to a discontinuous release of carbamazepine and certainly justifies further studies on the occurrence of this compound in Tucson's secondary effluent.

Table 7. Pharmaceuticals detected in Tucson Sweetwater wastewater and monitoring well samples

Use/origin	Compound	Tucson secondary effluent (ng/L)	WR-199 (ng/L)	WR-205 (ng/L)
Lipid regulator	Gemfibrozil	1235	n.d.	n.d.
	Clofibric acid	n.d.	n.d.	n.d.
	Fenofibrate	n.d.	n.d.	n.d.
Antiepileptic	Carbamazepine	n.d.	610	455
	Primidone	110	155	115
Analgesic/anti-inflammatory	Diclofenac	80	n.d.	n.d.
	Ibuprofen	3380	n.d.	n.d.
	Ketoprofen	45	n.d.	n.d.
	Naproxen	6280	<10	20
	Fenoprofen	35	n.d.	n.d.
	Propyphenazone	20	15	15
	Meclofenamic acid	n.d.	n.d.	n.d.
	Tolfenamic acid	n.d.	n.d.	n.d.

n.d. – not detected

Conclusions

Findings of this study indicate that most drugs, commonly found in other studies, were also present in secondary and tertiary treated effluents of selected wastewater treatment facilities in the Southwestern U.S. There was evidence that the hydraulic retention time (HRT) employed during wastewater treatment affects the remaining concentration of drugs especially of anti-inflammatory and lipid-regulating drugs. Concentrations of anti-inflammatory drugs varied three orders of magnitude between samples of not nitrified (HRT < 10 hrs.) and samples of nitrified/denitrified effluents (HRT > 10 hrs.). The lowest concentrations of anti-inflammatory and lipid-regulating drugs were determined in fully nitrified/denitrified effluents. Antiepileptic drugs represented the

most dominant drugs in treated wastewater with long hydraulic retention times and effluent samples exhibited similar concentration ranges regardless the wastewater treatment applied. Employing nanofiltration or reverse osmosis treatment guaranteed the rejection of all drugs investigated.
Soil-aquifer treatment was proven to efficiently remove anti-inflammatory and lipid-regulating drugs. Antiepileptic drugs (carbamazepine and primidone) persisted groundwater recharge and no clear indication was found pointing to removal during travel times of more than 6 years. Further studies are necessary to address the potential human health risk associated with antiepilectics found in groundwater recharge systems.

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Removal of Endocrine Disrupting Compounds Using Drinking Water Treatment Processes

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Abstract

Recently public concern has increased regarding industrial and environmental substances that may have adverse hormonal effects in human and wildlife populations. Although the list of potentially harmful substances is still being compiled and more sophisticated laboratory tests for detection of endocrine disrupting chemicals (EDCs) are being developed, an initial list of known EDCs has been made and an array of drinking water treatment processes has been evaluated for their ability to remove EDCs. Alkylphenols, bisphenol A, phthalates, polychlorinated biphenyls, dioxins, dibenzofurans as well as the pesticides methoxychlor, endosulfan, and DDT have been included in the initial list. In addition to the conventional water treatment processes of sedimentation, coagulation, and filtration we have also considered the potential effects of granular activated carbon, powdered activated carbon, nanofiltration, reverse osmosis and air stripping for the removal of EDCs. Our findings indicate that granular activated carbon (GAC) and powdered activated carbon (PAC) are the most effective processes for the removal of the selected group of EDCs and the laboratory data that was used in the Freundlich equation for determining the efficiency of GAC for EDC removal will be presented. In addition, additional analyses of the effectiveness for removing ethinylestradiol and melegesterol acetate through the use of GAC and PAC will also be presented.

Routes of Groundwater Contamination with Pharmaceuticals and the Removal Drug Residues During Water Treatment Processes

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Abstract

Recent investigations revealed that more than 30 different pharmaceuticals belonging to nearly all important medicinal classes could be found up to the µg/L range in rivers and streams. For instance, maximum concentrations were determined in German rivers up to 1.3 µg/L for carbamazepine (antiepileptic) and 1.2 µg/L for diclofenac (antiphlogistic). An possible contamination of soil and groundwater may be caused by the using of digested sludge from municipal STP on agricultural areas. Additionally, transport of drugs via bank filtration from highly contaminated surface water into groundwater is also a possibility as the infiltration of waste waters directly from leakages in sewers. Also drugs disposed together with domestic waste can reach landfill sites which could lead to groundwater contamination by leaching. A unique pathway for the contamination of soil and groundwater by medicinal residues, derived from human application, may be the disposal of raw sewage or STP effluents by spray and broad irrigation in agricultural areas.

About 233 groundwater samples from different potential routes were analyzed according to a potential groundwater contamination. In 1 % of the groundwater samples mainly located close to polluted small rivers or streams the concentrations are exceeding even 1 µg/L, and 15 % exhibited concentration levels above the limit of quantification (LOQ). Irrigation of treated sewage leads to groundwater contamination of up to 4 µg/L indicating that indirect reuse of treated sewage may be responsible for a appreciable contamination of groundwater. In a groundwater sample close to a domestic landfill site (another unique pathway) clofibric acid was detected as high as 11 µg/L, indicating the contamination of the aquifer by leaking waste deposition sites.

In few cases pharmaceuticals were found even in drinking water, due to the use of polluted surface water and ground water, contaminated with medicinal compounds by irrigation of treated sewage or bank filtration. Simultaneously, lab simulations of flocculation with Fe(III)chloride and/or ozonation of raw water were compared to the behavior in real water works. Flocculation does not appreciably remove the selected pharmaceuticals. Applying ozone concentrations in the range between 1.0 and 2.0 mg/L carbamazepine was totally and diclofenac, bezafibrate and clofibric acid were mainly eliminated. Therefore, ozonation is a powerful technique for the removal of pharmaceuticals in the water treatment.

Introduction

The fate of veterinary and human drugs after urinal or fecal excretion are quite different to each other (see Fig. 1). In general, excreted human pharmaceuticals have to pass through a sewage treatment plant (STP) prior to entering rivers or streams, whereas veterinary drugs are more likely to contaminate soil and groundwater (without previous wastewater treatment) when liquid manure is used for top soil dressing. After rainfall incidents, surface waters can be polluted with human or veterinary drugs by run-off from fields (esp. agricultural) treated with digested sludge or livestock slurries; groundwater can also be contaminated. The use of pharmaceuticals, especially antibiotics, in fish farms is a unique source for contamination of rivers and lakes, which receive the effluents of those ponds. Discharge of wastewater from pharmaceutical manufacturing into surface waters is another point source (Richardson and Bowron, 1985). Transport of drugs via bank filtration from highly contaminated surface water into groundwater is also a possibility, as is the infiltration of wastewater directly from sewer drain leakages. Drugs disposed off together with domestic waste can reach landfill sites, which can lead to groundwater contamination by leaching (Holm *et al.*, 1995). The use/disposal of raw sewage or STP effluents by spray and broad irrigation on agricultural areas is yet another route of introduction.

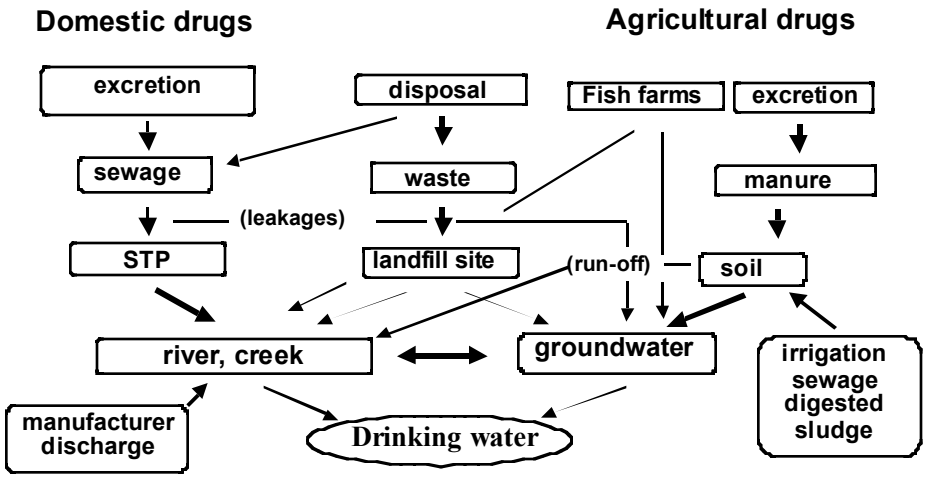

Fig. 1. Fate of pharmaceuticals in the environment

Groundwater Contamination

In Tab. 1 a survey is given about the detected pharmaceuticals in German surface water and groundwater. A comparison of the contamination for both matrices exhibits that frequently those compounds which are present in surface water can also be found in groundwater (Temes et al., 2001). In rivers 22 of 49 pharmaceuticals were determined above the LOQ, 13 of 49 were identified in groundwater. The data presented below clearly revealed that polar pharmaceuticals end up in the groundwater and will stay there probably for a very long time since microbial activity in groundwater is very low.

Tab. 1a: Occurrence of pharmaceuticals in German rivers and groundwater

Medicinal classes	Pharmaceuticals	LOQ (µg/l)	Occurrence in rivers	Occurrence in groundwater
Betablockers	Metoprolol	0.01	+	+
	Propranolol	0.01	+	-
	Betaxolol	0.01	(+)	-
	Bisoprolol	0.01	+	+
	Carazolol	0.01	+	-
	Timolol	0.01	-	-
	Nadolol	0.01	-	-
Sympathominetics	Fenoterol	0.01	-	-
	Terbutalin	0.01	-	-
	Salbutamol	0.01	-	-
	Clenbuterol	0.01	-	-
Lipid regulator/metabolites	Clofibric acid	0.01	+	+
	Clofibrate	0.03	-	-
	Fenofibrate	0.03	(+)	-
	Fenofibric acid	0.02	-	+
	Bezafibrate	0.03	+	+
	Gemfibrozil	0.02	+	(+)
	Etofibrate	0.03	-	-
Antiphlogistic	Diclofenac	0.01	+	+
	Ketoprofen	0.02	-	-
	Fenoprofen	0.02	-	-
	Ibuprofen	0.01	+	+
	Indometacin	0.01	+	+
	Naproxen	0.01	+	-
	Meclofenaminsäure	0.01	-	-
	Tolfenaminsäure	0.01	-	-
	Acetylsalicylic acid	0.02	-	-
	Phenazone	0.03	+	+
	Dimethylamiophenazone	0.03	-	+
Psychiatric drug	Diazepam	0.03	(+)	(+)
Antiepileptic	Carbamazepine	0.03	+	+
Vasodilator	Pentoxifyllin	0.03	(+)	-
Cyctostatic agent	Cyclophosphamide	0.03	(+)	-
	Ifosfamide	0.03	-	-

+ = detected, - = not detected, (+) = identified below LOQ

Tab. 1b: Occurrence of pharmaceuticals in German rivers and groundwater

Antibiotics	Pharmaceuticals	LOQ (µg/l)	Occurrence in rivers	Occurrence in groundwater
	Tetracyclin	0.05	-	-
	Chlortetracyclin	0.05	-	-
	Doxycyclin	0.05	-	-
	Oxytetracyclin	0.05	-	-
	Phenoxymethylpenicillin	0.05	-	-
	Oxacillin	0.05	-	-
	Nafcillin	0.05	-	-
	Cloxacillin	0.05	-	-
	Methicillin	0.05	-	-
	Dicloxacillin	0.05	-	-
	Roxithromycin	0.05	+	-
	Clarithromycin	0.05	+	-
	Erythromycin	0.05	+	-
	Sulfamethoxazol	0.05	-	-
	Trimethoprim	0.05	+	-
	Chloramphenicol	0.05	-	-

+ = detected, - = not detected, (+) = identified below LOQ

About 233 groundwater samples from different potential routes were analyzed according to a potential groundwater contamination. In 1 % of the groundwater samples mainly located close to polluted small rivers or streams the concentrations are exceeding even 1 µg/L, and 15 % exhibited concentration levels above the limit of quantification (LOQ). Antiphlogistics and betablockers are present in groundwater close to those small rivers (Fig. 2).

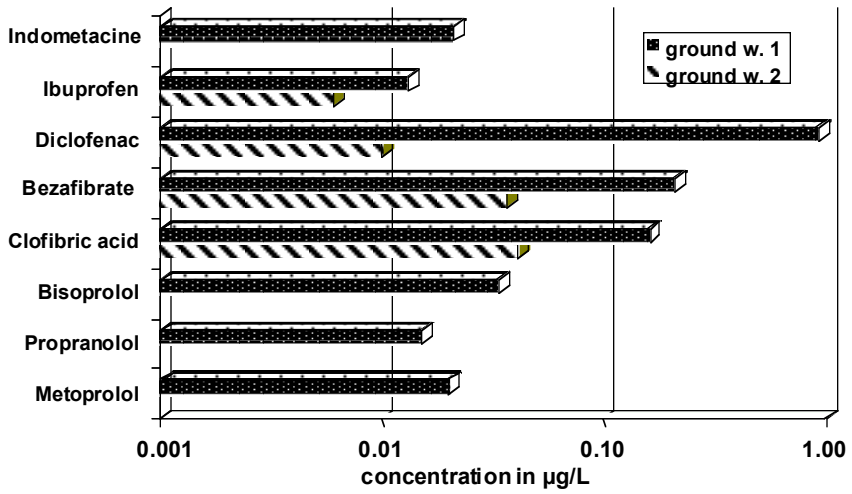

Fig. 2: Concentrations of pharmaceuticals in two groundwater samples from the vicinity of a highly polluted stream (groundwater 1: ca. 20 m; groundwater 2 ca. 100 m).

Fig 3-5 summarizes the concentration levels of the various medicinal classes in the groundwater sites investigated. The selected sites are predominantly influenced by infiltration of polluted water from small creeks which are located in their vicinity. Betablockers were detected in 22 % of the sites with concentration levels between 0.01-0.05 µg/L. However, 4 % exhibited contamination between 0.1-0.5 µg/L. The main detected pharmaceuticals are lipid regulators, antiphlogistics and their corresponding metabolites. Only 32 % of the groundwater sites were non polluted, which means that the remaining 68 % contained appreciable amounts of pharmaceuticals. Even 1 % exceeded 5 µg/L, mainly influenced by landfill sites. The total sum of the detected pharmaceuticals underlined the importance of lipid regulators and antiphlogistics for contamination.

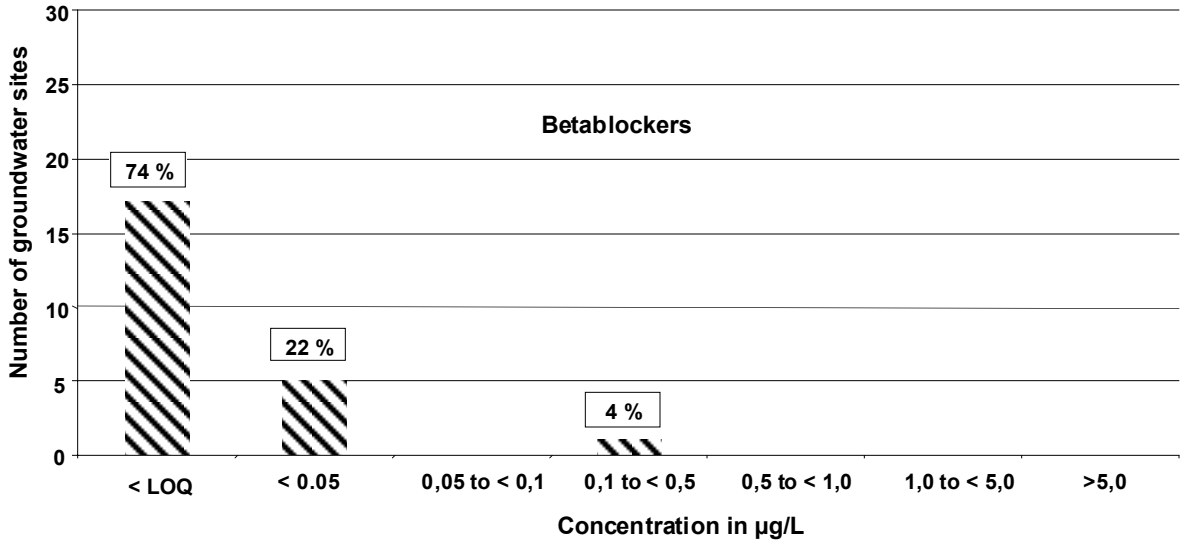

Fig. 3: Sum of betablockers in groundwater samples of 23 sites

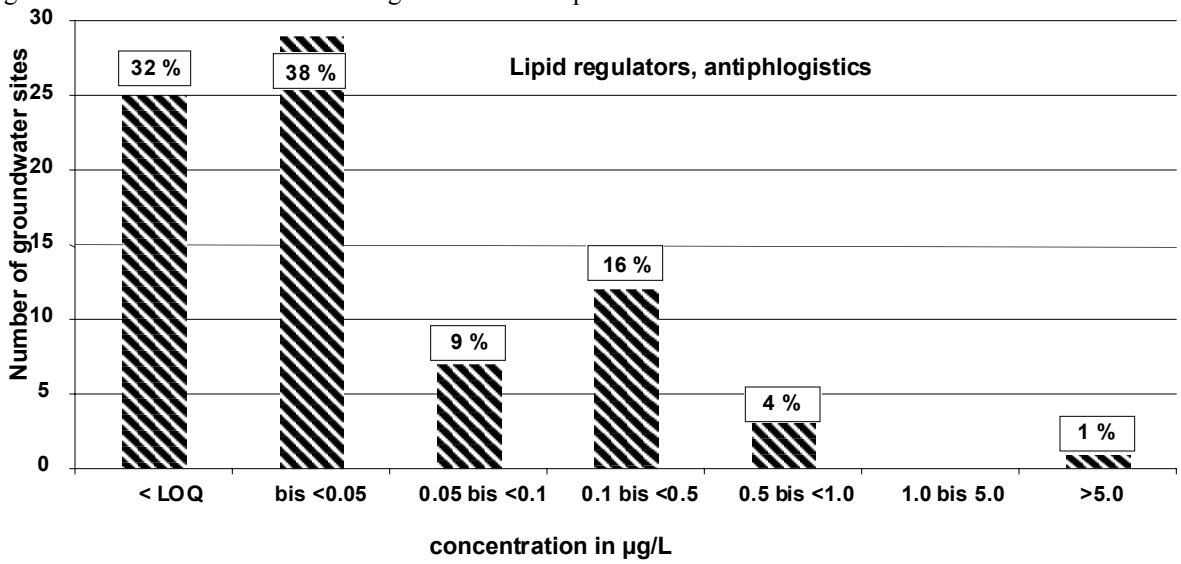

Fig. 4: Sum of lipid regulators and antiphlogistics in groundwater samples of 77 sites

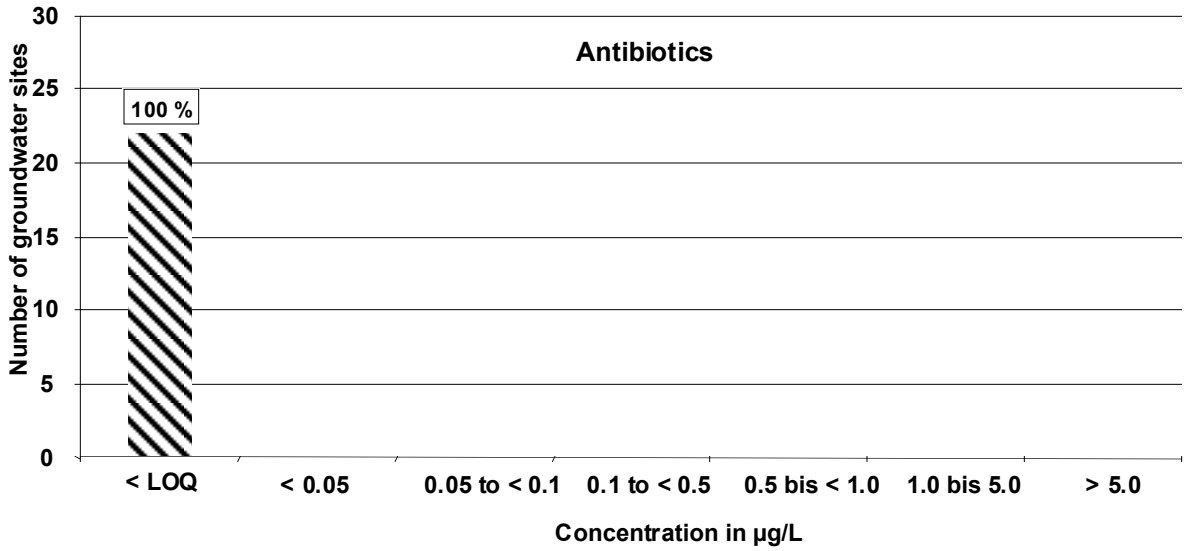

Fig. 5: Sum of antibiotics in groundwater samples of 22 sites

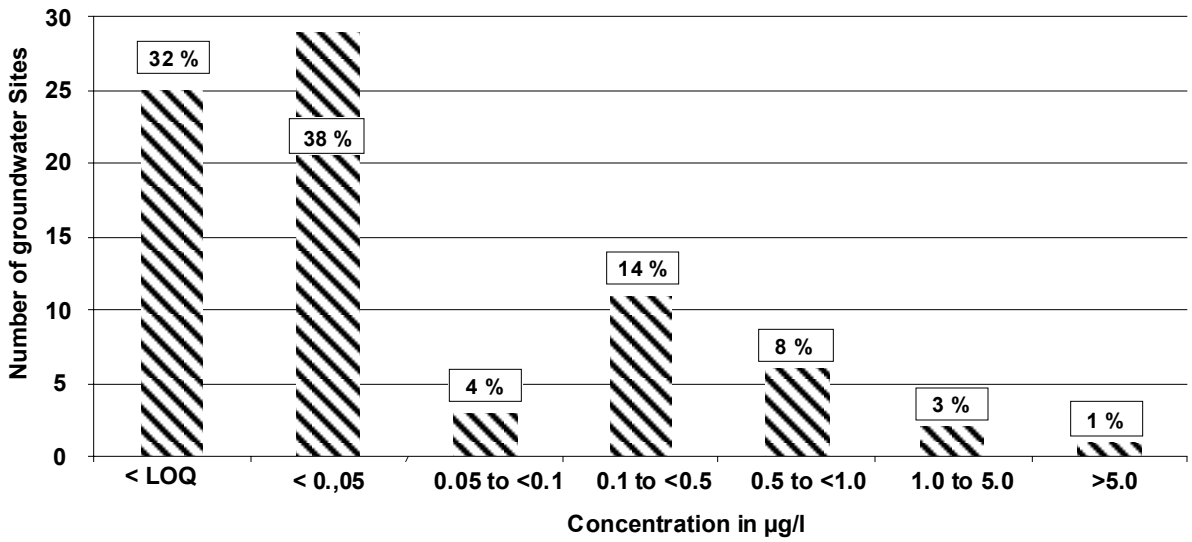

Fig. 6: Sum of pharmaceuticals in groundwater samples of 77 sites

Drinking water

In general, the target pharmaceuticals could not be identified in drinking water. In a few cases, pharmaceuticals were found in drinking water but always in concentrations below 0,10 µg/L. In drinking water, only 10 of 69 target pharmaceuticals were found (Table 2). Mainly clofibric acid and iodinated contrast media were detected above the LOQ. Applying ozone seems to be a powerful technique to remove many of the pharmaceuticals. Carbamazepine was totally eliminated, and diclofenac, bezafibrate, and clofibric acid were predominantly eliminated. Additionally, in waterworks the efficiency of granulated activated carbon (GAC) filtration for the removal of carbamazepine, diclofenac, and bezafibrate could be observed (*Ternes et al. 2002*). A comparison between the minimal daily dosis administered for therapeutic purposes (TD_{th}) with the maximal dosis taken by the consumption of drinking water (TD_{DW}) exhibited that the security factor defined as TD_{th}/TD_{DW} range from 3.5×10^5 to 1.1×10^8 (Ternes, 2001). For TD_{DW} the highest pharmaceutical concentrations ever found in drinking water was used. Thus, the probability that the therapeutic effects will be occur by drinking water consumption can be nearly ruled out.

Table 2: Comparison of administered minimal dosis TD_{th} for therapeutic purposes (Rote Liste, 1996; Forth *et al.,* 1996) with the maximal dosis taken by the consumption of drinking water (TD_{DW})

Substance	Daily dosis TD_{th} (therapeutic) in mg/d	Drinking water maximal detected concentration in µg/l	Daily dosis TD_{DW} by drinking water consumption in µg/d	security factor TD_{th}/TD_{DW}
Diatrizoate	ca. 30000	0,089	0,27	$1{,}1\times10^{8}$
Clofibric acid	250	0,27	0,81	$3{,}6\times10^{5}$
Bezafibrate	200	0,027	0,08	$2{,}5\times10^{6}$
Diclofenac	25	0,006	0,02	$1{,}3\times10^{6}$
Ibuprofen	200	0,003	0,01	$2{,}0\times10^{7}$
Fenofibric acid	100	0,042	0,13	$0{,}8\times10^{6}$
Carbamazepine	200	0,030	0,09	$2{,}2\times10^{6}$

Summary and Conclusions

In general the mean levels of contamination are about one orders of magnitude higher than in the groundwater. The infiltration of highly polluted rivers is a major source for groundwater pollution with drug residues. Especially, small rivers used as receiving waters of STPs contribute mainly under infiltration conditions.

In addition to the small rivers leakages in sewer drains result in concentration levels up to several micrograms. However, these are local contamination in contrast to the infiltration of rivers over a long line.

An important source for groundwater contamination can be seen in leackages of sewer drains of cities, since the raw wastewater with elevated concentrations are directly introduced into the aquifer.

Polar pharmaceutical residues will enter groundwater on appreciable routes and leads to sometimes enormous concentrations up to 4 µg/l. Due to the low microbial activity in groundwater the pollution will probably remain for a long time. Thus, the use of those groundwater for drinking water production requires treatment to remove or totally eliminate the drug residues to prevent drinking water contamination. Since currently waterworks which are using groundwater as feeding water the applied treatment processes consist only few steps such as flocculation or de-acidification, an appreciable reduction of the pharmaceuticals cannot be expected. For those waterworks a contamination of the drinking water cannot be ruled out, if the raw water exhibit a pollution by pharmaceuticals.

While lipophilic compounds accumulate in fish, sediment etc., we have to notify that polar persistent compounds such as pharmaceuticals will not disappear from the environment, they end up in polar matrices such as sea water and groundwater. Therefore there is a urgent necessity to control the mass flow of pharmaceuticals into the environment otherwise we have to treat and purify our essential groundwater resources in the future for elevated extent. Further, we should be aware that the contamination we are currently detect in groundwater is caused by approximately the last 50 years. What will be the groundwater levels in 500 years, if the medicinal development will further growing?

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2001 and 2002 Conferences

Ground Water Expo Petroleum Hydrocarbons and Organic Chemicals
in Ground Water: Conference and Exposition
November 14-16 ◆ Houston, Texas

Annual Meeting and Conference
December 7-9 ◆ Nashville, Tennessee

Fractured Rock Aquifers 2002
March 13 –15, 2002 ◆ Denver, CO

Midwest FOCUS Conference
April 11-12, 2002 ◆ Chicago, IL

MTBE in Ground Water: Assessment, Remediation Technologies, and Public Policy Conference
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