Environ Sci Pollut Res (2010) 17:250–260
DOI 10.1007/s11356-009-0234-1

CHEMICAL AND BIOLOGICAL ENVIRONMENTAL MONITORING • SERIES • RESEARCH ARTICLE

Effect-related monitoring: estrogen-like substances in groundwater

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Received: 24 February 2009 / Accepted: 9 August 2009 / Published online: 4 September 2009

Abstract
Background, aim, and scope Concentration monitoring as a basis for risk assessment is a valid approach only if there is an unambiguous relation between concentration and effect. In many cases, no such unambiguous relation exists, since various substances can exert the same effect with differing potencies. If some or all of these substances contributing to a biological effect are unknown, effect-related monitoring becomes indispensable. Endocrine-disrupting substances in water bodies, including the groundwater, are a prominent example of such a case. The aim of the investigations described here was to detect hormonally active substances in the groundwater downstream of obsolete landfills by using the E-screen assay and to possibly assign the biological effect to individual chemical compounds by means of instrumental analyses carried out in parallel.
Materials and methods Grab samples of the groundwater were collected downstream from abandoned landfills and prepared by liquid/liquid extraction. The total estrogenic activity in these samples was determined in vitro by applying the E-screen assay. The human breast cancer cells (MCF-7) used in the E-screen proliferate in response to the presence of estrogenically active compounds. Expressed in concentration units of the reference substance 17β-estradiol (E2), the test system allows the quantification of estrogenicity with a limit of detection (LOD) in the range of 0.1 ng/L. Aliquots of the samples were screened using gas chromatography/mass spectrometry (GC/MS) in order to quantify known estrogenically active substances and to identify unknown compounds. Estrogen-positive samples were extracted at different pH values, split into acidic, neutral, and basic fractions and analyzed by GC/MS, searching for individual components that display estrogenic activity.
Results and discussion Estrogenic activity exceeding the LOD and the provisional benchmark of 0.5 ng E2/L was found at three out of seven abandoned waste disposal sites tested. The low concentrations of known xenoestrogens such as bisphenol-A, nonylphenols, or phthalic acid esters determined by GC/MS, however, were not sufficient to explain the detected activity. Neither natural nor synthetic hormones have caused the activity because these chemical structures are readily degradable and cannot persist in abandoned landfills for decades. The highest activity in the E-screen assay was found in the acidic fractions. Hydroxy-polychlorinated biphenyls (PCBs), hydroxylated polycyclic aromatic hydrocarbons (PAHs) and hetero-PAHs, as well as alkylphenols could be identified as further compounds with possible hormonal activity.
Conclusions Estrogenically active substances may occur in the groundwater below obsolete landfills, especially those that contain PCBs or waste from gasworks. These substances are not part of analytical programs routinely applied to contaminated sites and may therefore escape detection and assessment. Analyses using the E-screen assay and GC/MS in parallel have shown that the total estrogenic activity found in groundwater samples is to be ascribed to a

Responsible editors: Heinz Rüdel, Schmallenberg; Winfried Schröder, Vechta; Karl Theo v.d. Trenck, Karlsruhe; Gerhard Andreas Wiesmüller, Münster

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multitude of individual compounds, some of which cannot be quantified due to lack of standard substances or assessed due to lack of a standardized procedure for determination of their estrogenic potency. By comparison with provisional guide values for estradiol (0.5 ng/L) and ethynylestradiol (0.3 ng/L), the damaging potential of the total estrogenic activity in groundwater samples can in fact be assessed, but specific remediation measures are impossible unless the hormonal activity can be attributed to individual chemical substances.

Recommendations and outlook On the one hand, further analyses of samples taken from possible pollution sources should be conducted in order to characterize the extent of groundwater pollution with xenoestrogens. On the other hand, the most potent individual compounds should be identified according to their estrogenic potency. To this end, bioassay-directed fractionation and structure elucidation should be carried out with concentrated samples.

Keywords 17β-estradiol · Contaminated site · Endocrine disruptors · E-screen assay · Gas chromatography/mass spectrometry (GC/MS) · Groundwater · Guide value · Landfill · Xenoestrogens

1 Background, aim, and scope

The ultimate aim of environmental policy is to identify and eliminate disturbing factors that endanger the ecosystem and/or the resources for human life. When the disturbing factor is a chemical substance, its damaging effects are of concern rather than its mere presence. Therefore, concentration monitoring is a valid approach only if there exists an unambiguous relation between concentration and effect. In many cases, no such unambiguous relation exists, since various substances can exert the same effect with differing potencies. If some or all of these substances contributing to the biological effect are unknown, effect-related monitoring becomes indispensable. Endocrine-disrupting substances in water bodies, including groundwater, are a prominent example of such a case.

Approximately 2000 abandoned waste disposal sites are suspected to have dangerous effects on groundwater quality in the German state of Baden-Württemberg alone. Specific disposal characteristics are often unknown, and observation is based on guide values for conventional pollutants (BMU 1999).

The aim of the investigations described here was to detect hormonally active substances in the groundwater downstream of obsolete landfills by using the E-screen assay and to assign the biological effect to individual chemical compounds by means of instrumental analyses [high resolution gas chromatography (GC) coupled with mass selective detection (MS)] carried out in parallel.

2 Materials and methods

2.1 Selection of samples

For this purpose, the State Institute for Environment, Measurements and Nature Conservation of the German state of Baden-Württemberg (LUBW, formerly LfU BW) commissioned the company ARCADIS, Karlsruhe and the University of Stuttgart to collect groundwater samples from some disused landfills in the state and to analyze them for estrogenic activity and for organic chemicals known to display such activity. Originally, the sites had been exploited as gravel pits and were later used to deposit excavated soil, building debris, domestic, commercial, and industrial waste (starting before World War I and continuing well into the 1970s). All of the selected deposits are devoid of bottom sealing.

Criteria for the selection of the landfills were as follows: availability of best possible information from previous investigations concerning the pollution potential, and the pollutant emissions, a broadest possible range of different chemicals deposited (ammonium as indicator of domestic waste, aromatic hydrocarbons, polycyclic aromatic hydrocarbon (PAH), phenols, plasticizers, volatile chlorinated hydrocarbons), differing pollutant sources, and appropriate sampling points. Seven locations were selected according to the criteria indicated above. Samples were collected from the seven sites in the year 2003, and the locations displaying estrogenic activity as well as one negative reference site were sampled again in 2004. These repeated samples were fractionated to narrow down the number of possible compounds and investigated by appropriate trace analytical methods to pinpoint individual active ingredients.

2.2 Description of the sites sampled during the second year

Site 1 contains a deposited volume of 90,000 m^3 (see Table 1). Between 1925 and 1956 especially waste from gasworks and benzene production and from the US armed forces was deposited in a former sand pit. In the groundwater, the values for PAH, phenols and aromatic hydrocarbons exceed the guide values (BMU 1999).

At **site 2**—a former gravel pit with 80,000 m^3 of deposited volume—polychlorinated biphenyls (PCBs)-containing capacitors were deposited among other objects between 1958 and 1972. Accordingly, PCBs could be determined in the lower microgram per liter range in the groundwater.

Site 3 contains the largest volume of 290,000 m^3. Domestic, commercial, and industrial waste (70%) as well

Table 1 Estrogenic activity in groundwater samples

Site	Deposition characteristics	Sampling well	EEQ [ng/L] (RPE[a])				
			2003 Total sample	2004 Total sample	2004 Acidic fraction	2004 Neutral fraction	2004 Alkaline fraction
1	Waste from gasworks, benzene production	P1	n.d.	–	–	–	–
		P3	2.42 (25.8%)	1.78 (23,7%)	1.33 (27.8%)	n.d.	n.d.
2	Capacitors containing PCBs	P3	2.12 (34.3%)	–	–	–	–
		P4	2.46 (73.1%)	1.19 (74.8%)	0.88 (82.2%)	0.29 (68.4%)	n.d.
3	Excavated soil and building debris (30%), domestic, commercial and industrial waste (70%)	B3	2.03 (22.1%)	2.07 (26.7%)	2.86 (33.6%)	n.d.	n.d.
		B4	n.d.	–	–	–	–
4	Excavated soil, building debris, domestic and commercial waste, sewage sludge, motor vehicle waste	B2	n.d.	n.d.	n.d.	n.d.	n.d.

EEQ 17β-estradiol equivalents, *n.d.* not detected, (–) not measured

[a] RPE, relative proliferative effect: <10%: not determinable; 10%–25%: weak agonist; 25%–80%: partial agonist

as excavated soil and building debris (30%) were deposited here starting before World War I and again from 1965 to 1976.

These three sites that tested positive for estrogenic activity in 2003 and one negative site for comparison (**dumpsite 4**) were reinvestigated in a new sampling campaign in the following year. This site contains 55,000 m³ of excavated soil, building debris, domestic and commercial waste, sewage sludge, and motor vehicle waste deposited from the early 1950s to round about 1974. Site 4 was selected because of its conspicuous ammonium concentrations in the groundwater of up to 14 mg/L.

2.3 Sample preparation for the E-screen assay

Grab samples of the groundwater were collected at recorded observation wells. After the onsite determination of typical indicator parameters (temperature, pH, conductivity, and visual characterization), the samples were transported in amber glass bottles with ground-glass stoppers at 4°C; sample preparation was performed immediately after delivery. The samples (1 L) were acidified (sulfuric acid, pH 2.5) and extracted twice with a mixture of *n*-heptane and diethyl ether (80 mL, 80:20 *v*/*v*). The combined organic extracts were dried (anhydrous sodium sulfate), rotavaporated to a volume of 2 mL, and transferred into 5-mL vials with screw caps. After adding 50 µL dimethyl sulfoxide (DMSO) as solubilizing agent, the extracts were evaporated to 50 µL (nitrogen stream, 40°C). During the second year, aliquots of the samples were fractionated into acidic, neutral, and basic portions in order to facilitate the allocation of an activity to a certain group of substances and in order to preclude interfering effects of other groups of substances. The acidified ground water sample was extracted with *n*-heptane/diethyl ether as above. After separating the organic extract containing acid and neutral compounds (acidic/neutral fraction), the aqueous phase was rendered alkaline (pH 11) and extracted twice with 50 mL of toluene (basic fraction). To separate acid and neutral compounds of the *n*-heptane/diethyl ether phase, a liquid/liquid extraction with aqueous potassium hydroxide (2 M, 2×20 mL) was performed. Phenolic and acid substances are dissolved in the aqueous phase; neutral compounds remain in the organic solvent (neutral fraction). The acid fraction was obtained by extracting the alkaline phase with toluene after acidification. All the fractions were treated as described for the whole samples. Demineralized and tap water were assayed as blank values along with groundwater samples. In both cases, no estrogenic effects were observed.

2.4 E-screen assay

The extracts of the groundwater samples were tested in vitro by applying the E-screen assay, developed by Soto et al. (1995), following the descriptions of Körner et al. (1999a) with modifications (Schultis 2005). The human breast cancer cells (MCF-7) used in this in vitro test system proliferate in response to the presence of estrogenically active compounds. The proliferation depends on estrogen receptor activity. In contrast to other test systems, the E-screen determines a biological endpoint. Estrogenically active substances not only need to bind to the estrogen

receptor but have to trigger consecutive events also stimulated by natural hormones. The MCF-7 cells were maintained under saturated humidity in Dulbecco's modified Eagle's medium with 5% fetal calf serum (37°C, 5.0% CO_2) and passed weekly. To start the assay the cells were trypsinized, counted, and plated into 96-well tissue culture plates (approximately 2,300 cells per well). One day after seeding, the culture medium was replaced by Dulbecco's experimental medium without phenol red [charcoal dextran-treated fetal calf serum (CDFCS) media]. The fetal calf serum present in the experimental medium is stripped of steroids using charcoal-dextran. To obtain the standard dose–response curve, the cells were exposed to a dilution series of the natural hormone 17β-estradiol (10^{-14}mol/L–10^{-10}mol/L, eight wells per concentration). The MCF-7 cells of one column (eight wells) were spiked with experimental medium only as control. For assaying the samples, the extracts (in DMSO) were diluted in 5 mL CDFCS medium and dilution series added to the cells. The range of concentrations applied to the cells was adapted to obtain complete dose–response curves (approximately from 0.1-fold up to 40-fold of the samples). The cells were incubated for 5 days, after which they were washed with phosphate-buffered saline buffer, fixed with trichloroacetic acid (10%, on ice, 30 min), rinsed with water, and dried at 40°C. The cell protein was stained with sulforhodamine B as described by Shekan et al. (1990). After 10-min incubation, the excess dye was washed off with acetic acid (1%). Dried again, the dye was resuspended in Tris buffer (10 mM, pH 10.5, 100 µL per well). The extinction was measured with a microplate reader at 550 and 630 nm; calculation and analysis of the dose–response functions was performed using the program TableCurve 2D.

2.5 Calculation of estrogenic activity

The estrogenic activity of a test substance can be calculated via the inflection points (EC_{50}) of the dose response curves measured for the standard 17β-estradiol (E2) and the test substance. Provided that concentration and molecular weight of a test substance are known, dimensionless 17β-estradiol equivalent factors (EEF) can be determined:

$$\mathrm{EEF} = \mathrm{EC}_{50}(\mathrm{E2}, \mathrm{mol/L})/\mathrm{EC}_{50}(\text{test substance}, \mathrm{mol/L}) \quad (1)$$

The EEF of the standard 17β-estradiol is defined as unity, comparable to the definition of dioxin toxic equivalents in relation to the most toxic congener 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Using EEFs and concentrations of single compounds, estrogenic activities of complex mixtures can be calculated. The additivity of hormonal effects as a basic requirement for this approach is documented sufficiently (Soto et al. 1994, 1995; Körner et al. 1999a,b; Körner 2000). EEFs of xenoestrogens like the 4-nonylphenols, bisphenol-A, or some esters of phthalic acid are in the range of 10^{-4}–10^{-7} (Körner 2000). On assaying mixtures or samples containing unknown or unidentified substances, it is possible to determine 17β-estradiol equivalent (EEQ) concentrations:

$$\mathrm{EEQ} = \mathrm{EC}_{50}(\mathrm{E2}, \mathrm{ng/L})/\mathrm{EC}_{50}(\mathrm{sample}, \mathrm{ng/L}) \quad (2)$$

The estrogenic activity of an environmental sample reflects the sum of effects elicited by a complex mixture. The parameter includes added estrogenic effects of individual active compounds present in the sample but may also be influenced both by anti-estrogenic and general toxic effects. Activities detected with the E-screen do not provide any information on the chemical structure or the concentration of compounds present. The EEQ only describes the activity determined in a sample in concentration units of the standard 17β-estradiol (E2). The lowest EEQs detectable with the E-screen are in the range of 0.1 ng/L [limit of detection (LOD)].

By comparing the maximal cell proliferations induced by a test substance and the standard E2, their relative proliferative effect (RPE) is calculable:

$$\mathrm{RPE} = [\mathrm{proliferation}_{\max}(\mathrm{sample})/\mathrm{proliferation}_{\max}(\mathrm{E2})]\ 100\% \quad (3)$$

The RPE defines total agonists of E2 between 80% and 100% relative proliferation. Partial and weak agonists induce a cell proliferation from 25% up to 80%, or 10% to 25%, respectively. Examples of total agonists are natural and synthetic hormones and derivatives (estrone, ethynylestradiol, etc.). Most xenoestrogens (bisphenol-A, 4-nonylphenols, etc.) are partial agonists of the standard E2. RPEs <10% are defined as not significant (no estrogenic effect). As noted above, the proliferation determined in environmental samples may reflect the sum of estrogenic, anti-estrogenic, and toxic effects.

2.6 Sample preparation for GC/MS analysis

The groundwater samples assayed with the E-screen were analyzed in parallel using gas chromatography/mass spectrometry (GC/MS). In contrast to the E-screen sample preparation, internal standards were added to the groundwater. The extracts obtained by liquid/liquid extraction were dried with anhydrous sodium sulfate, rotavaporated to 2 mL, transferred into 5-mL vials with screw caps, and concentrated to a volume of 100 µL (nitrogen stream, 40°C) prior to GC/MS analysis. The online derivatization reagent trimethylsulfonium hydroxide (0.5 M in methanol) was used for methylation of the acidic fractions containing phenolic compounds and acids. Analyses were carried out using an Agilent 6890 gas chromatograph directly coupled with an

Agilent 5973N mass selective detector. Chromatographic separation was performed on a DB-5 ms column (30 m× 0.25 mm×0.25 µm, Agilent J&W). The extracts were analyzed both in single ion monitoring mode and in scan mode (*m*/*z* 50–650). Quantification of target compounds was carried out via external calibration (10 points) for bisphenol-A and tetrabromobisphenol-A employing internal standards and using the isotope dilution method. The threefold standard deviation of the background noise was chosen as LOD, and the sixfold standard deviation of the background noise as limit of quantitation (LOQ).

3 Results and discussion

3.1 Findings with the E-screen assay and instrumental analysis

In the course of the first sampling campaign, an estrogenic activity exceeding the detection limit of the assay could be determined in the groundwater downstream of three out of seven selected landfills. This activity expressed as EEQ was found at a concentration around 2 ng/L, a range that was also determined in the effluents of municipal sewage treatment plants (STPs) or in small rivers serving as receiving waters (Bolz 2000; Körner 2000; Kuch and Ballschmiter 2001; Kuch et al. 2003). The groundwater samples that tested positive were taken from sites with widely differing deposition characteristics (Table 1).

In the second campaign, the results of the previous year could be confirmed (see Table 1). In the total samples from sites 1, 2, and 3, estrogenic activities between 1.2 and 2.1 ng EEQ/L were determined. The groundwater underneath the reference site 4 again displayed no activity. Fractionating of the samples demonstrated the estrogenic activity to reside mainly in the acidic fractions. This indicates that the detected activity was caused by phenolic and/or acidic compounds. Only the sample taken from site 2 displayed estrogenic activity in the neutral fraction.

The RPE is a measure for the magnitude of the response of the tested substances relative to the reference substance 17β-estradiol (=100%). Three samples showed RPEs between 22% and 34%; the response magnitude of their ingredients thus corresponds to that of weak agonists. An RPE of 73% was identified in only one sample (P4) from site 2. This sample, therefore, displayed a response magnitude somewhere between those of partial and full agonists of the reference substance. The measurements conducted in the following year revealed RPEs in the same range, and sample P4 from site 2 again showed an RPE corresponding to the presence of partial to complete agonists. As noted above, the proliferation determined in environmental samples may reflect the sum of estrogenic, anti-estrogenic, and toxic effects. Assignment of these effects to specific classes of agonists is not possible.

Parallel to measuring the estrogenic activity, GC/MS investigations were already carried out during the first sampling campaign and successfully demonstrated the presence of industrial chemicals well known for their hormonal activity, such as bisphenol-A, 4-nonylphenols, and the ubiquitous phthalic esters (Table 2). The concentrations of the xenoestrogens bisphenol-A or the 4-nonylphenols amounted up to 330 or 460 ng/L, respectively, in the present study. Especially in the case of the phthalic esters as well as of the polymer starting material bisphenol-A, one has to be aware of possible high blank values. A contamination with these compounds may occur during the various steps of sampling as well as clean up.

These compounds display EEF values of the order of 10^{-4} to 10^{-7} and are thus less active than steroid hormones by several orders of magnitude (Körner 2000). The EEQ can be calculated from the concentrations of the measured compounds and their EEF values. For example, in order to explain the total activity of 2 ng EEQ/L solely through the presence of the potent xenoestrogenic "4-nonylphenols," these would have to occur in concentrations of 20,000–50,000 ng/L. An estimation of the EEQ from the sum of the single activities of the known xenoestrogens identified in the samples shows that the concentration of these xenoestrogens was far from sufficient to explain the findings established with the E-screen assay.

GC/MS screening showed that the total samples from **site 1** contained a great number of PAH, hetero-PAH, and phenolic compounds that can be classified as potential estrogenic compounds solely on the basis of structural analogies with known xenoestrogens or do indeed display a documented estrogenic activity (Santodonato 1997; Khim et al. 2000; Machala et al. 2001; Fertuck et al. 2001; Yang and Yin 2002; Villeneuve et al. 2002). In the acidic fraction hydroxybiphenyls, naphthols, alkylnaphthols, hyroxydibenzofurans, hydroxyfluorenes, and hydroxycarbazoles could be identified above all and, in lesser quantities, cresols and other alkylphenols besides a multitude of other phenolic components. In this context, alkylphenols, hydroxydibenzofurans, hydroxybiphenyls, and other hydroxy-PAH warrant particular mention. The sum of the concentrations of the three isomeric hydroxybiphenyls (2-, 3-, and 4-hydroxybiphenyl) alone amounted to 1.8 µg/L, the known estrogen 4-hydroxybiphenyl contributing 1.2 µg/L. The estrogenic activity of 1.3 ng EEQ/L in the acidic fraction (see Table 1) can be caused by the numerous phenolic components. However, it is impossible to assign this finding to certain single compounds because the estrogenic potencies of the identified components are unknown or only assumed. The neutral and basic fractions from site 1 displayed no estrogenic activity in the E-screen assay.

Table 2 Compounds identified by GC/MS analysis in groundwater samples tested for estrogenic activity in 2004

Site	Conventional parameters from previous groundwater monitoring	Sampling well	Instrumental analysis – Identified compounds		
			Acidic fraction	Neutral fraction	Alkaline fraction
1. Sand pit	High PAH, hetero-PAH, phenols and aromatic hydrocarbons in the groundwater downstream	P3	Alkylphenols (chain C_1, C_2, C_3), hydroxybiphenyls (2-, 3- and 4-hydroxybiphenyl=1.8 µg/L), 1-naphthol, 2-naphthtol, alkylnaphthols (C_1, C_2, C_3), hydroxydibenzofurans, hydroxyfluorenes, hydroxycarbazols	PAHs and alkylated PAHs, dibenzofuran, dibenzothiophene, carbazole, benzo-naphthofuranes, benzo-naphthothiophens, benzo-naphthocarbazoles	Naphthylamines, 4-aminofluorenone, traces of *N*-heterocyclic compounds such as carbazole and methylcarbazoles
2. Gravel pit	High PCBs in the groundwater downstream	P4	4-hydroxybiphenyl, Cl_1–Cl_3 -hydroxy-PCBs (ca. 1.9 µg/L), Bisphenol-A (507 ng/L), Bisphenol-F (193 ng/L), phthalic acid (280 ng/L), terephthalic acid (50 ng/L), 4-hydroxy-benzoic acid (1100 ng/L), (chlorinated) biphenylcarboxylic acids	PCBs (Cl_1–Cl_4, 940 ng/L)	*N*-butyl-benzene sulfonamide, *N*-(3-chloropropyl)benzene-sulfonamide, propyphenazone
3. Landfill	High ammonium and DOC in the groundwater downstream	B3	Aliphatic dicarboxylic acids, phthalic acid, terephthalic acid, benzene polycarboxylic acids, Bisphenol-A (290 ng/L), butylated hydroxytoluene (BHT, 422 ng/L), 4-*t*-butylphenol, dehydroabietic acid	Phthalic acid esters, mineral hydrocarbons, PAHs	–
4. Dumpsite	High NH_4^+-concentrations up to 14000 µg/L indicating oxygen consuming degradation of organic material	B2	Bisphenol-A (80 ng/L), BHT (72 ng/L)	Cholesterol, cholesta-3,5-diene, cholest-4-en-3-one, 4-hydroxy-benzoic acid methyl ester	aromatic sulfonamides, propyphenazone

Fractions devoid of estrogenic activity: **site 1**, in the neutral fraction PAH, hetero-PAH (benzo- and naphtho-condensed furans, thiophenes, and pyrrols), alkylated PAH, etc., were identified. These substances can be regarded typical for deposits, which accrue from the processing of anthracite/tar. As true bases, the naphthylamines and 4-aminofluorenone were accumulated in the alkaline fraction. Nitrogen heterocycles such as carbazole and methylcarbazoles were carried over into the basic fraction of the groundwater sample P3 to a minor extent. **Site 2**, the alkaline fraction did not display estrogenic activity. **Site 3**, in the neutral fraction only mineral oil hydrocarbons and PAH in low concentrations, and the ubiquitous plasticizers could be detected. In the basic fraction no conspicuous or potentially estrogenic compounds could be identified. **Site 4**, neither the total samples nor the fractions from site 4 contained any estrogenic activity

The extracts were specifically searched for the presence of different pharmaceutically active ingredients and industrial chemicals with known estrogenic activity. Examples are diclofenac, ibuprofen, propyphenazone, and clofibrate, whose presence is characteristic of more recent disposal sites. Modern pharmaceutically active ingredients were not assumed to be detectable in the old waste deposits investigated in this study. The presence of modern pharmaceuticals would have to be taken as indicating a more recent contamination, as would the presence of natural or synthetic hormones used as contraceptives. Moreover, their presence could indicate the mixing of different groundwater currents. Compounds with proven estrogenic activity such as 4-*t*-octylphenol, various stabilizers, and additives such as butylhydroxyanisole were also specifically analyzed. Disinfectants such as triclosan selected for this study also constitute a typical micro-contamination of sewage treatment plant (STP) effluents, surface waters, but also leachates of dumpsites. These substances can be demonstrated in various compartments of the environment as ubiquitous contaminants but were absent in our samples

Site 2 was selected among other reasons because of the lower µg/L concentrations of PCBs present in the groundwater (see Table 2). In the GC/MS investigations, the neutral fraction from sampling position P4 (site 2) was also essentially dominated by the occurrence of PCBs. Their concentration as the sum of all detected PCBs amounted to 940 ng/L with prevailing frequencies of the lower chlorinated congeners ($Cl_{n<4}$) and a maximum at the dichlorobiphenyls. Interestingly, an estrogenic activity (0.29 ng/L) could be determined in the neutral fraction of the groundwater sample P4 from site 2, as opposed to the samples taken at the other sites (see Table 1). The activity in this sample may possibly be caused by the presence of PCBs, which are known from several analyses to display estrogenic activity (Gülden et al. 1997). Hydroxylated degradation products of PCBs, especially hydroxy-PCBs with two chlorine atoms, could be identified in the acidic fraction besides bisphenol-A and bisphenol-F. The non-chlorinated 4-hydroxybiphenyl and the mono- and trichlorinated hydroxybiphenyls were also detectable in lower concentrations. The polychlorinated hydroxybiphenyls are toxic substances whose occurrence at least in the aquatic environment is attributed to the degradation of polychlorinated biphenyls (Safe et al. 1998; Hovander et al. 2002). Besides a weak estrogenic activity, an interaction with regulation cycles controlled by thyroid hormones could also be demonstrated for the hydroxybiphenyls (Gierthy et al. 1997; Kester et al. 2000; Kuroki et al. 2001; Legler et al. 2002; Buckman et al. 2004; Miyazaki et al. 2004; Machala et al. 2004). The concentrations of the hydroxylated PCBs could only be measured through external calibration of 4-hydroxybiphenyl because other standard substances were not available. This semiquantitative procedure, which tends to underestimate the true values, yielded a concentration of 1.9 µg/L for the sum of hydroxy-PCBs. The profile of the hydroxy-PCB homologues largely agreed with that of the PCB homologues found in the neutral fraction. Non-chlorinated, mono-, and dichlorinated biphenylcarboxylic acids could be identified as further PCB derivatives in the acidic fraction of site 2. The estrogenic activity found in the groundwater sample P4 from site 2 can be ascribed with some probability to the PCBs and the sum of the phenolic components with known and assumed estrogenicity (see Tables 1 and 2).

The total samples from **site 3** exhibited estrogenic activities of about 2 ng/L in the E-screen assay in both years. In the acidic fraction, some compounds were identified, which, in groundwater samples from other sites, were present only to a small extent or not at all, such as a number of dicarboxylic acids starting with hexanedicarboxylic acid (adipic acid) up to decanedicarboxylic acid and benzene polycarboxylic acids. The occurrence of bisphenol-A (290 ng/L), phthalic acid, terephthalic acid, butylhydroxytoluene, and alkylphenols like 4-*t*-butylphenol leads to the conclusion that plastic materials or production waste has to be considered as the source. Furthermore, dehydroabietic acid, a resin acid which is used, e.g., in paper production, and miscellaneous homologues were demonstrated. However, the estimated effect caused by the known xenoestrogens is not sufficient to account for the total estrogenic activity of 2.86 ng/L.

Screening of the groundwater sample from the reference **site 4** (no estrogenic activity), in contrast to other samples, revealed the presence of compounds with a steroid structure such as cholesterol, cholesta-3,5-diene, and cholest-4-en-3-one, which are contained in excrement and constitute typical ingredients of municipal sewage sludges. This site was operated from the early 1950s to 1974, and municipal sewage sludges were indeed taken there in 1974 and covered with chlorinated lime. Known estrogenic phenolic compounds, which could be detected, are bisphenol-A and butylhydroxytoluene. Furthermore, miscellaneous aromatic sulfonamides and the active pharmaceutical agent propyphenazone were identified.

3.2 Assessment criteria

Not all sites tested were contaminated by estrogenic activity. No such activity was detected at four other abandoned waste disposal sites. Neither could estrogenic activity be detected by Sonzogni et al. (2006) employing the E-screen assay in groundwater samples taken next to clearly contaminated surface waters or septic effluents. However, in our investigation, a total estrogenic activity in the order of magnitude of 2 ng 17β-estradiol equivalents per liter (EEQ/L) could be established with the E-screen assay at four groundwater sampling wells of three disposal sites (see Table 1).

The detection of an estrogenic activity in the groundwater raises the question of how this finding should be assessed. The minimal requirements needed for any assessment of contaminated environmental media are guide values, which take account of background concentrations; action levels are advantageous but not indispensable (vd Trenck et al. 1994; vd Trenck 1997; vd Trenck and Jaroni 2001; Rahtkens and vd Trenck 2007). A provisional benchmark of 0.5 ng/L was derived for the natural estrogen 17β-estradiol (E2) considering effect levels, assessment factors, background concentrations, and analytical detection limits. The environmental quality standard of 0.5 ng/L proposed by the German Federal Environment Agency (UBA 2007) served as a starting point. UBA reports for E2 a lowest observed effect concentration (LOEC) of 4.7 ng/L for the induction of vitellogenin synthesis in rainbow trout (*Onchorhynchus mykiss*) and divides this LOEC by an assessment factor of 10. A long-term study with fathead

minnows in an experimental lake established 5–6 ng EEQ/L as clear effect level. The male fish in this experiment displayed signs of feminization in conjunction with an increased vitellogenin synthesis. The production of the egg yolk protein vitellogenin in male fish disturbs the function of the kidney, suppresses sperm production, and leads to tissue destruction in the testes. Ultimately, these symptoms caused an almost complete extinction of the population (Kidd et al. 2007). Supportive evidence for a guide value of 0.5 ng E2/L can be derived from results obtained with cell tests. Routledge and Sumpter (1996) and Miller et al. (2001) reported a no observed effect level of 3 ng/L in the recombinant yeast bioassay. Meerts et al. (2001) established an EC_{50} of 2.7 ng/L for E2 binding to the estrogen receptor of a human breast cancer cell line.

EE2 (17α-ethynylestradiol) was reported to be more potent in fish than the natural estrogens estradiol and estrone because it persists longer in the body and accumulates 650-fold in the soft tissues and 10,000-fold in bile. Therefore, a concentration of 0.1 ng/L is sufficient to induce vitellogenin in fish (detection limit of the biological test, Aerni et al. 2004), and 1–10 ng/L can jeopardize reproductive success (Nash et al. 2004). Pickering and Sumpter (2003) report that E2 and EE2 display approximately the same potency in the yeast assay with recombinant human estrogen receptors, but the latter is about tenfold more potent in whole fish. According to data compiled by the German Federal Environment Agency (UBA 2007), the lowest effect concentrations for EE2 were measured in a multi-generation study with zebrafish (*Danio rerio*) for the endpoint "rate of fertilization," the no observed effect concentration (NOEC) being 0.3 ng/L and the LOEC 1.1 ng/L. However, the environmental quality standard of 0.03 ng/L based on this NOEC by applying an assessment factor of 10 is lower than the LOQ (0.1 ng/L) in the biological test (Aerni et al. 2004; Nash et al. 2004) and therefore not practicable. A more relevant provisional benchmark for EE2 can be derived from a life cycle test (289 days) with fathead minnows (*Pimephales promelas*) evaluated by Grist et al. (2003). These authors obtained a NOEC of 0.2 ng/L and a LOEC of 1.0 ng/L for the population growth rate in this experiment. The geometric mean of these two values (0.5 ng/L) approximates the true effect threshold. As there is reason to assume that the effect data include the most sensitive organism (Aerni et al. 2004; Duis and Knacker 2003; Nash et al. 2004), a reduced assessment factor of 2 suffices and yields a figure of 0.25, rounded to 0.3 ng/L, to be recommended as a more feasible provisional guide value for EE2. Several observations support this benchmark: The data assessed by Grist et al. (2003) were taken from a test conducted by Länge et al. (2001) who observed histological changes of the gonads at 4 ng/L (LOEC) but none at 1 ng/L (NOEC histology). An effect threshold of 0.5 ng/L is also obtained as the lower 95% confidence limit of the EC_{20} for the decrease in the population growth rate and is therefore suggested by Grist et al. (2003) as a conservative estimate of a NOEC. A zero population growth rate results at 3.11 ng/L. These results are confirmed by outdoor experiments from Ontario, where the addition of EE2 to a lake eradicated a population of 7,000 fathead minnows. The measured concentration of EE2 amounted to 5–6 ng/L (Pelley 2003). The exposure of zebra fish to 5 ng EE2/L for 210 days decreased the reproductive success by 56% because the males of the F_1 generation showed normal male reproductive behavior but were lacking functional testes because of defective sexual differentiation. The NOEC was 0.5 ng/L (Nash et al. 2004).

3.3 Background levels

Model calculations for the effluent of a typical Swiss STP resulted in an E2 concentration of 1.6 ng/L stemming from human excreta. Actual measurements averaged 2.0 ng/L and thus confirmed the predicted concentration (Suter 2002). Along the same lines, an EE2 concentration of 3.0 ng/L was calculated and confirmed by measurements distributed around a mean of 1.5 µg/L in the STP effluent (Suter 2002). The EEQ of E2, estrone, and EE2 combined (with the natural estrogens predominating) are known to usually range from 1 to 10 ng/L in British STP effluents and from 1 to 5 ng/L in British rivers (EU 2003). No estrogenic activity can be determined in pristine rivers or in uncontaminated groundwater with a LOQ of 0.2 ng/L. For practical reasons, the range of 0.1–0.2 ng EEQ/L has to be considered as background activity because lower concentrations cannot be detected or quantified, respectively.

Therefore, there is little latitude between background activity and the guide values of 0.3 ng/L for EE2 or 0.5 ng/L for E2, respectively. In the case of EE2, the application of a default assessment factor of 10 according to the Technical Guidance Document (EC 2003) is unnecessary because fish are the most sensitive organisms with respect to this synthetic estrogen (Aerni et al. 2004; Duis and Knacker 2003; Nash et al. 2004), and it would render the result useless because it leads to an environmental quality standard below the detection limit.

3.4 Assignment of activity to chemical structure

The approaches undertaken to attribute the determined biological effects to certain chemical compounds or classes of compounds consistently demonstrate the gaps that may open up in assessing a complex contamination via the contributions of individual substances. The present investigations indeed succeeded in identifying compounds that are known to exert estrogenic effects in all samples.

However, the concentrations of these compounds were far from sufficient to explain the positive findings. One must be aware that the instrumental methods applied here (GC/MS) to identify potentially active compounds are subject to a high degree of selectivity in the chromatographic separation and detection of these compounds. Compounds contributing significantly to the total activity may go undetected. This problem, which occurs especially on application of GC/MS methods, has also been described by Hollert et al. (2005), who could explain only 7–15% of the estrogen receptor-mediated vitellogenin synthesis in isolated rainbow trout hepatocytes as being due to known estrogens identified by chemical analyses. This discrepancy is probably a consequence of the inapplicability of GC or thermal instability of the compounds, constricted polarity windows, or insufficient detectable mass range and is essentially inherent to all instrumental methods not only in ultra trace analysis. This is also true of conventional parameters and guide values, which serve as a basis for the evaluation of groundwater pollution. Positive signals or exceeded guide values may indicate biological effects but cannot necessarily be assigned to a certain substance. Biological effects of individual substances can add up to a gross effect, even if the contributing substances occur in concentrations below their LOD or LOQ by instrumental analytical techniques. Kortenkamp et al. (2007) conclude in their comprehensive review that "on the basis of published evidence, it is difficult to rule out the possibility of mixture effects from low level dose multiple exposure" even in the case of dissimilarly acting chemicals. Theoretically, the total estrogenic activity of a sample may indeed be caused by one single substance in a measurable concentration, but it is more likely that the total effect will result from the combined action of a multitude of individual substances possibly occurring in concentrations beyond their limit of determination.

Another obstacle is the fact that specific biological effects have been measured only for a limited number of substances. The activity of all other substances can only be estimated based on structural analogies once they have been identified.

4 Conclusions

The results indicate that the groundwater downstream of landfills (especially those containing PCBs or waste from gasworks) may contain substances that display a biological effect in very low concentrations. These substances usually remain undetected because they are not covered by the analytical program normally applied for the assessment of contaminated sites.

The E-screen assay used here is well validated to quantify 17β-estradiol with a limit of 0.2 ng/L. An estrogenic potential has been demonstrated in the groundwater in some locations, but the substances responsible for this activity remain largely unidentified.

GC/MS analyses applied in conjunction with the E-screen assay have shown that the total hormonal activity is due to a great number of individual substances whose activity is marginal but whose additive action may exceed the guide value based on the effect threshold. Some estrogenic compounds such as PAHs, PCBs, and phenolic compounds were identified. The presence of other, unidentified compounds such as hydroxylated PCBs, hydroxylated PAHs, and heterocycles is likely. Their contribution would be included in the total estrogenic activity. The search for these substances takes instrumental analytical methods such as GC/MS to their limits. Therefore, the samples should be concentrated, especially for the detection of steroid hormones which occur in very low concentrations.

Due to provisional guide values for estradiol and ethynylestradiol, the damaging potential of the total estrogenic activity in groundwater samples can be assessed, the but lack of knowledge of individual compounds contributing to the hormonal activity presently precludes application of specific chemical remediation measures. Many substances could be identified but not assessed, either because their biological effects have not been determined or because effects were determined in different in vivo or in vitro test systems that are not fully comparable.

5 Recommendations and outlook

The contamination of groundwater by emissions of hormone-like substances from obsolete landfills may be significant. Therefore, the extent of the problem should be explored by further analyses of samples taken downstream from possible pollution sources. In choosing further sampling sites the location in water protection areas should be taken into consideration besides the kind of waste deposited. Individual compounds should be identified according to their estrogenic potency. To this end, bioassay-directed fractionation and structure elucidation should be conducted with concentrated samples.

Acknowledgements We are grateful for the expert editorial support by Anthony Rackstraw, Weinheim, and project management by Heike Schuler, ARCADIS CONSULT GmbH, Karlsruhe. The LUBW is acknowledged for financing this project.

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