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Removal of Estrogenic Activity from Municipal Waste Landfill Leachate Assessed with a Bioassay Based on Reporter Gene Expression

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The leachate of a municipal waste landfill was tested for estrogenic activity using a reporter-gene-based bioassay with a human breast-cancer-derived cell line (MVLN cells). The leachate was processed with two different membrane-employing processes operating in parallel. One process consists of aerobic biological degradation, ultrafiltration, and subsequent adsorption to activated carbon. The second process was a reverse osmosis treatment of the raw leachate. Both processes are common in the treatment of landfill leachate. Here, the efficacy of the two processes to remove "estrogenicity" was compared. Both treatment processes removed more than 97% of the estrogenic activity, calculated as estradiol equivalents (EEQs), but they were not equally effective. After adsorption to activated carbon, no estrogenicity was detected, whereas concentrated effluent of the reverse osmosis treatment still elicited an estrogenic response in the bioassay. On the basis of chemical analysis, it is proposed that bisphenol A was responsible for the majority of estrogenic activity in the raw and treated leachate. Although the contribution of treated leachate to the estrogenic load on the aquatic environment seems to be low compared to that of sewage treatment works, the high estrogenic activity in raw landfill leachate stresses the necessity for the appropriate treatment of these leachates.

Introduction

Since Purdom et al. (*1*) first reported estrogenic effects in fish exposed downstream from wastewater treatment plants (WWTPs), much effort has been undertaken to identify compounds potentially interfering with the hormonal systems of both animals and humans. Besides the ongoing identification of these so-called endocrine disrupting compounds (EDCs), the release and fate of EDCs in the aquatic environment has been investigated in several national and international research projects (*2*−*11*). Because sewage effluents are suspected to be the major source for EDCs in the aquatic environment (*11*), the elimination of these substances in WWTPs is of special interest. Results from different countries indicate that WWTPs employing activated sludge processes remove known EDCs from wastewaters to varying degrees. Removal of the natural and synthetic estrogens is generally greater than 85% (*12*). Advanced treatment methods, such as activated charcoal filtration, seem to remove a larger proportion of EDCs compared to that removed by conventional activated sludge treatments (*13*). WWTPs using activated sludge treatment are not only the most often employed systems for the cleaning of municipal sewage, especially in larger cities, but also with regard to EDCs the most often investigated type of treatment plants. However, much less attention has been paid to removal of these compounds from leachates of municipal waste dumping or landfill sites, which are another source of EDCs to the environment. These leachates can contain high concentrations of organic and inorganic compounds, depending on the age of the site and the type of waste deposited (*14*−*17*). Some known xenoestrogens such as bisphenol A (*15*, *16*, *18*−*20*) and nonylphenol (*16*, *19*) have been reported to be present in leachate from municipal landfill sites. Although most landfill sites in Europe possess a bottom sealing to prevent contact with the environment, there are still uncontrolled landfills where the leachate is able to infiltrate groundwater and surface waters. The results of an investigation of the fish crop in a small remote Swedish lake is the first example of a proposed endocrine effect in wildlife caused by the leachate of an uncontrolled municipal dumping site, situated uphill from the lake (*21*). Noaksson et al. (*21*) reported a reduction in the gonadosomatic index in both female and male roach (*Rutilus rutilus*) and perch (*Perca fluviatilis*), reduced plasma steroid levels in female perch and roach, and a high percentage of sexually immature female perch. This example stresses the necessity for leachate control, which is already the target of increasing regulatory activity in Europe (*14*). Landfill leachate is often only pretreated and then discharged to municipal WWTPs for further treatment (*14*). Because of the typically high chemical oxygen demand and low biological degradability, landfill leachate is often treated with a combination of advanced methods (*22*, *23*), e.g., membrane technology, which are less often used in the case of municipal sewage. The leachate investigated in this study was treated in parallel with two different processes, both employing membranes and both belonging to the most often used processes for the treatment of waste landfill leachate in Europe (*14*). The aim of this study was to compare two membrane-based treatment processes for their ability to eliminate EDCs.

Experimental Section

Landfill Leachate Treatment Plant. The leachate used in this study was from a municipal landfill in West Germany which was in operation from 1983 to 1998. The leachate is routinely treated with two different membrane processes in parallel (Figure 1). Each process treats approximately half of the collected leachate, about 5−7 m^3/h per process. One process starts with aerobic biological degradation followed by biomass removal by ultrafiltration. The permeate of the ultrafiltration is further purified by adsorption to granulated activated carbon before discharge to the municipal WWTP. The second treatment process is a two-step reverse osmosis, where the retentate of the first step is further concentrated under high-pressure in a second step. The permeates of both steps are combined and then discharged to the municipal WWTP.

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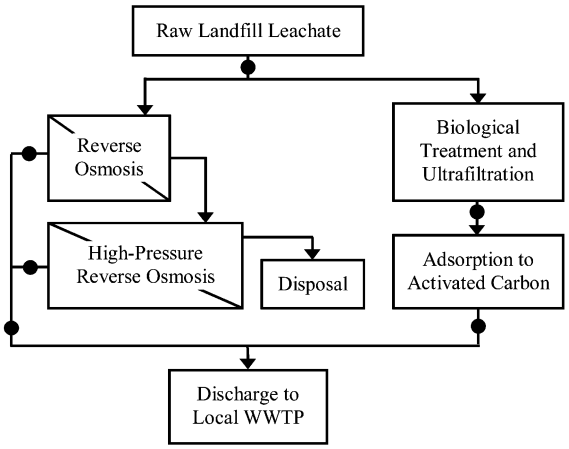

FIGURE 1. Schematic drawing of the two treatment processes for the investigated municipal waste landfill leachate with sampling points (dots).

Sample Collection and Extraction. On five different days in 2001, grab samples of volumes between 250 mL and 1 L were taken at several steps within the treatment processes (Figure 1). The samples were stabilized by addition of 4 M HCl to a final pH of 2, cooled, and extracted on the same day as collection. The extraction was based on that of Körner et al. (*9*−*11*), who used it for the detection of estrogenic activity in wastewater by a bioassay as well as for chemical analysis of xenoestrogens. Raw leachate was filtered through a GF/C glass fiber filter (Whatman), and the filter was washed with acidified water, freeze-dried, and extracted for 4 h with acetone (purum, Fluka, Germany) in a Soxhlet apparatus. Aqueous samples were extracted using solid-phase columns (200 mg Isolute ENV+, International Sorbent Technology, UK) and eluted with acetone. Acetone was removed under a gentle stream of nitrogen, but not to total dryness. The extracts were resuspended in ethanol (absolute, Riedel-de Haen, Germany), filter-sterilized (0.2 μm PTFE filters, Roth, Germany), reduced to a final volume of 1 mL, and stored at −20 °C until analysis. Laboratory blanks (demineralized water, Millipore) were co-extracted on all sampling dates.

Cell Culture and Bioassay. Human breast cancer cells stably transfected with an estrogen-responsive reporter-gene coding for firefly luciferase were used to detect estrogenic activity. These MVLN cells were developed and characterized by Pons et al. (*24*). The suitability of the MVLN-cell bioassay for the detection of estrogenic activity in complex samples such as wastewater, sludge, and sediments has been described in published literature (*25*−*27*). Cell culture and bioassay procedures were based on these previously described methods. Briefly, MVLN cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with Hams F-12 nutrient mixture (Sigma-Aldrich, Germany) supplemented with 10% fetal bovine serum (FBS, Greiner, Germany), 1 mg/L insulin (Sigma-Aldrich, Germany), and 1.0 mM sodium pyruvate (Sigma-Aldrich, Germany). For the bioassay, a suspension containing 75 000 cells/mL was prepared with test medium (cell culture medium supplemented with dextran−charcoal stripped fetal bovine serum (DC-FBS) instead of FBS, Hyclone, Logan, UT). Cells were seeded into 96-well plates with 250 μL of the cell suspension per well. After 24 h incubation the cells were dosed with 2.5 μL of the extracts or dilutions thereof. All samples or dilutions were assessed in triplicate in at least three independent assays. Extracts were diluted with ethanol and tested at least at three different concentration factors in order to achieve dose−response curves in the bioassay. The concentration factor relates the exposure concentration in the bioassay to the original concentration of the samples (concentration factor = exposure concentration/concentration of original sample). Therefore, a concentration factor above one means that the exposure in the bioassay was higher than in the original sample and a concentration factor below one corresponds to the testing of a diluted sample. In every assay, three full dose−response curves were performed with the standard 17-β-estradiol (E2, Sigma-Aldrich, Germany) at seven concentrations ranging from 4.1 to 1000 pM. After an exposure of 72 h, the assay was terminated by removal of the test medium and washing with phosphate buffered saline (PBS) containing 1 mM Ca^{2+} and Mg^{2+}. Plates were kept at 4 °C until luminescence measurement, which was done within 3 h of assay termination. Luminescence was measured with a Spectrafluor Plus (Tecan, Austria) using LucLite cocktail (Packard Instruments). Thereafter, the protein content per well was determined by the method described by Udenfriend et al. (*28*) using fluorescamine. Additionally, a protein standard curve with bovine serum albumin was measured in each assay. The protein content and microscopic inspection of the cells before assay termination were used together to detect outliers and as an index of cytotoxicity (*25*).

Chemical Analysis. Raw leachate and effluents taken at one sampling date in December 2001, in parallel to the sampling for the bioassay, were also chemically analyzed for bisphenol A and nonylphenol. This analysis was performed at the laboratory of the Department of Environmental Engineering at the Aachen University, Germany. The method used for identification and detection by capillary column gas chromatography/mass spectrometry (GC/MS) after derivatization, and subsequent liquid/liquid extraction with cyclohexane, is published elsewhere (*29*).

Data Evaluation and Statistics. Luminescence and fluorescence values were both corrected by subtracting the mean values of the blank controls (at least three blank and three solvent controls per plate). The mean maximum response of the positive control (1000 pM 17-β-estradiol) was set to 100% and the relative light units (RLUs) of all other samples were expressed relative to this maximum response in the same assay (RLU in %E2max). RLUs of the estradiol standard curve were logit- and concentrations log-transformed, and a linear regression was calculated for each assay taking only the three median concentrations into account, which were regularly in the linear range of the dose−response curve. From the equation of this linear regression the estradiol equivalent (EEQ) was calculated for each extract dilution measured in the same assay, and the results from different assays were pooled to calculate the mean EEQ of each treatment step for each sampling date. Only replicates with both an RLU value between 20 and 80% of the maximum response achieved with estradiol and a protein content of more than 75% of the negative control were included in the calculation of EEQs. This method to calculate EEQs was preferred to other methods in which the relation between the EC50 of the standard substance and the EC50 of the sample are reported as EEQ (e.g., refs *10*, and *19*), because EEQ calculation was then also possible for samples which did not reach a response of greater than 50% of the maximum response observed for E2. Still, some assumptions underlying the calculation of relative potencies (*30*, *31*) may be violated by these complex environmental samples, e.g., parallel dose−response curves and equal efficacy of standard and sample. Additionally, multiple comparisons versus solvent control treatments were performed with E2 standard curves and with response curves of the samples to determine minimal concentrations which were different from the controls (analysis of variance with Dunnett's test, or, when normal distribution or equal variances were not given, Dunn's test). To calculate the expected responses in the bioassay based on the determined bisphenol A concentrations, standard curves with bisphenol A (99.9%, Promochem, Wesel, Germany) were performed in

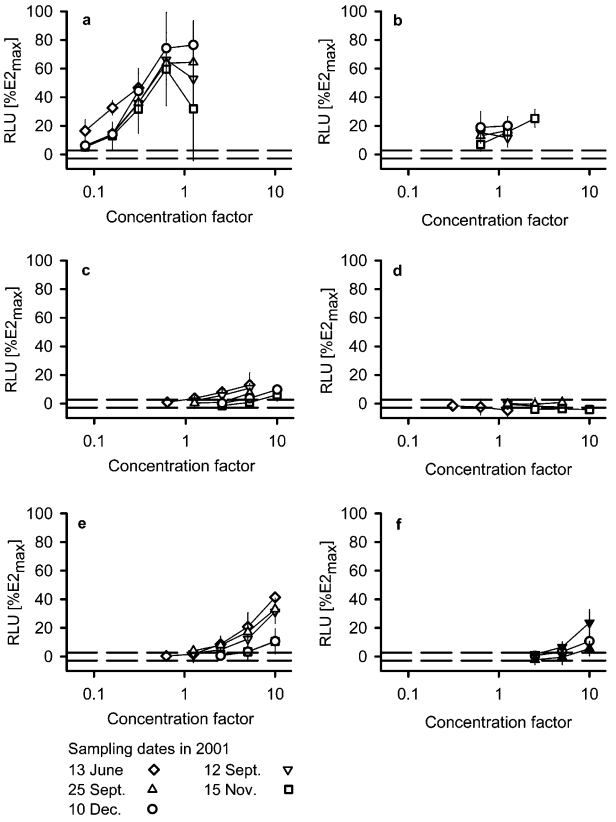

FIGURE 2. Estrogenic activity (luminescence relative to the maximum achievable response with 17-β-estradiol, RLU in %E2max) in raw and treated municipal waste landfill leachate, detected with the reporter gene based MVLN-cell bioassay. One sample per site was taken at five sampling dates in 2001; given are means and standard deviations ($n = 2-4$ independent assays). Dashed lines indicate the 3-fold standard deviation of solvent controls. (a) Solid-phase extracts and (b) Soxhlet extracts of raw leachate, (c) after biological treatment and ultrafiltration, (d) after subsequent adsorption to activated carbon, (e) final effluent of reverse osmosis treatment, and (f) effluent of the first step (triangles up) and the second step (triangles down) and the combined final effluent (circles) of the reverse osmosis process, all sampled in December 2001.

several independent assays and the relative potency (RP) was calculated as RP = (minimum dose of E2 to achieve maximum response)/(minimum dose of bisphenol A to achieve maximum response). All calculations were done with Microsoft Excel 1997, statistics and linear regression were conducted with SigmaStat for Windows 2.03 (Microsoft Corporation).

Results and Discussion

Raw Leachate. Dose-dependent estrogenic activity was detected by the MVLN-cell bioassay in the solid-phase extracts of raw landfill leachate (Figure 2a). The activity was greatest at a concentration factor of 0.63, with a maximum response of 77% of the response achieved with 1000 pM 17-β-estradiol. No full agonistic response could be achieved, probably because of cytotoxicity caused by the undiluted leachate (above concentration factor 1). The variability in estrogenic activity between different sampling dates was low, indicating a uniform leachate composition over time (Table 1). Seasonal effects were unlikely to occur because the leachate of this covered landfill is not influenced by rainfall and the treatment processes are indoors. Soxhlet extracts exhibited estrogenic responses different from those of controls at concentration factors of 0.63 or 1.25 (Table 1). The detected estrogenic activity in the Soxhlet extracts

TABLE 1. Estradiol Equivalents (EEQ in ng/L) Calculated from the MVLN-Cell Bioassay for Raw and Treated Landfill Leachate (the Lowest Concentration Factor with a Reporter Gene Induction Significantly Different from that of Solvent Controls is Given in Parentheses ($p < 0.05$, Dunnett's Test)

	sampling date[a]				
sample	**June 13**	**Sept. 12**	**Sept. 25**	**Nov. 15**	**Dec. 10**
raw leachate					
SPE extract	66.4 (0.08)	48.0 (0.63)	45.4 (0.63)	39.4 (0.63)	56.6 (0.31)
Soxhlet extract	n.a.	8.2 (0.63)	7.5 (n.s.[b])	5.9 (1.25)	10.9 (0.63)
biological treatment and ultrafiltration	2.3 (5.0)	< 0.7 (5.0)	< 0.7 (n.s.)	< 0.7 (10.0)	< 0.7 (10.0)
activated carbon adsorption	< 0.7 (n.s.)	< 0.7 (n.s.)	< 0.7 (n.s.)	< 0.7 (n.s.)	n.a.
reverse osmosis	1.6 (5.0)	1.3 (5.0)	1.5 (5.0)	0.8 (n.s.)	< 0.7 (10.0)
first step	n.a.	n.a.	n.a.	n.a.	< 0.7 (n.s.)
second step	n.a.	n.a.	n.a.	n.a.	1.1 (10.0)

[a] One sample per sampling date was analyzed for each treatment step, unless indicated with n.a.: data not available. [b] n.s.: Not significant up to the highest tested concentration factor.

TABLE 2. Elimination Rates of the Treatment Methods Related to the Total Estradiol Equivalent (EEQ in ng/L) in Raw Leachate Calculated from the MVLN-Cell Bioassay

	sampling date				
	June 13	**Sept. 12**	**Sept. 25**	**Nov. 15**	**Dec. 10**
raw leachate					
total EEQ (sum of both extracts)	≥66.4	56.2	52.9	45.3	67.5
% elimination					
biological treatment and ultrafiltration	≥96.5	> 98.8	> 98.7	> 98.5	> 99.0
activated carbon adsorption	> 98.9	> 98.8	> 98.7	> 98.5	n.a.
reverse osmosis	97.6	97.7	97.2	98.3	> 99.0

demonstrated that estrogenic compounds were either bound to suspended matter in the raw leachate or adsorbed to the filter material during filtration. These results deviate from the findings of Desbrow et al. (*3*) who found no estrogenic activity in particulate matter or glass fiber filters after the filtration of WWTP effluents. The EEQs from both extracts (particulate and dissolved) were added together to calculate the EEQs for the raw landfill leachate (Table 2), resulting in a mean EEQ over all sampling dates of 57.7 ng/L and a standard deviation of 9.4 ng/L. To our knowledge the only other report of estrogenic activity in landfill leachate estimated an EEQ of 4.8 ng/L and a response of 72% of the maximum achievable response with E2 (*19*). Although different bioassays and EEQ calculation methods were used and leachates from different landfills can be expected to be different, the estrogenic activity of leachates from a German and a Japanese waste landfill differed only by a factor of 10. The kind of waste deposited in this Japanese landfill were not reported, but chemical analysis revealed concentrations of bisphenol A and nonylphenol in the lower μg/L range and also detected estradiol in the raw leachate.

Treated Leachate. After aerobic biological treatment and subsequent ultrafiltration, concentrated extracts exhibited low estrogenic activity (Figure 2c). The activity was significantly different from that of controls for four samples at concentration factors of 5 or 10 (Table 1). Only the sample taken in June showed a reporter gene induction high enough

to calculate the EEQ; all other samples were below an EEQ of 0.7, which would correspond to a response of 20% E2max at a concentration factor of 10. After adsorption to activated carbon, the second step in this treatment path, no significant estrogenic activity was found in up to 10-fold concentrated extracts. Consequently, no EEQ could be calculated and the EEQ in Table 1 is given as below 0.7 ng/L. Thus, this treatment process reduced the estrogenic activity of the raw leachate to a level where no estrogenic response could be detected with the MVLN-cell bioassay. Expressed in EEQs, the elimination was above 98.5% at all sampling dates (Table 2). After a comparable treatment of sewage in an activated sludge process and subsequent activated carbon filtration, Körner et al. (*13*) estimated an EEQ of 0.21 ng/L. Our study of landfill leachate treatment confirms these results in that advanced treatment with activated carbon is able to reduce estrogenic activity to a very low level.

The reverse osmosis treatment of raw leachate eliminated at least 97.2% of the EEQ (Table 2). However, there was still estrogenic activity in the final effluent (Figure 2e), because the response of four out of five samples was different from that of control treatments at a concentration factor of 10 and in three samples even at a concentration factor of 5 (Table 1). The EEQs of these effluents were lower than the maximal observed EEQ of the biologically treated leachate, but more samples showed a significant estrogenic activity. The separate sampling of the two treatment steps of the reverse osmosis process revealed that the permeate of the second step, the high-pressure reverse osmosis of preconcentrated leachate, had a higher estrogenic activity than the permeate of the first step and, therefore, contributed more to the estrogenic activity in the final effluent (Figure 2f). The low number of replicates (number of assays in which respective extracts were tested, 3 or 4) hindered in some cases the detection of differences because of low statistical test power. The extraction method used was chosen in order to concentrate all potential estrogenic substances within a broad range of polarity, but the extraction efficiency of the method for known xenoestrogens was not assessed in this study. Therefore, results have not been corrected for recovery, and the data presented may underestimate the estrogenic activity in the original samples. Because of the detection limit of about 10 pM E2 in the bioassay (corresponding to about 0.3 ng E2 in 1 L of effluent sample), very low estrogenic activities may not be found with this method.

Identification of Xenoestrogens in the Leachate. A bisphenol A concentration of 3.61 mg/L was determined in the raw leachate. The previously reported concentrations of bisphenol A in landfill leachates range from nondetectable to 17.2 mg/L with a median concentration of 269 μg/L (*18*). Thus, the concentration in the leachate investigated in this study was in the upper range of concentrations reported for Japanese landfills. The sources of bisphenol A in landfill leachates were proposed to be deposited waste plastics (*15*, *18*), which are also present in this landfill. A full dose−response curve with bisphenol A was achieved in the bioassay and a relative potency (RP) of 7.1×10^{-5} compared to E2 was determined. The lowest detectable bisphenol A concentration in the bioassay was 3.65×10^{-6} M, whereas the lowest tested E2 concentration different from the control was 10.24×10^{-12} M ($p < 0.05$). Maximal induction of the reporter gene occurred at 1×10^{-9} M estradiol (Figure 3) and half-maximal induction (EC50) occurred at 89.0×10^{-12} M with a standard deviation of 19.9×10^{-12} M ($n = 21$ assays). On the basis of the relative potency of bisphenol A, an EEQ of 0.3 μg/L E2 was calculated from the measured bisphenol A concentration. The EEQ of the parallel sample determined by the MVLN-cell bioassay was 65.2 ng/L E2 (Table 2). Although the use of different extraction methods (solid phase extraction and liquid/liquid extraction, respectively) does not allow a direct comparison

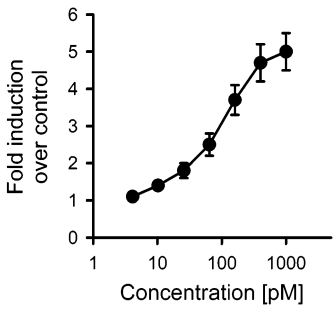

FIGURE 3. Induction of the reporter gene in estrogen-sensitive MVLN cells by 17β-estradiol relative to negative controls. Given are means and standard deviations ($n = 21$).

of bioassay-established EEQ and EEQ predicted from chemical analysis, the amount of bisphenol A present was alone sufficient to have caused the response observed in the bioassay. However, the presence of other estrogenic substances (e.g., phytoestrogens or synthetic estrogens) cannot be excluded, because we could not perform a chemical analysis of steroids in these complex samples. The elution with acetone in the SPE could lead to high amounts of unknown co-extracted substances, which may have suppressed the response in the bioassay. Further experiments are underway to find a suitable method to reduce these presumed matrix effects by a cleanup of the extract without reducing the content of estrogenic substances. In treated leachate, bisphenol A was present only in the permeate of the high-pressure reverse osmosis step at a concentration high enough to expect a response in the bioassay. In this case, the measured concentration of 46.2 μg/L led to a predicted EEQ of 3.9 ng/L E2, whereas the bioassay-derived EEQ was 1.1 ng/L E2. Again, mainly bisphenol A seemed to be responsible for the detected estrogenicity. Nonylphenol, another known xenoestrogen, was not detected in any of the raw or treated leachate samples collected on this date.

Regardless of the actual compounds causing the response, both treatment processes eliminated more than 97% of the estrogenic activity observed in the raw landfill leachate. However, the process using activated carbon adsorption as the final step showed a better performance, reducing the estrogenicity to the level of the negative controls. The remaining EEQ (up to 1.6 ng/L) in the effluent of the reverse osmosis process is below the typical predicted value for effluents from activated sludge treatment works of 5 to 5.5 ng/L (*12*) and slightly below the recently reported median EEQ of 1.9 ng/L in effluents of 12 WWTPs with activated sludge treatment in southwestern Germany (*13*). Taking into account the much lower volume of landfill leachate compared to that of WWTP effluents, the contribution of treated leachate to the estrogenic load on the aquatic environment seems to be low. However, the initial estrogenic activity was within the reported range of 1 to 120 ng/L in WWTP influents (*10*, *32*, *33*) and stresses the necessity for the appropriate treatment of landfill leachates.

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