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Endocrine disruptors in bottled mineral water: Estrogenic activity in the E-Screen☆

Martin Wagner*, Jörg Oehlmann

Department Aquatic Ecotoxicology, Faculty of Biological Sciences, Goethe University Frankfurt am Main, Siesmayerstr. 70 A, D-60054 Frankfurt am Main, Germany

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ABSTRACT

Human exposure to endocrine disruptors is well documented by biomonitoring data. However, this information is limited to few chemicals like bisphenol A or phthalate plasticizers. To account for so-far unidentified endocrine disruptors and potential mixture effects we employ bioassays to detect endocrine activity in foodstuff and consequently characterize the integrated exposure to endocrine active compounds.

Recently, we reported a broad contamination of commercially available bottled water with estrogenic activity and presented evidence for the plastic packaging being a source of this contamination. In continuation of that work, we here compare different sample preparation methods to extract estrogen-like compounds from bottled water. These data demonstrate that inappropriate extraction methods and sample treatment may lead to false-negative results when testing water extracts in bioassays.

Using an optimized sample preparation strategy, we furthermore present data on the estrogenic activity of bottled water from France, Germany, and Italy: eleven of the 18 analyzed water samples (61.1%) induced a significant estrogenic response in a bioassay employing a human carcinoma cell line (MCF7, E-Screen). The relative proliferative effects ranged from 19.8 to 50.2% corresponding to an estrogenic activity of 1.9–12.2 pg estradiol equivalents per liter bottled water.

When comparing water of the same spring that is packed in glass or plastic bottles made of polyethylene terephthalate (PET), estrogenic activity is three times higher in water from plastic bottles. These data support the hypothesis that PET packaging materials are a source of estrogen-like compounds. Furthermore, the findings presented here conform to previous studies and indicate that the contamination of bottled water with endocrine disruptors is a transnational phenomenon.

1. Introduction

In 1991 scientists from diverse disciplines gathered at the Wingspread Conference Center to structure and define the phenomenon of endocrine disruption. They came to the consensus that "a large number of man-made chemicals [...] have the potential to disrupt the endocrine system of animals, including humans" [1]. Since then, research in this multidisciplinary field and thus knowledge about endocrine disruptors is steadily expanding. Many current aspects concerning the effects of endocrine disruptors on different hormonal pathways [Kato, this issue; Watson, this issue; vom Saal, this issue; Baker, this issue; Blumberg, this issue], organs [Prins, this issue; Miyagawa, this issue], individuals [Rubin, this issue], and populations [Woodruff, this issue; Hayes, this issue] are comprehensively documented in this Special Issue of The Journal of Steroid Biochemistry and Molecular Biology.

Characterizing the exposure to endocrine disruptors has been recognized as a crucial aspect for the prediction of actual health effects in the Wingspread Consensus [1]. Two decades later, the Endocrine Society renewed the demand for making the screening for exposures a research priority in its Scientific Statement on endocrine disruptors [2]. However, understanding the complexity of human exposure to man-made chemicals, including endocrine disruptors, is compromised by the overwhelming number of compounds in use and the technical limitations in their detection. Thus, exposure science is forced to focus on few chemicals as proxies for the total exposure, like for example bisphenol A and phthalates for which excellent biomonitoring data are available [3,4]. Whether these compounds adequately represent the total exposure and consequently the total toxicity remains, nevertheless, questionable in

Abbreviations: b.d.l., below detection limit; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EEQ, estradiol equivalents; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MCF7, Michigan Cancer Foundation cell line 7; PET, polyethylene terephthalate; PTFE, polytetrafluoroethylene; RPE, relative proliferative effect; SPE, solid phase extraction; YES, Yeast Estrogen Screen.

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* Corresponding author. Tel.: +49 69 798 24900; fax: +49 69 798 24748.
E-mail address: wagner@bio.uni-frankfurt.de (M. Wagner).

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face of a "universe of toxicants" as Daughton phrases it [5]. In this respect, recent advances in mixture toxicity contribute to a more holistic appraisal of the effect assessment in toxicology [6–8]. For exposure assessment on the other hand, that same holistic view is desirable but obstructed by the limitation of analytical tools to elucidate the entire chemical universe including an unknown number of yet-to-be identified compounds.

Bioanalytical techniques (i.e. bioassays) can help to overcome this shortcoming because they characterize the actual biological effect of a complex sample and thus integrate the effects of unidentified compounds and potential mixtures. Ecotoxicology takes advantage of that by routinely employing in vitro bioassays to assess the endocrine activity in environmental samples (e.g. effluents from sewage treatment). In human toxicology this practice is far less common, and only scarce data is available for endocrine activity of human matrices [9–13] or foodstuff as main route of exposure [14–16].

In this context, we focus on bottled mineral water and characterize its total estrogenic burden using bioassays. Since limited in vitro evidence for the presence of estrogen-like chemicals in bottled water is available [17–19], the present study aims to provide additional data on that issue. In our previous study, we employed a yeast-based bioassay (Yeast Estrogen Screen, YES) to determine the estrogenicity of bottled water. Therefore, we decided to reassess our findings using an additional bioassay that is based on a human cancer cell line (MCF7, E-Screen). Developing and employing an optimized sample preparation method, we here report a broad contamination of commercially available bottled water with estrogen-like compounds. When comparing water from the same spring that was packed in glass or plastic bottles, estrogenicity was significantly higher in samples from PET bottles. This corroborates our hypothesis that the plastic packaging is one source of so-far unidentified endocrine disruptors in bottled water.

2. Materials and methods

2.1. Samples

Bottled mineral water was purchased at local retailer stores. In total, the analyzed water samples comprised 18 products (coded as samples 1 to 18) from 13 different companies, including water from five bottlers that was packed in glass and plastic bottles made of PET (samples 1 + 2, 3 + 4, 5 + 6, 7 + 8, 9 + 10). With the exception of one so-called table water (bottled tap water), the products are marketed as so-called mineral water. These products originate from natural springs and are not processed or altered beyond deferrization. The springs of the products are located in different geographic regions in France, Germany, and Italy. Of each product, a sufficient number of bottles from the same lot (n = 10–12) was purchased and stored at 4 °C prior to analysis.

2.2. Optimization strategy for sample preparation

Sample preparation methods, like the extraction of water samples by solid phase extraction (SPE), are normally optimized for the analytical detection of specific chemicals. In case of bioassays, that also include effects of unknown compounds and mixtures, an adaptation of those methods is needed. Here, we apply a tiered approach to develop an optimized sample preparation procedure by comparing different methods of (1) sample treatment and (2) solid phase extraction and (3) apply the optimized procedure to a broader range of samples. The first two steps were carried out with tap water as procedural blank and one bottled water (sample 18) that has been repeatedly shown to be estrogenic in previous experiments.

2.3. Sample treatment

Evaporation of sample extracts is a common procedure to reduce the extract volume or exchange a solvent. In in vitro bioassays extracts are often evaporated directly on the microtiter plates to eliminate a particular solvent. To investigate whether evaporation of extracts during sample preparation results in a loss of estrogenic activity, we extracted tap water and bottled water (sample 18) via SPE using reversed phase C18 columns (C18-HD, 24 mg, 3 M, St. Paul, MN). The SPE columns were conditioned twice with 4 mL acetone and equilibrated twice with 4 mL tap water. 1.5 L water sample was loaded on each column using a vacuum manifold and a maximum flow rate of 12 mL/min. One set of columns containing tap water or bottled water was dried under a gentle stream of nitrogen for 30 min. After that, these columns were eluted with 4 mL acetone, and extracts were evaporated to dryness under nitrogen and redissolved in 100 μL dimethyl sulfoxide (DMSO, method A). From the second set of columns residual water was removed by applying vacuum for 1 min (method B). Compared to method A, 100 μL DMSO was added to the extracts before evaporation. Due to its high melting point DMSO functions as a so-called keeper that retains volatile compounds during evaporation [20]. Again, nitrogen was used to remove acetone yielding residual extracts in 100 μL DMSO. All extracts were stored in glass vials with PTFE caps at −20 °C prior to analysis in the E-Screen.

2.4. Comparison of different solid phase extraction methods

In the next step of optimization six different SPE sorbents were compared. In addition to the silica phase (C18) described above, copolymer sorbents were used because of their higher capacity and selectivity for polar compounds. These SPE sorbents include the copolymers N-vinylpyrrolidone-divinylbenzene (Oasis HLB, 200 mg, Waters, Milford, MA) as well as styrene-divinylbenzene (Bakerbond SDB[1], 200 mg, J.T. Baker, Deventer, Netherlands; SDB[XC], 15 mg, 3 M, St. Paul, MN) and its hydroxylated form (Isolute ENV+, 200 mg, Biotage, Uppsala, Sweden). The sixth sorbent consists of an amorphous carbon molecular sieve (ENVI-Carb Plus, 400 mg, Supelco, Bellefonte, PA) that is optimal for the enrichment of highly polar compounds from water samples.

The general procedure for solid phase extraction of tap and bottled water was performed with the six different sorbents as described above. Each sorbent was conditioned according to the manufacturer's recommendation: C18 (2 × 4 mL acetone, 2 × 4 mL tap water), HLB/ENV+/SDB[1]/Carb (2 × 4 mL methanol, 2 × 4 mL tap water), and SDB[XC] (4 mL 1:1 ethyl acetate:methylene chloride, 2 × 4 mL methanol, 2 × 4 mL tap water). 1.5 L tap water or bottled water was applied to each column. In case of bottled water (sample 18) the content of twelve individual bottles was mixed in equal parts to create one uniform sample for all extraction procedures. Columns were shortly dried under vacuum and eluted with 4 mL acetone (C18) or 4 mL methanol (all other sorbents). The resulting sample extracts (containing 100 μL DMSO as keeper) were concentrated under nitrogen and kept in glass vials with PTFE caps (−20 °C) prior to analysis in the E-Screen.

2.5. Optimized sample preparation procedure

Based of the previous experiments, an optimized method was used to extract a broader spectrum of bottled water. 1.5 L of 18 different products were degassed in an ultrasonic bath. C18 columns were conditioned with 2 × 4 mL acetone and 2 × 4 mL tap water. Water samples were drawn through the columns with a flow rate of 12 mL/min and directly eluted with 4 mL acetone and 4 mL methanol consecutively in glass vials containing 50 μL DMSO. Ace-

tone and methanol was removed under a gentle stream of nitrogen yielding final extracts in 50 μL DMSO.

2.6. E-Screen

In the E-Screen, estrogen-dependent proliferation of MCF7 cells is used to assess the estrogenic potential of chemicals and environmental samples [21,22]. The cell culture conditions and assay procedure have been described previously [20,23] and are used here with minor modifications. In brief, MCF7 cells (a kind gift by Dr. A. Soto, Boston, MA) were seeded on 96-well microtiter plates at an initial density of 1500 cells/well. Hormone-free cell culture medium (DMEM w/o phenol red) containing 5% charcoal stripped fetal bovine serum, 20 mM HEPES, 2 mM L-glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 2.5 μg/mL amphotericin B was used. After one day allowing the cells to attach, the cell culture medium was replaced with 100 μL medium containing the adequate controls (blank, negative/solvent/positive control) and the sample extracts in eight replicates each. 17β-Estradiol was used as positive control in concentrations ranging from 10 nM to 1 fM. Sample extracts (in DMSO) were diluted 200-fold (optimization experiments) or 400-fold (optimized procedure) with medium, resulting in a final solvent concentration of 0.5% or 0.25% (v/v). In these concentrations, DMSO did not exhibit any effects compared to untreated controls. After five days of incubation, proliferation was determined using resazurin (Alamar Blue) as a marker for metabolic activity [24,25]. For that, a stock solution of 0.1 mg/mL resazurin sodium salt (CAS 62758-13-8) was prepared in PBS (w/o Ca^{2+}/Mg^{2+}) and filtered sterile. 30 μL of resazurin solution were added to each well. After incubation over night absorbance was measured photometrically at 595 nm and 540 nm wavelength.

2.7. Data analysis

In the E-Screen, the percentage reduction of resazurin is proportional to the cell number and was calculated according to AbD Serotec [26] for all controls and samples. In each experiment a dose response curve for 17β-estradiol (positive control) was generated using a four-parameter logistic function. The percentage reduction of resazurin was normalized to the solvent control (0%) and the maximal response induced by 17β-estradiol (100%). This provides so-called relative proliferative effects (RPE) as described by Soto et al. [22]. Here, the presented RPE refer to the estrogenic effect of the water extracts in the experiments, not the original water samples. Since the water samples were concentrated by factor 15,000 or 30,000 via SPE and diluted in the E-Screen 200- or 400-fold, the final concentration factor was 75 for all samples. Accordingly, the RPE reported here refer to a volume of 100 μL per well and are therefore equivalent to a sample volume of 7.5 mL of the respective water sample.

In addition to the RPE, estradiol equivalents (EEQ) were calculated by non-linear interpolation from dose response curve of 17β-estradiol in the respective experiment as described by Wagner and Oehlmann [17]. Derived EEQ were corrected for the concentration factor of 75 and reported as pg/L of the original water sample. The limit of quantification calculated from the mean effect of the solvent control plus three times the standard error was 33.6 pg EEQ/L for the E-Screen and 0.45 pg EEQ/L for the sample extracts accordingly.

Data analysis was performed using GraphPad Prism 5.03 (GraphPad Software Inc., San Diego, CA). Nonparametric Kruskal–Wallis tests (with Dunn's multiple comparison test) were applied to compare the medians of data sets. For pair-wise comparison of bottled water from glass and PET bottles we used nonparametric Mann–Whitney tests. A p value of <0.05 was regarded as significant. All presented data comprise of mean ± standard error of the mean (SEM).

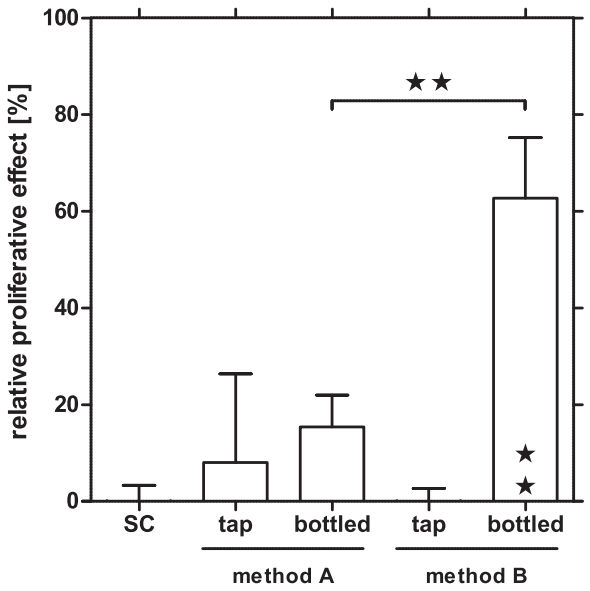

Fig. 1. Effect of sample evaporation on the estrogenic activity of tap and bottled water. The estrogenicity in the E-Screen is significantly higher in bottled water extracted without evaporation (method B) compared to evaporated extracts (method A) and the solvent control (SC, $^{**}p < 0.01$).

3. Results

3.1. Effect of sample evaporation on the estrogenic activity

The aim of the first set of experiments was to determine if specific steps of sample preparation lead to a loss of estrogenicity. Therefore, the common procedure of drying of SPE columns after extraction and the complete evaporation of sample extracts thereafter (method A) was compared to a procedure that minimized these evaporation steps (method B). The results of this comparison indicate that tap water as procedural blank did not induce significant estrogenic effects in the E-Screen, irrespective of the sample treatment procedure (Fig. 1). Extraction of bottled water (sample 18) according to method A (complete evaporation of the extracts) resulted in an increased but not statistically significant estrogenic response in the E-Screen (15.4 ± 6.58% RPE). Compared to that, extraction of the same bottled water according to method B (including DMSO as keeper) yielded extracts with a pronounced and statistically significant estrogenic activity (62.8 ± 12.5% RPE). In case of bottled water, the difference between the two sample preparation procedures is statistically significant ($p = 0.008$).

3.2. Comparison of the extraction efficacy of different SPE methods

To determine the extraction efficacy for estrogenic activity from bottled water we compared six different SPE sorbents. The extraction was performed according to method B from the previous experiments because a sample preparation that minimizes the evaporation of volatile compounds proved to be suitable. In case of all extractions, neither the used solvents and the extracts from empty SPE cartridges, nor the procedural blanks (extracted tap water) contained significant estrogenic activity (data not shown). This indicates that the extraction procedure itself did not result in an estrogenic contamination of the samples.

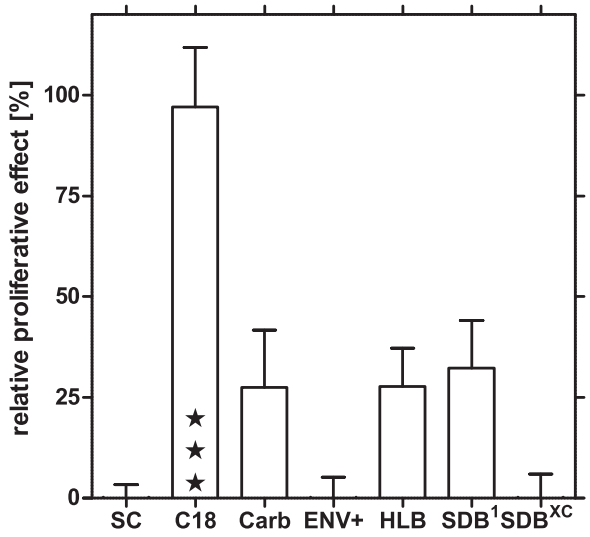

Fig. 2. Efficacy of different SPE methods to extract estrogenic activity from bottled water. From all sorbents (C18, Carb, ENV+, HLB, SDB^1, SDB^{XC}), only C18 extracted significant levels of estrogenicity compared to the solvent control (SC, ***$p < 0.001$).

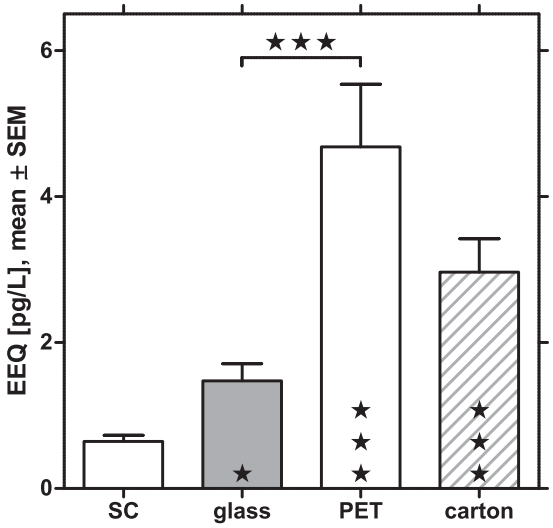

Fig. 4. Influence of the packaging material on the estrogenic activity of bottled water. Pooled analysis of products from the same springs bottled either in glass (sample 1, 3, 5, 7, 9) or plastic (PET, samples 2, 4, 6, 8, 10). Water bottled in PET contains significantly higher estrogenicity compared to water from glass (***$p < 0.001$). Significant differences compared to the solvent control (SC, *$p < 0.05$, ***$p < 0.001$).

SPE sorbents on the basis of styrene-divinylbenzene (SDB^1 and SDB^{XC}) and hydroxylated styrene-divinylbenzene (ENV+) did not extract significant estrogenic activity from bottled water (Fig. 2). The same hold true for sorbents consisting of an amorphous carbon molecular sieve (Carb) or a N-vinylpyrrolidone-divinylbenzene copolymer (HLB). Compared to that, the C18 sorbent yielded an extract of the same bottled water that was significantly estrogenic in the E-Screen (97.1 ± 14.8% RPE, $p < 0.0001$). Taken together, the SPE method employing a traditional silica-based sorbent (C18) and a sample preparation that minimizes evaporation steps is the most effective for the extraction of estrogen-like compounds from bottled water.

3.3. Estrogenic activity of bottled water

Based on the previous experiments, we applied the optimized sample preparation procedure to 18 different products of bottled water. In the E-Screen, 11 of the 18 extracted bottled waters (61.1%) induced a significant estrogenic response (Fig. 3, pooled data from three independent experiments). In these samples relative proliferative effects ranged from 19.8 (sample 12) to 50.2% (sample 17) of the maximal effect induced by 17β-estradiol (pooled data from three independent experiments). In four samples of bottled water (products 1, 2, 4, and 9) we did not detect any proliferative effects compared to the appropriate solvent controls, indicating the absence of estrogen-like chemicals in these extracts. Extracts of three other products (3, 11, and 15) induced an elevated proliferation of MCF7 cells (RPE: 9.17–12.1%) that was statistically not significant.

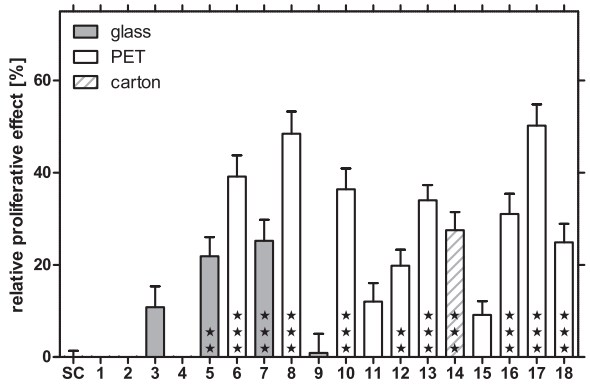

Fig. 3. Estrogenic activity of different bottled water products (samples 1–18) in the E-Screen. The first five pairs of samples each originate from the same springs and are bottled in glass and plastic (PET). Significant differences compared to the solvent control (SC, **$p < 0.01$, ***$p < 0.001$).

The relative proliferative effect provides information on the effect of the sample extract in the E-Screen (equivalent to 7.5 mL of bottled water). To quantify the estrogenic activity of the original water samples we derived estradiol equivalents (EEQ) that are related to 1 L of bottled water (Table 1). In samples with significantly elevated estrogenicity these ranged from 1.9 ± 0.25 to 12.2 ± 3.57 pg EEQ/L. The average estrogenic activity of bottled water calculated from all analyzed samples is 3.33 ± 0.30 pg EEQ/L ($n = 432$).

3.4. Influence of the packaging material on estrogenicity

To investigate the effect of the packaging material on the estrogenic activity of bottled water, we included five pairs of products in our sample set that were available in glass as well as plastic (PET) bottles. Each sample pair (1 + 2, 3 + 4, 5 + 6, 7 + 8, 9 + 10) originated from the same natural spring. This enables the comparison of the different packaging materials. Water from two bottlers (1 + 2, 3 + 4) did not contain significantly elevated levels of estrogenic activity, irrespective of the packaging material (Fig. 3.). However, in all other cases, estrogenicity was significantly higher in water from PET bottles compared to the appropriate sample from glass bottles (5 vs. 6 $p = 0.0078$, 7 vs. 8 $p = 0.0018$, 9 vs. 10 $p < 0.0001$). Estradiol equivalents calculated for these samples (Table 1) were 63.7–87.2% higher in water from PET vs. glass bottles. A pooled analysis of these sample pairs is provided in Fig. 4. The average estrogenic activity of glass-bottled water is 1.48 ± 0.23 pg EEQ/L. In water from the respective PET bottles, we derived estradiol equivalents of 4.68 ± 0.86 pg EEQ/L. Again, estrogenicity is significantly higher in water from PET material compared to samples from glass bottles ($p = 0.0009$).

In water provided in a cardboard beverage carton (sample 14) we also detected significant estrogenic activity in the E-Screen (2.96 ± 0.46 pg EEQ/L). For this kind of packaging, a comparison to

Table 1
Estrogenic activity of bottled water in the E-Screen (data from three independent experiments).

Sample	Packaging			Estrogenic activity (mean ± SEM)		
	Material	Volume	Type	RPE (%)	EEQ (pg/L)	*n*
1	Glass	0.7 L	Reusable	b.d.l.[a]	b.d.l.	24
2	PET	1 L	Single-use	b.d.l.	b.d.l.	24
3	Glass	0.7 L	Reusable	10.8 (4.52)	1.64 (0.39)	24
4	PET	1 L	Reusable	b.d.l.	b.d.l.	24
5	Glass	0.75 L	Reusable	21.9 (4.14)	2.12 (0.49)	24
6	PET	1 L	Reusable	39.2 (4.61)	5.84 (1.15)	24
7	Glass	0.75 L	Reusable	25.2 (4.57)	2.94 (0.85)	24
8	PET	1 L	Single-use	48.5 (4.84)	12.2 (3.57)	24
9	Glass	1 L	Reusable	0.88 (4.20)	0.62 (0.21)	24
10	PET	1.5 L	Single-use	36.4 (4.54)	4.86 (0.82)	24
11	PET	1.5 L	Single-use	12.1 (3.98)	1.18 (0.25)	24
12	PET	1 L	Reusable	19.8 (3.45)	1.90 (0.25)	24
13	PET	1.5 L	Single-use	34.0 (3.32)	4.20 (0.68)	24
14	Carton	1 L	Single-use	27.5 (3.94)	2.96 (0.46)	24
15	PET	1.5 L	Single-use	9.17 (2.98)	0.96 (0.19)	24
16	PET	1.5 L	Single-use	31.1 (4.36)	4.24 (0.92)	24
17	PET	1.5 L	Single-use	50.2 (4.60)	11.3 (1.97)	24
18	PET	1.5 L	Single-use	24.9 (4.00)	2.49 (0.51)	24

[a] b.d.l., below detection limit.

other materials is difficult because these products are rarely available in glass bottles.

4. Discussion

4.1. Sample preparation for bioassays: what you extract is what you see

Preparation techniques for water sample are commonly optimized for the enrichment and analytical determination of one specific compound. Here, we employed an optimization strategy to adapt traditional solid phase extraction methodology for the isolation of estrogenic activity from bottled water. In contrast to chemical analysis, the target compound(s) responsible for the biological effects detected in bioassays are often unknown. In this case, an optimization of sample preparation therefore relies on maximizing the biological effect observed in the bioassay.

For estrogen-like compounds from bottled water our data demonstrate that evaporation steps during the sample preparation lead to a considerable loss of estrogenicity. This observation is in accordance with results from a previous study: Boehmler et al. [27] detected estrogenic activity in extracts of one brand of mineral water in the E-Screen (25.7% RPE) when DMSO was used as keeper. When the same extract was evaporated to dryness without DMSO, the estrogenic activity considerably decreased to 9.5% RPE. From these findings we can conclude that the estrogen-like compound(s) in bottled water are volatile and may be lost during sample evaporation.

The comparison of different methods of solid phase extraction furthermore indicates that the choice of sorbent determines the effect detected in the bioassay. This is due to the selective enrichment of bioactive compounds by the different sorbents. SPE matrices based on copolymers or carbon spheres are often regarded as “gold standard” for the extraction of organic compounds from aqueous samples. In case of bottled water, these sorbents are less effective in extracting estrogenicity compared to a traditional silica based matrix (C18). This might be due to an insufficient retention of estrogen-like compounds or an additional enrichment of anti-estrogenic substances. The latter holds true for a sorbent based on hydroxylated styrene-divinylbenzene (ENV+): bottled water extracts yielded with this type of SPE column do not induce estrogenic effects in the E-Screen but contain potent anti-estrogenic activity in a yeast-based bioassay that might mask potential estrogenic effects (unpublished data). On the one hand, this demonstrates that a complex mixture of diverse-acting endocrine disruptors is present in bottled water. On the other hand these findings also reveal the limitations of commonly employed sample extraction techniques. A specific sorbent is able to extract only a finite fraction of the broad spectrum of bioactive compounds from a complex sample. In case of bottled water the C18 sorbent retains estrogenicity more effectively compared to sorbents that are selective for polar chemicals. This indicates that this method is preferable to extract estrogen-like compounds from bottled water and that the respective compounds are more likely to be non-polar.

Taken together, the application of inappropriate sample preparation techniques may lead to false-negative results in bioassays due to a loss of volatile compounds or an ineffective enrichment of target compounds during extraction. This demonstrates the need to develop adapted sample preparation methods when the biological effect of samples with unknown (mixtures of) bioactive compounds is characterized in bioassays.

4.2. Estrogenic activity in bottled water: growing body of evidence

Employing an optimized sample preparation method, our results demonstrate a broad contamination of bottled water with estrogen-like chemicals. Eleven of the 18 investigated products significantly induced estrogenic effects in a human cancer cell line (E-Screen) with proliferative effects from 19.8 to 50.2% compared to 17β-estradiol (1.9–12.2 pg EEQ/L).

In addition to this study, there are four published reports on estrogenicity in bottled water emphasizing that its contamination with endocrine disruptors is more than a singular phenomenon. Boehmler et al. [19] investigated extracts of 37 German bottled water products (packed in PET, carton, and glass) in the E-Screen. With a relative proliferative effect of more than 14% RPE, eight of 37 bottled waters were estrogenic. In a second campaign, Boehmler et al. [19] tested these eight products again and found seven products to be estrogenic. The RPE of positive samples in the E-Screen ranged from 16 to 71%. Interestingly and in accordance to our study, tap water was classified as estrogen-negative. Though not published under peer review, the nonprofit organization Environmental Working Group commissioned a study of bottled water

from the United States. Experiments conducted at the University of Missouri provide evidence that extracts of one of ten tested products induce a relative proliferative effect of 78% RPE in the E-Screen [28].

With regard to the broad spectrum of products analyzed in the three available E-Screen studies, the consistent detection of estrogenic effects of bottled water is remarkable. Unfortunately, the reported relative proliferative effects are not suitable for a quantitative comparison because Boehmler et al. [19] and Naidenko et al. [28] did not report in which concentration factor they tested the respective water extracts. To overcome this shortcoming, we performed a reanalysis of Boehmler et al.'s unattributed raw data to estimate estradiol equivalents according to the method used in this study. Although Boehmler et al.'s study was originally not designed for quantitative analysis, estradiol equivalents of the recalculated water samples were in the same range (pg EEQ/L) as in our study.

Besides these results from human cell culture experiments, there are two studies that employ a yeast-based bioassay (YES) to investigate estrogenicity in bottled water. Although two slightly different yeast strains [29,30] were used in these investigations, YES systems generally determine estrogenic activity as activation of the human estrogen receptor alpha.

In our previous work we tested untreated water samples of 20 products of bottled water (packed in PET, carton, and glass) and found that twelve samples contained significantly elevated levels of estrogenic activity [17]. In positive samples, the estrogenic potency ranged from 2.64 to 75.2 ng EEQ/L. Notwithstanding the fact that our current sample set comprehends mostly other products than the previous one, the ratio of samples that are estrogenic in the two different bioassays is remarkably similar. Approximately 60% of products was tested positive in the YES and the E-Screen. For samples investigated in both studies qualitative results are completely consistent for both assays.

In bottled water from Italy (SPE extracts of nine products, all packed in PET) Pinto and Reali [18] detected 0.9–23.1 ng EEQ/L. The authors defined a cut-off value of 10% of the effect of 10 nM 17β-estradiol and accordingly classified eight of nine products as weakly estrogenic. Compared to the findings by Wagner and Oehlmann [17], the low activity reported by Pinto and Reali [18] is somewhat surprising. It might be attributed to the lack of sensitivity of the used bioassay as pointed out by Sax [31] or the loss of volatile estrogen-like compounds during sample preparation as discussed previously. Apart of these negligible quantitative differences, Pinto and Reali's study adds up to the growing body of literature demonstrating the broad contamination of bottled water with endocrine disruptors.

Compared to the data from E-Screen, the higher estrogenic activity of bottled water in the YES is unexpected, since the E-Screen is a more sensitive tool for detecting estrogenicity (in case of our studies by a factor 50). The potent estrogenic activity we determined in untreated bottled water in the YES [17] gives rise to the assumption that only a fraction of estrogen-like compounds actually present in bottled water is extractable. Such compound(s) might be too polar to be retained by common SPE sorbents. This demonstrates a fundamental dilemma when monitoring effects of complex samples containing unidentified bioactive compounds: whatever preparation technique is employed, absolute recovery of all chemicals present in such samples is simply not achievable. Therefore, only an unknown portion of the sample's actual toxicity can be assessed [32]. Bearing this limitations in mind, it is notable that studies from four different institutions, performed with two different bioassays and samples from France, Germany, Italy, and the United States come to the same conclusion: bottled water is broadly contaminated with endocrine disrupting, estrogen-like chemicals.

4.3. Sources of estrogenic contamination

Interestingly, the public debate about the estrogenicity of bottled water did not center around the pure fact that there is a contamination with endocrine disruptors but the question where this is originating from. In principle, there are three possible sources of contamination as discussed previously: the spring itself, the production process and the packaging material [17]. So far, only few systematic investigations are available on this issue. Boehmler et al. [19] analyzed water that was sampled directly at the spring of several bottling plants. In the E-Screen approximately half of these spring waters were classified as estrogenic (4–62% RPE). This gives rise to the assumption that mineral water springs itself (and consequently the aquifer) are contaminated with estrogen-like chemicals. Given the fact that according to the bottler's information, the products investigated here originate from deep wells (100–700 m depth), an infiltration of organic compounds appears unlikely. Still, an anthropogenic contamination of ground water is far from impossible. The widespread introduction of pharmaceuticals and chemicals from personal care products in the environment (including ground water) has been demonstrated (as reviewed in Ref. [33]). In this respect and because of the far-reaching consequences for human and environmental exposure, investigating the potential infiltration of endocrine disruptors in the aquifer is imperative.

In our work, we focus on food packaging materials as source of exposure to endocrine disruptors. Using a yeast-based in vitro assay, we confirmed that the estrogenic activity of bottled water from PET containers was approximately twice as high compared to products from glass bottles [17]. This raised the hypothesis that the estrogenicity was caused by chemicals migrating from the plastic packaging. To pursue this question, we conducted an in vivo experiment with an estrogen-sensitive molluskan model that was bred in water bottles made of glass and PET. The reproductive output of animals housed in plastic bottles was doubled compared to specimen from control groups and glass water bottles. This provided evidence for the leaching of endocrine disrupting compounds from water bottles made from PET.

The present data from E-Screen experiments further support this hypothesis. When comparing products bottled from the same spring, water packed in PET exhibits a 60–90% higher estrogenic activity compared to the respective product in glass bottles. This difference is especially pronounced for the spring of which samples 9 and 10 originated. Whereas the water from glass bottles did not contain any significant estrogenic activity, we detected prominent estrogenicity in the same product from plastic bottles. Taken together, data from three different bioassays indicate that PET bottles are one source of estrogen-like compounds in mineral water. However, additional migration studies are needed to improve our understanding of the leaching of bioactive compounds from plastic packaging. The fact that food contact materials are an existing (but underestimated) source of endocrine disruptors receives increasing scientific attention and is discussed in depth in this Special Issue [Muncke, this issue]. This is more than a hypothetical scenario as Muncke [34] recently documented by compiling a list of 50 known or suspected endocrine disruptors that are authorized for the use in food contact materials in the European Union and the United States. In addition to this, the utilization of recycled plastic for food contact materials might be a source of contamination [31].

The endocrine activity of two products packed in glass bottles (samples 5 and 7) implies that there are additional sources of estrogenicity in bottled water. Besides a contamination of the spring or during the production, the bottles' closure might contribute to the endocrine activity. In the light of these findings, it is probable that there are multiple sources of contamination of

the final product. Since empirical data concerning the spring and the packaging are available, scientific focus should be set on the production process as potential source of endocrine disruptors in bottled water.

The question where the estrogenic contamination of bottled water is coming from is interwoven with the elucidation of the identity of respective chemical(s). So far, no substantial progress has been made concerning this issue. As discussed previously, it is unrealistic that the estrogenic activity in bottled water is caused by one single known endocrine disruptor since the estrogenic potency of those compounds is too low [17]. This view is supported by Franz and Welle [35], who employed a theoretical migration model to calculate that far too high levels of the nonylphenol or bisphenol A are hypothetically needed to explain the observed estrogenic effects. However, this argument is not feasible to exclude the packaging as potential source of contamination because it is based on a scenario that does not take the complexity of the sample's chemical composition into account. Given the vast number of so-far unidentified chemicals in food and food contact materials (non-intentionally added substances, break-down products, etc.) it appears likely that the effects observed in bottled water are caused by either a mixture of several weakly estrogenic compounds or an unknown bioactive substance with high estrogenic potency.

4.4. Exposure to estrogen-like chemicals from bottled water

From the available date we can conclude that bottled water is a source of human exposure to chemicals that mimic estrogen in vitro. A quantitative evaluation of exposure to estrogenic activity (e.g. as total daily intake of estradiol equivalents) might prove to be a suitable integrated criterion to include unknown endocrine disruptors and mixture effects in exposure assessment. Unfortunately, up to date this is hindered by methodological differences in sample preparation, bioassays, and data analysis. From a qualitative point of view, the steadily increasing consumption of bottled water, the high ratio of estrogen-positive products in different studies, and the broad range of samples included therein, leads to the assumption that exposure to endocrine disruptors from bottled water is a transnational phenomenon. Based on a daily consumption of 1–2 L [18], the total daily intake of estrogenicity from bottled water can be estimated to be in a range of picogram to nanogram estradiol equivalents. This intake could result in a low dose but long term exposure to estrogen-like compounds that affects a broader population, including potentially sensitive subpopulations (infants, pregnant women, and women with breast cancer). However, an actual assessment of potential effects to human health requires the identification of the chemical(s) responsible for the observed estrogenic effects. In that context, the combination of bioanalytical and analytical techniques provides a powerful tool to create a more holistic understanding of the complex human exposure to endocrine disruptors.

Conflict of interest

The authors declare to have no conflict of interest.

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