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Characterizing field sediments from three European river basins with special emphasis on endocrine effects – A recommendation for *Potamopyrgus antipodarum* as test organism

Claudia Schmitt [a,*], Jan Balaam [b], Pim Leonards [c], Rikke Brix [d], Georg Streck [e], Anita Tuikka [f], Lieven Bervoets [g], Werner Brack [e], Bert van Hattum [c], Patrick Meire [a], Eric de Deckere [a,h]

[a] University of Antwerp, Department of Biology, Ecosystem Management Research Group, Universiteitsplein 1, 2610 Wilrijk, Belgium
[b] Ctr. Environ., Fish./Aquacult. Sci., CEFAS Lowestoft Laboratory, Pakefield Road, Lowestoft, NR33 0HT, United Kingdom
[c] Institute for Environmental Studies, VU University Amsterdam, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands
[d] Department of Environmental Chemistry, IDAEA-CSIC, C/Jordi Girona 18-26, 08034 Barcelona, Spain
[e] UFZ-Helmholtz Centre for Environmental Research, Department Effect-Directed Analysis, Permoserstrasse 15, 04318 Leipzig, Germany
[f] University of Eastern Finland, Department of Biology, P.O. Box 111, 80110 Joensuu, Finland
[g] University of Antwerp, Department of Biology, Department of Biology, Ecophysiology, Biochemistry and Toxicology Research Group, Groenenborgerlaan 171, 2020 Antwerp, Belgium
[h] University of Antwerp, Institute of Environment and Sustainable Development, Universiteitsplein1, 2610 Wilrijk, Belgium

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ABSTRACT

The assessment of endocrine disrupting potentials of field sediments has until now been mostly limited to classical chemical analysis, *in vitro* assays and *in vivo* bioassays performed with vertebrates. There is an urgent need for easy, cheap and reproducible invertebrate tests which may be applied in certain monitoring activities. Since the mudsnail *Potamopyrgus antipodarum* is known to be tolerant to natural stressors, but also sensitive to endocrine disrupting chemicals, it is very likely that this organism could be suitable for the assessment of endocrine effects of e.g. field sediments. Within this study the endocrine potential of sediments in three European river basins was assessed. The yeast estrogen screen (YES) and a sediment contact test with *P. antipodarum* were performed. Furthermore, analyses of physico-chemical properties and concentrations of heavy metals, PAHs, organotins, natural steroids and alkylphenols were done. In the sediment contact test, the reproduction of the snail was promoted by a part of the sediments. This phenomenon could not be explained by their physico-chemical properties. However, at some of those sites a high estrogenic activity was detected in the YES, leading to the assumption that endocrine disrupting compounds could be responsible for those effects. This assumption could be confirmed to some extent with partially high concentrations of xeno-estrogens (e.g. nonylphenol) at the certain sites. Our study demonstrates the applicability of the test with *P. antipodarum* for a variety of sediments and once again points out the need of suitable *in vivo* biotests for the risk assessment of field sediments.

1. Introduction

One of the biggest problems concerning toxicity in aquatic ecosystems are not only the well known "highly toxic compounds" but also compounds which are affecting the growth and reproduction of aquatic animals in low concentrations, mostly even below their chemical detection limits. Those compounds, such as for example the rather heterogenic group of endocrine disrupters are potent in very low concentrations and their effects can be additive in mixtures of compounds with the same mode of action (Silva et al., 2002). Within the last years, a lot of novel *in vitro* assays have been developed and applied for the assessment of possible endocrine effects of sediments (Kase et al., 2009; Le Blanc et al., 2009) but also well established assays like the yeast estrogen screen (YES) (Routledge and Sumpter, 1996) or the E-Screen (Soto et al., 1995) are regularly applied. High throughput *in vitro* tests are necessary and useful for a first screening, but it has to be considered that they are lacking ecological relevance and factors like bioavailability and metabolism of certain compounds cannot be taken into account (Beresford et al., 2000). Thus, in order to draw conclusions about the endocrine disrupting potential of sediments, the above mentioned *in vitro* tests should always go along with suitable *in vivo* biotests. Those *in vivo* biotest should be easy, cheap and applicable for a great variety of samples and preferably have clear mechanistic endpoints. The organism should be tolerant

* Corresponding author.
E-mail address: claudia.schmitt@ua.ac.be (C. Schmitt).

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to "natural stressors" but sensitive to endocrine disrupting compounds. Putting all this together, snails are very promising candidates. Prosobranch snails in particular have been reported to be excellent test organisms for the determination of endocrine disrupting effects (Oehlmann et al., 2000; Duft et al., 2007; Matthiessen, 2008). The fact that their hormone system appears to be quite unique for invertebrate species and is to some extent comparable to those of vertebrates (Thornton et al., 2003) may provide new alternatives for the replacement of vertebrate animal testing (Jobling et al., 2004). The parthenogenic mudsnail *Potamopyrgus antipodarum* is known to be very sensitive to endocrine disrupting compounds in the laboratory, whereby reproduction increased after exposure to estrogens (Duft et al., 2003b; Jobling et al., 2004) and decreased after exposure to androgens (Duft et al., 2003a). Furthermore, their reproduction appeared to be even more sensitive than the induction of estrogenic gene expression in the YES assay (Schmitt et al., 2008). At the same time, *P. antipodarum* is very tolerant to variations in e.g. temperature, salinity and sediment composition (Alonso and Castro-Díez, 2008). Thus, considering all of these characteristics, it is most likely that also the endocrine "potential" of a huge variety of field sediments could be detected with this species.

Within this study, a comprehensive approach was followed, where the endocrine potential of freshwater and brackish sediments in three different river basins was assessed with *in vitro* and *in vivo* bio assays, accompanied by chemical analysis of metals, PAHs, organotin compounds, estrogens and alkylphenols.

2. Material and methods

2.1. Sediment sampling

The sampling sites are spread over Europe and include three large river basins, namely the Elbe in the Czech Republic, the Scheldt in Belgium and The Netherlands and the Llobregat in Spain. For each basin, two rivers each with two sampling sites were selected, whereby those two sites were predefined in "reference sites" and "hotspots". This classification was done according to historical monitoring data, mostly from local water agencies. For the sites in the Elbe basin, data were used from the database of water agency in the Czech Republic: Czech Hydrometeorological institute (CHMU; www.chmu.cz). For the sites at the Scheldt basin the data from the flemish environment agency (VMM; www.vmm.be) were evaluated and the basis for the selection of the sites at the Llobregat is given in (Prat and Rieradevall, 2006). A detailed overview of the sampling locations and their predefined classification as "reference sites" and "hot spots" is given in Table 1. Sediment samples were taken with an Ekman grab sampler, whereby for each site, three replicates were pooled and mixed in one big container. In order to remove large particles or debris, all sediments were sieved (ø 2 mm). Samples were sent to the appropriate laboratories immediately. For the sediment contact test, sediments were stored dark at 4 ± 1 °C for 14 d. For chemical analyses and the yeast estrogen screen (YES), sediments from the Scheldt and Elbe basin were homogenized, freeze dried and sieved to 63 µm. Samples from the Llobregat were treated similar but sieved to 2 mm since these sediments exhibited mainly a coarse texture. Samples were stored at −32 °C until analysis.

2.2. Sediment analysis

Sieved sediments were analyzed for dry weight (105 °C, >16 h) and loss of ignition (550 °C, 2 h) organic carbon, nitrogen, black carbon and grain size in triplicate as described in Sormunen et al. (2010).

Chemical analyses of the sediments were carried out for metals, organotin compounds, PAHs, natural steroid estrogens and alkylphenols. For practical reasons, no analyses were done for the brackish sites at Han and Tern. For the reference site from the Bilina (Jir) and for the hot spot of the river Llobregat LL4, no complete datasets are available.

For the analysis of metals, sediments were digested in a microwave with HNO_3/HCl. As, Cd, Cr, Co, Cu, Ni, Pb and U were analyzed using a mass spectrometer with inductively coupled plasma (ICP-MS 7500c series, Agilent). Mn and Zn were analyzed with optical emission spectrometry with inductively coupled plasma (ICP-OES, Perkin–Elmer, OPTIMA 3000), while Hg was determined using atomic absorption spectrometry/cold vapour technique (Perkin–Elmer, 4100 ZL). All heavy metals were determined as total content of the sample.

Four organotin compounds (mono-, di-, tri- and tetra-butyltin) were analyzed using a gas chromatograph coupled to an atomic emission detector (GC–AED) with a method similar as described in Mothes and Wennrich (2000). Prior to analysis, organometals were derivatized with sodium tetraethylborate (Ashby and Craig, 1989; Craig and Mennie, 1994). The analytical system consists of a HP5890 GC (Agilent) and a G2350 AED (Joint Analytical Systems).

Analyses of estrogens and alkylphenols have been described in detail by Brix et al. (2009). In brief, sediments were extracted by pressurized liquid extraction (PLE) on a Dionex ASE 200 (Dionex, Idstein, Germany). Extracts were subsequently cleaned by solid phase extraction (SPE). Chemical analysis was performed on an LC–MS system consisting of an Alliance 2690 LC pump equipped with an autosampler (Waters, Milford, MA, USA) and a Quattro LC triple-quadruple mass spectrometer from Micromass (Manchester, UK). The mass spectrometer was employed in negative and positive ionization mode and two SRM transitions were recorded per compound.

PAHs were extracted from sediment with hexane:acetone (3:1, v/v) using an Dionex ASE200 system (Dionex, Idstein, Germany).

Table 1
Details of the sampling sites. Used codes, corresponding river and water system, location with coordinates and predefined status according to the MODELKEY database, hot spots are colored grey.

Code	Basin	River	System	Location	Coordinates	Status
Par	Elbe	Elbe	Freshwater	Pardubice, Czech Republic	50°02′35.93″N/15°46′17.14″E	Reference
Pre	Elbe	Elbe	Freshwater	Prelouce, Czech Republic	50°02′24.76″N/15°35′33.46″E	Hot spot
Jir	Elbe	Bilina	Freshwater	Jirkov, Czech Republic	50°30′15.79″N/13°40′13.72″E	Reference
Most	Elbe	Bilina	Freshwater	Most, Czech Republic	50°30′15.79″N/13°40′13.72″E	Hot spot
Run	Scheldt	Groot Schijn	Freshwater	Rundvoort, Belgium	51°13′23.89″N/04°32′43.19″E	Reference
Een	Scheldt	Groot Schijn	Freshwater	Eenhoorn, Belgium	51°13′23.49″N/04°32′48.01″E	Hot spot
Han	Scheldt	Scheldt	Estuarine	Hansweert, The Netherlands	51°26′38.06″N/03°59′56.82″E	Reference
Ter	Scheldt	Scheldt	Estuarine	Terneuzen, The Netherlands	51°20′54.48″N/03°50′22.97″E	Hot spot
A1	Llobregat	Anoia	Freshwater	Jorba, Spain	41°35′49.22″N/01°32′46.85″E	Reference
A3	Llobregat	Anoia	Freshwater	Martorell, Spain	41°28′27.64″N/01°55′37.10″E	Hot spot
LL4	Llobregat	Llobregat	Freshwater	Barcelona, Spain	41°20′57.25″N/02°02′47.84″E	Hot spot

The extract was further cleaned with alumina oxide (8% water deactivated) using pentane as eluents, followed by the removal of sulphur with tetrabutylammoniumhydrogensulfate reagents. This extract was fractionated with silica (1.5% water deactivated) using hexane and hexane: diethylether. Final fractions were transferred to isooctane and analyzed by gas chromatography with mass selective detection (GC–MSD, Agilent 6890 GC with 5973 MSD) using a BPX-5 column (054113, SGE, Australia). The GC oven temperature program used for the PAH analysis was: 80 °C (1 min), 30 °C min^{-1}, to 150 °C, (0 min), 5 °C min^{-1} to 220 °C, 2 °C min^{-1} to 290 °C, followed with 10 °C min^{-1} to 320 °C (5 min).

2.3. Yeast estrogen screen

Samples were tested for *in vitro* estrogenic activity using the YES that has been fully validated and used in effects directed analysis (EDA) type investigations (Thomas et al., 2004). The bioassay was carried out using methods described previously (Routledge and Sumpter, 1996). Sediment samples were extracted with ASE using dichloromethane (1:1, v/v) and cleaned with gel permeation chromatography. The extracts (50 μL in ethanol) were added to the microtitration test plate at a range of concentrations and then allowed to evaporate to dryness at room temperature. An assay medium, which consisted of the chromogenic substrate and a growth medium that had been inoculated with yeast cells containing the human estrogen receptor, was added to the plate. The plate was then incubated for three days at 32 °C. On the third day any change in the color of the chromogenic substrate was read colourimetrically using a UV/Vis plate-reader (Bio-Tek instruments, Inc.) at an absorbance of 540 nm for color and 620 nm for the turbidity. A dilution series of 17ß-estradiol (E2) as a positive control and estrogenic standard together with a solvent blank, were assayed alongside each batch of samples.

2.4. Sediment contact test

P. antipodarum used for building up our own laboratory culture were kindly provided by the Aquatic Ecotoxicology group of Prof. Jörg Oehlmann (Goethe University, Frankfurt/Main, Germany). The snails were bred in 10 L glass aquaria, filled with reconstituted water (OECD, 2006) with a pH-value of 7.9–8.4 and a conductivity ranging between 500–600 μS cm^{-1}). They were kept at 15 ± 1 °C and a 16:8 h light:dark cycle. The experiment was conducted in a static system in 1 L glass beakers under the same conditions as used for the breeding stock and all samples were included in the same experiment. All beakers were filled with 50 g of wet field sediment and 800 mL of reconstituted water. In order to ensure that the snails were exclusively exposed to the sediments, no external "clean food" was added. To guarantee sufficiently aerobic conditions in the samples, water was aerated with a glass pasteur pipette, seven days prior to the insertion of the test organisms. As control sediment, quartz sand (grain size 100–400 μm) containing 5% fine grounded beech leaves (*Fagus sylvatica*) as carbon source (equivalent to 1.95% TOC) was used (Duft et al., 2007). Groups of prosobranch snails (80 sexually mature snails per sample) with a shell height of 3.8 ± 0.5 mm were exposed to the field and the control sediment. After 28 and 56 days, mortality and reproduction success was assessed by counting the embryos in the brood pouch of 20 randomly selected maternal snails per treatment.

2.5. Statistical analyses

The estrogenic equivalent values in the YES were determined by comparing responses with that of the E2 standard. All equivalent E2 values of a sample were divided by their relevant concentration factors to produce equivalent E2 values for the original raw water sample. All values falling in the linear range of the sample response curve were averaged to produce the final equivalent E2 value (ng E2 kg^{-1}) of the sample.

For the yeast estrogen screen and the sediment contact tests, mean values of the different sites were analyzed using the Mann–Whitney test for significant differences ($p < 0.05$). Correlations between the snails reproduction and the physico-chemical characteristics of the sediment and the concentrations of compounds were all performed using Pearson correlation. All statistical analyses were performed using the software Prism 4.03 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Sediment characteristics

The physico-chemical sediment characteristics differed significantly between the selected sediments (Table 2). While for example the freshwater reference site of the Scheldt basin (Run) and the reference site from the Elbe (Par) showed a higher percentage of dry weight than their corresponding hot spots, the opposite was true for the Llobregat and the Bilina sites. The loss of ignition varied between 2.70% dw for the hot spot of the Llobregat and 16.1% dw for the one of the Bilina. Beside the hot spot of the river Bilina where 7.10% dw organic carbon were detected, concentrations varied in other samples between 0.60 and 4.20% dw. Nitrogen content was most of the time higher at the hot spots. However, at the two estuarine sites of the Scheldt basin, a nitrogen content of 0.03% dw was determined for the references site Han. No nitrogen could be detected at the hot spot Ter. Concentrations of black carbon were also most of the time higher in the polluted sediments, but again, the estuarine part of the Scheldt and the sites of the Llobregat showed a different pattern. Both of them contained more black carbon in the reference sediments. The biggest variance within all samples was detected for the particle size distribution of the different sediments, which ranged from 10.0% fine particles at Run up to 75.3% at Pre.

3.2. Chemical analyses

The concentration of metals, organotin compounds, natural steroid estrogens, alkylphenols and PAHs are shown in Table 3. Concerning the metals, the two most polluted sites were the hot spot Pre at the Elbe with a total concentration of 1.62 mg metals g^{-1} sediment dw and the hot spot of the Schijn Een with a total concentration of 1.27 mg metals g^{-1} sediment dw. In general, the concentrations of pollutants were most of the time higher at the hot spots compared to the reference sites. Exceptions were the sites at the Llobregat River, where no clear distinction could be made between concentrations of heavy metals at the site reference site A1 and the hot spot A3. For the organotin compounds the difference between hot spots and reference sites were obvious in each case. Only for tetrabutyltin, the concentrations did not differ a lot between the two sites of the rivers. Highest concentrations were measured for tributyltin, with maximum 83.2 ng g^{-1} sediment dw at the hot spot Pre and for dibutyltin with 34.3 ng g^{-1} sediment dw at the hot spot Een.

Concentrations of all measured steroid estrogens were below the detection limits. The xeno-estrogenic compounds alkylphenols and -ethoxylates were present in all basins, whereby the ethoxylates were mostly detected in the Elbe basin and the alkylphenols in the Scheldt and Llobregat basin. The most polluted site concerning this group of compounds was the freshwater hot spot Een, where amongst others, a concentration of 0.91 ng nonylphenol g^{-1} sediment dw were measured.

Table 2
Characteristics of sediments on a dry weight basis (mean ± SD; $n = 3$; n.a. = not analyzed; n.d. = below detection limit), hot spots are colored grey; dw = dry weight; loi = loss of ignition; OC = organic carbon; N = nitrogen; BC = black carbon.

	Par	Pre	Jir	Mos	Run	Een	Han	Ter	A1	A3	LL4
dw (%)	35.9 ± 0.03	30.7 ± 0.04	28.3 ± 0.02	32.1 ± 0.20	65.5 ± 0.90	24.2 ± 0.10	61.1 ± 0.02	46.3 ± 0.10	58.4 ± 0.10	66.3 ± 0.40	76.4 ± 0.30
loi (%dw)	10.6 ± 0.20	12.2 ± 0.50	13.3 ± 0.20	16.1 ± 0.40	3.20 ± 0.10	14.8 ± 0.10	6.50 ± 0.40	9.00 ± 0.40	6.60 ± 1.00	4.60 ± 0.10	2.70 ± 0.20
OC (%dw)	3.20 ± 0.30	4.20 ± 0.20	3.70 ± 0.30	7.10 ± 1.60	1.50 ± 0.20	4.20 ± 0.60	1.10 ± 0.10	0.60 ± 0.03	3.80 ± 0.50	1.20 ± 0.06	0.70 ± 0.20
N (%dw)	0.20 ± 0.04	0.30 ± 0.01	0.20 ± 0.04	0.30 ± 0.10	0.10 ± 0.01	0.30 ± 0.05	0.03 ± 0.03	n.d.	n.d.	n.d.	n.d.
C:N (g/g)	14.5	13.5	19.0	20.8	15.0	14.9	34.4	n.a.	n.a.	n.a.	n.a.
BC (%dw)	0.17 ± 0.02	0.40 ± 0.10	0.05 ± 0.01	0.10 ± 0.03	0	0.11 ± 0.01	0.02 ± 0.01	0	0.60 ± 0.10	0.03 ± 0.03	0.02 ± 0.01
Particles <63 μm (%)	61.9 ± 3.80	75.3 ± 8.40	67.3 ± 5.93	36.4 ± 1.54	10.0 ± 0.60	49.7 ± 3.00	62.0 ± 2.00	35.5 ± 1.10	71.1 ± 1.50	31.1 ± 0.80	15.8 ± 1.10

Table 3
Concentrations of heavy metals, organotin compounds, PAHs, estrogens and alkylphenols in the sediments, hot spots are colored grey, n.a. = not analyzed.

	Par	Pre	Jir	Mos	Run	Een	A1	A3	LL4
Heavy metals (μg g^{-1} sediment dw)									
As	14.4	42.9	n.a.	56.7	13.8	26.3	8.40	5.20	12.8
Cd	0.83	4.12	n.a.	1.62	0.19	4.15	0.16	0.16	0.34
Cr	53.2	255	n.a.	66.6	12.5	57.6	84.9	35.6	83.1
Cu	31.2	157	n.a.	104	9.50	88.4	23.2	32.7	88.4
Hg	0.16	4.40	n.a.	0.85	0.06	0.30	0.05	0.06	0.21
Ni	27.0	47.3	n.a.	83.3	4.50	23.5	23.2	16.2	47.8
Pb	26.3	97.1	n.a.	35.7	9.00	92.8	18.6	12.6	28.6
U	1.38	3.20	n.a.	3.17	0.33	0.73	3.22	0.65	1.55
Zn	189	512	n.a.	317	58.0	609	74.0	67.0	233
Mn	483	496	n.a.	352	44.0	369	333	314	448
Organotin compounds (ng Sn g^{-1} sediment dw)									
Monobutyltin	22.6	12.8	9.70	17.8	4.02	18.0	5.40	7.20	5.10
Dibutyltin	28.2	29.0	2.30	14.2	1.40	34.3	0.50	2.70	8.35
Tributyltin	6.10	83.2	0.35	3.10	0.13	10.8	0.26	2.60	1.30
Tetrabutyltin	0.06	0.13	0.10	0.06	0.05	0.07	0.03	0.04	0.05
Steroid estrogens (ng g^{-1} sediment dw)									
Estradiol-17-glucuronide	<0.09	<0.09	n.a.	<0.09	<0.09	<0.09	<0.09	<0.09	<0.09
Estrone-3-sulfate	<0.06	<0.06	n.a.	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06
Estriol	<0.05	<0.05	n.a.	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Estradiol	<1.00	<1.00	n.a.	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00
Ethynyl estradiol	<1.00	<1.00	n.a.	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00
Estrone	<0.50	<0.50	n.a.	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50
Diethylstilbestrol	<0.10	<0.10	n.a.	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
Alkylphenols and alkylphenol ethoxylates (ng g^{-1} sediment dw)									
Nonylphenol	0.003	0.001	n.a.	0.001	0.04	0.91	0.09	0.03	0.30
NPE1C	0.00	<0.004	n.a.	0.00	<0.004	0.15	<0.004	<0.004	<0.004
Octylphenol	<0.005	<0.005	n.a.	<0.005	<0.005	0.01	<0.005	<0.005	<0.005
NPE1O	<0.007	<0.007	n.a.	<0.007	<0.007	0.07	n.a.	n.a.	n.a.
NPE2O	0.01	0.04	n.a.	0.14	<0.004	0.01	<0.004	0.01	n.a.
NPE3O	0.02	0.06	n.a.	0.09	<0.001	0.01	n.a.	n.a.	n.a.
PAHs (ng g^{-1} sediment dw)									
Naphthalene	50.0	265	n.a.	461	13.0	58.0	7.00	7.00	n.a.
Acenaphthylene	16.0	27.0	n.a.	55.0	0.50	13.0	<0.30	0.40	n.a.
Acenaphthene	6.00	9.00	n.a.	76.0	4.00	16.0	<0.30	<0.30	n.a.
Fluorene	74.0	111	n.a.	156	4.00	33.0	1.00	0.90	n.a.
Phenanthrene	731	937	n.a.	1036	41.0	178	<6.00	<4.00	n.a.
Anthracene	97.0	2793	n.a.	169	11.0	62.0	1.00	0.90	n.a.
Fluoranthene	1216	1358	n.a.	1142	83.0	414	3.00	7.00	n.a.
Pyrene	759	1115	n.a.	1225	60.0	366	4.00	7.00	n.a.
Benz(a)anthracene	420	786	n.a.	439	51.0	213	2.00	5.00	n.a.
Chrysene	480	934	n.a.	495	44.0	283	2.00	6.00	n.a.

Highest concentrations of PAHs were measured at the sites in the Elbe basin. The most polluted site was the hot spot Pre at the Elbe with a total concentration of 3849 ng PAH g^{-1} sediment dw, followed by the other hot spot in that river basin at Mos with 5254 ng PAH g^{-1} sediment dw. The freshwater hot spot of the Scheldt basin at Een contained 1636 ng PAH g^{-1} sediment dw and the hot spot A3 at the Llobregat basin was less polluted with PAHs, whereby a total value of 34.0 ng PAH g^{-1} sediment dw was calculated. In general, highest concentrations were measured for Flouranthene, Pyrene and Anthracene.

3.3. Yes

The yeast estrogen screen, which is shown in Fig. 1 revealed a quantifiable estrogenic activity, measured as ng E2 equivalents (eq) kg^{-1} sediment dw for seven of the eleven samples including both reference sites and hot spots. In general, for each river basin, the hot spots showed higher estrogenic potential compared to their corresponding reference site. For the Elbe basin, the hot spot Most at the river Bilina was the only site which showed a detectable estrogenic effect whereby 75.8 ng E2 eq kg^{-1} sediment dw

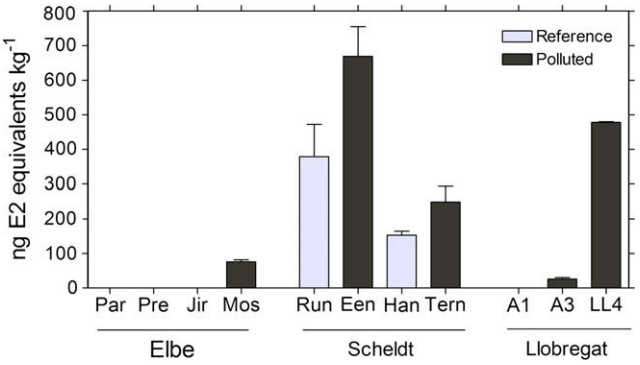

Fig. 1. Estrogen equivalence values (mean ± SD) calculated for extractions of sediments of the river basins Elbe, Scheldt and Llobregat; $n = 2$; for abbreviations see Table 1.

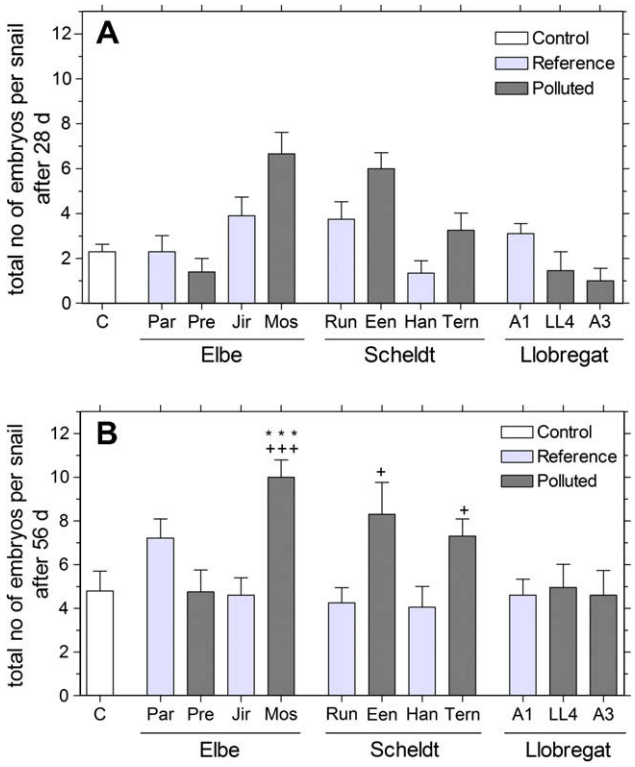

Fig. 2. Mean number (±SEM) of *P. antipodarum* embryos per adult snail, exposed for 28 (A) and 56 (B) d to sediments of the river basins Elbe, Scheldt and Llobregat; $n = 20$; ★ significant differences to control; + significant differences to corresponding reference site; + $p < 0.05$, ★★★/+++ $p < 0.001$; Mann–Whitney test; for abbreviations see Table 1.

were calculated. For both of the Llobregat hot spots, estrogenic effects were measured with the highest value of 478 ng E2 eq kg^{-1} sediment dw at LL4. Overall the Scheldt basin showed the highest estrogenic potency. While 379 ± 93.2 ng E2 eq kg^{-1} sediment dw were found for the freshwater reference site of the Scheldt basin, almost twice as high concentrations were calculated for the hot spot Een (669 ± 85.6 ng E2 eq kg^{-1} sediment dw). Compared to the freshwater part, the marine part of the Scheldt showed lower estrogenic potency, with about two times higher concentrations at the hot spot (249 ± 44.9 ng E2 eq kg^{-1} sediment dw) than at the reference site (153 ± 11.5 ng E2 eq kg^{-1} sediment dw).

3.4. Sediment contact test

The results of the sediment contact test with *P. antipodarum* after 28 and 56 d, which are shown in Fig. 2 reveal a similar picture. The reproduction of the snails was increased by the polluted sediments at three of the six rivers. While the differences in reproduction were not significant after 28 d of exposure (Fig. 2A), high significant differences were detected after 56 d (Fig. 2B). At that point if time, the reproduction of the snails in the reference sediments was with between four and five embryos per snail at the same level as observed for the artificial sediment control. In contrast, three of the polluted sediments led to a significantly increased reproduction (Mann–Whitney test $p < 0.001$–0.05) compared to their corresponding reference site and/or the control sediment. This phenomenon was observed for the sites at the rivers Schijn, Scheldt and Bilina but not for the Elbe and Anoia/Llobregat.

In addition, the growth of the snails appeared to be unaffected by the sediments and no mortality was observed in any of the sediments during the whole experiment (data not shown).

4. Discussion

While comparing the results of the YES carried out using extracts of the sediments with the results of the *Potamopyrgus antipodarum* sediment contact test, some parallels are obvious. In both tests, the sediments from the hot spots at the rivers Bilina, Schijn and Scheldt led to an increasing reproduction ($p < 0.05$ Mann–Whitney test) and increasing estrogenic activity compared to their reference sites. The sediment contact test showed this trend already after 28 d, but significant differences were only obvious after 56 d of exposure. Regarding the YES, it has to be taken into account that, due the test design and the calculation of E2 equivalents, no well-funded statistical analyses could have been carried out. However, obviously increased *in vitro* estrogenicity together with a higher reproduction of *P. antipodarum* has been detected for three of the hot spots. Though, there are some differences concerning the intensity of effects *in vivo* and *in vitro*. While the site Mos showed the highest effect in the sediment contact test, the E2 equivalent values which were calculated in the YES are relatively low. Those differences could be explained with compounds whose endocrine effects cannot be detected with the YES (e.g. androgens/anti-androgens). Recent studies have shown that e.g. PAH can have anti-androgenic potency (Weiss et al., 2009). The anti-androgenic compounds may also lead to a higher reproduction in the snail, but cannot be detected with the YES, because the estrogen receptor in the target. In contrast, for the polluted site of the river Llobregat LL4 an increased estrogenicity was detected *in vitro* but no effects on the snail's reproduction were observed at this site. One reason for this phenomenon could be differences in bioavailability of compounds, which is higher in the YES than in the raw field samples. For the YES a total extraction of the sediment was performed, while for the contact test, *P. antipodarum* is only exposed to the bioavailable compounds. Another possible reason could the masking of estrogenic or anti-androgenic effects with their antagonists, which has been recently demonstrated by Weiss et al. (2009).

While comparing the results of the biotests with the results from the sediment analyses, two issues are important. First, the chemical analysis of metals, organotin compounds, estrogens, alkylphenols and PAHs reflects a partially very high contamination of especially the hot spots. Thus, the classification which has been done according to the monitoring data prior to the study is confirmed by the real measured concentrations. Secondly, while the results of the field sediment contact test with *P. antipodarum* and the *in vitro* YES test also indicate differences between hot spot and reference sediments, the physico-chemical properties do not show the same distinction. Differences in sediment dry weight,

loss of ignition, C:N ratio and particle size distribution occur between all sites and samples. They are not correlated with the assay results (Pearson correlation, $p > 0.05$) and do not show a clear pattern. Although the organic carbon content, as a measure of food quantity for the snail assay, is often higher at the polluted sites, this is not true for the site Ter, were a high reproduction together with a low organic carbon content was observed. Thus, the assumption that food supply could play an important role for the reproduction of *P. antipodarum* cannot be confirmed. This again proves the robustness of *P. antipodarum* against natural stressors as it was already shown by Alonso and Castro-Díez (2008). It is thus most likely that certain groups of compounds may be responsible for the increase in reproduction at the three polluted sites. From the literature it is known that *P. antipodarum* is a very sensitive organism concerning endocrine disrupting compounds (Duft et al., 2007) and even an upregulation of the snails' estrogen receptor under exposure to 17α-ethinylestradiol (EE2) has been reported recently by Stange et al. (2009). Some studies reported a stimulated reproduction after exposure to field samples (water or sediment) (Oetken et al., 2005; Mazurovà et al., 2008) and direct evidence for estrogenic exposure as a cause could be provided (Jobling et al., 2004). Thus, keeping in mind the estrogenic activity which has been observed in the YES, one can assume that certain endocrine disrupting compounds may play an important role in order to explain the increase in the reproduction of *P. antipodarum*. The first suspects are of course natural/synthetic estrogens and xeno-estrogens like for example alkylphenols. Although no steroid estrogens could be detected in any of the sediments, they still could be of importance, because those compounds are already potent in low concentration ranges (Thorpe et al., 2003). Presuming a worst case scenario and taking the detection limits as real concentrations, an E2 equivalence concentration can be calculated for the sites by means of summing up the known relative potencies of estrone, estradiol, and EE2 as described in Rutishauser et al. (2004). This calculation resulted in an E2 equivalence concentration of 2380 ng kg^{-1} sediment dw. Taking into account the maximum E2 equivalence concentration of 669 ng kg^{-1} sediment dw which has been calculated with the YES (at the site Een), the steroid estrogens could be one of the main causes for the observed effects. In contrast to the steroid estrogens, the alkylphenols could be detected in various sediments. The estrogenic activity of nonylphenol is very well known (Bonefeld-Jorgensen et al., 2007) and the snail's sensitivity (with a LOEC of 0.01 μg nonylphenol g^{-1} sediment dw) has already been reported by Duft et al. (2003b). Summarizing those facts, it is quite likely that the observed effects at the site Een could be due to the contamination with nonylphenol. Another remarkable fact is the apparently high tolerance of *P. antipodarum* towards partially very high concentrations of heavy metals and organotin compounds. Especially at the hot spot Pre at the Elbe, the concentrations of Cd, Cu and tributyltin should have an impact on the snail's survival, growth or reproduction (Dorgelo et al., 1995; Duft et al., 2003a). However, no significant correlations (Pearson correlation, $p > 0.05$) were calculated between those parameters. Thus, as already stated by Hollert et al. (2005), chemical analysis of field samples on its own is very limited and may not tell us the whole story. The focus should always be on integrated approaches like e.g. effect directed analysis, including the outcome of different bioassays, biomarker studies and other bioanalytical tools (Brack, 2003).

5. Conclusions

The sediment contact test with *Potamopyrgus antipodarum* has shown its applicability for a variety of sediments from different geographical regions. The observed increased reproduction at the hot spots was not caused by variances in physico-chemical properties but rather confirmed by the yeast estrogen screen and partially also by measured concentrations of xeno-estrogens (nonylphenol). *P. antipodarum* is a very promising test organism regarding the risk assessment of endocrine disrupting compounds. Biotests using this organism should be further developed and tested for their adaptability for e.g. effect directed analysis or *in situ* experiments.

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