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Prosobranch snails as test organisms for the assessment of endocrine active chemicals—an overview and a guideline proposal for a reproduction test with the freshwater mudsnail *Potamopyrgus antipodarum*

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Abstract Recently, prosobranch snails have been recommended as promising candidates for test organisms for the assessment of endocrine active chemicals. Three prosobranch snail species, the freshwater mudsnail *Potamopyrgus antipodarum*, the freshwater ramshorn snail *Marisa cornuarietis*, and the marine netted whelk *Nassarius reticulatus* are portrayed and their respective biotests are presented together with results of laboratory experiments and biological effect monitoring surveys in the field. All characterized species are highly sensitive toward xeno-androgens [triphenyltin (TPT), tributyltin (TBT), methyltestosterone (MT) and fenarimol (FEN)], and xeno-estrogens [bisphenol A (BPA), octylphenol (OP), ethinylestradiol], and show effects at environmentally relevant, rather low concentrations in laboratory experiments. For exposure to the xeno-androgen TPT, EC_{10} values range between 15.9 and 29.0 ng as Sn/L (sediment 0.03 µg as Sn/kg), for TBT, EC_{10} values are found between 3.42 and 37.8 ng as Sn/L (sediment 2.98 µg as Sn/kg) and effect concentrations for FEN are calculated as 18.6 ng/L (EC_{10}) and 0.19 µg/kg (EC_{50} sediment; EC_{10} not calculable). Exposure to xeno-estrogens yielded EC_{10} values of 13.9 ng/L (0.19 µg/kg) for BPA, a NOEC of <1 µg/L (EC_{10} of 0.004 µg/kg) for OP and a NOEC of 1 ng/l (EC_{10} sediment of 2.2 µg/kg) for ethinylestradiol. Responses to androgens comprised the development of imposex and the reduction of fertility or embryo production, effects of estrogens included the stimulation of egg production and embryo production, and the increased weight of glands. Also, biological effect monitoring studies with *P. antipodarum* and *N. reticulatus* in several rivers or estuarine areas revealed the capacity of the biotests to detect an androgenic or estrogenic potential of sediment samples. A comparison of the three test species with regard to sensitivity and practical aspects in routine application favors the freshwater mudsnail *P. antipodarum* for a standardized procedure, and this reproduction test will be introduced into the OECD guideline program for standardization in the near future.

Keywords Biotest · Endocrine disruptors · Prosobranch snails · *Potamopyrgus antipodarum* · *Marisa cornuarietis* · *Nassarius reticulatus* · Standard test

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Introduction

A variety of xenobiotics in the environment have been shown to induce adverse effects in animals and humans by interfering with their endocrine functions with different modes of action. These so-called endocrine disruptors, endocrine modulators or hormone-mimetic substances have been associated with a decrease in sperm counts in man (Swan et al. 2000), increased frequencies of sex-hormone dependent cancers (breast, testis, prostate, etc.), genital abnormalities, premature

puberty in females, and increased occurence of endometriosis in humans (Gist 1998). The European Commission defines endocrine disruptors as ''exogenic compounds which negatively affect the health of an intact organism or its offspring by interference with its endocrine function''. Endocrine disrupting chemicals (EDCs) are environmental chemicals, which directly or indirectly influence the hormonal system and may be active at low concentrations.

Our knowledge about the effects of these substances, especially on invertebrates, is limited. Therefore, the development and standardization of biotests using invertebrates, which are sensitive to endocrine disrupting chemicals, faces driving attention, and is broadly supported by international committees, e.g. the ''Ad hoc OECD Expert Group on Invertebrate Testing''. Whole-sediment biotests with sediment-dwelling organisms as test species are especially rare and of particular interest since it is essential to obtain information on effects in the sediment compartment, the so-called ''memory of the water''. This meets an urgent demand, since analytical measurements in water, sediment and tissue are rather time-consuming, and expensive and besides, restricted to the respective detection limit. Hence, biomonitoring represents a more economical and more relevant method.

Recent studies recommend molluscs and particularly snails as most sensitive organisms concerning effects of endocrine disruptors in invertebrates (e.g. Matthiessen and Gibbs 1998). During the EDIETA workshop on Endocrine Effects in Invertebrates in the Netherlands in 1999, insects and prosobranch snails were presented as most promising candidates in the development of tests for the assessment of endocrine disruptors (deFur et al. 1999).

Prosobranch snails are important members of aquatic habitats and possess a high ecological relevance for marine and freshwater ecosystems. Their hormonal system is for a large part comparable to that of vertebrates (and humans), which makes them particularly qualified and promising test organisms for the identification of endocrine disrupting chemicals. A detailed description of the hormonal system of prosobranchs is beyond the scope of this article but outlined by Lafont and Mathieu (2006) and Janer and Porte (2006). Besides, prosobranch snails probably represent the best-documented and population-relevant case of endocrine disruption in wildlife, with the development of imposex by tributyltin (TBT) being one of the most prominent examples of the effects of an endocrine disrupting substance (for review see Matthiessen and Gibbs 1998; deFur et al. 1999). There is now an understanding of the effects at all levels of organization from molecules to populations and possibly communities, all of which present opportunities for the observation of effects in field samples. In recent years however, most endocrine disruptor research was focused on estrogens, and here mainly on vertebrate testing. This work aims at giving a comprehensive overview of effects of both xeno-estrogens and xeno-androgens on prosobranch snails as important invertebrate representatives, and at deriving recommendations for the application of the most suitable biotest as routine standard test.

Portrait of three candidate prosobranch snail species

The freshwater mudsnail *Potamopyrgus antipodarum*

The freshwater mudsnail *P. antipodarum* (Gray 1843) belongs to the phylum Mollusca, class Gastropoda, sub-class Prosobranchia, order Mesogastropoda, and family Hydrobiidae. It originates from New Zealand, but has been introduced to other parts of the world with ballast water of ships. In Europe, the snail is native for over 150 years, yet a ''newcomer'' to European freshwater ecosystems. Since then, the species was known in Europe as *Potamopyrgus jenkinsi* (Ponder 1988). Typical habitats are running waters from small creeks to streams, lakes, and estuaries, where its reproduction is often very intensive (Kinzelbach 1995; Cope and Winterbourn 2004). The shell height of adult snails averages about 4.3 mm and can reach up to 6 mm (Fig. 1). *P. antipodarum* is predominantly living in freshwater, but it is also able to survive and reproduce in brackish water with a salinity up to 15% (Jacobsen and Forbes 1997). Mudsnails prefer living in or on soft sediments of standing or slowly

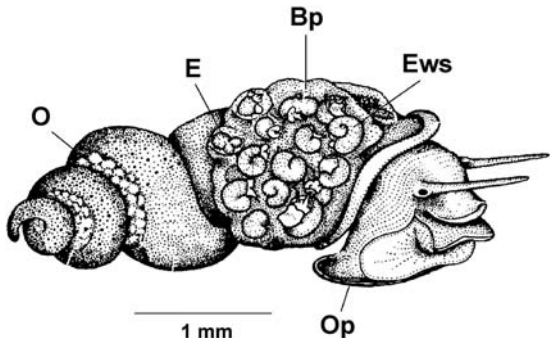

Fig. 1 *Potamopyrgus antipodarum*, female, after removal of the shell (modified after Fretter and Graham 1994). *O* ovary, *Bp* brood pouch, *E* ''new'' embryo without shell (unshelled), *Ews* embryo with shell, *Op* operculum

flowing waters as well as in estuarine areas on the coasts. Being an euryhaline species they exist also in some regions of the Baltic Sea and in estuaries of the North Sea and Atlantic in co-existence with other hydrobiid (mudsnail) species. During dry or cold periods, mudsnails live completely buried in the sediment. They feed on detritus, algae, and bacteria, which are rasped from the surface of plants, stones or sediment. In their ancestral distribution area, populations exhibit an almost balanced ratio of males to females with a sympatric coexistence of biparental and parthenogenetic populations. European populations consist almost entirely of female snails reproducing parthenogenetically. In this way a single snail is capable of establishing an entire population. In Europe, male snails are found only rarely (Wallace 1979; Ponder 1988) and have never been observed in our laboratory culture. Hauser et al. (1992) found that there are three genotypes in Europe (Euro A, Euro B, and Euro C) of which Euro A is spread in freshwater ecosystems in continental Europe, Euro B is present in estuaries of the Baltic Sea, and Euro C is found in Great Britain.

Reproduction occurs throughout the year, although there are periodical fluctuations concerning the quantity of offspring (Scholz 2003; Schmitt et al. 2006). *Potamopyrgus antipodarum* performs a very distinct kind of brood care. The eggs grow in the anterior part of the pallial oviduct section, which is transformed into a brood pouch. Older embryos are situated in the anterior and younger embryos in the posterior part of the brood pouch. Embryos are released through the female aperture when the egg shell tears open. This kind of reproduction is called ovovivipary (Fretter and Graham 1994). After removing the shell of the snail, embryos can easily be seen through the epithelia (Fig. 1). By opening the brood pouch and subsequently removing and counting the embryos, the reproductive output of each female is easy to determine.

The laboratory culture was initiated with snails originating from a natural population in the Gievenbach, a small creek in Dörente, near Ibbenbüren (North Rhine-Westphalia, Heinsberg, Germany). It was shown that this population (like other populations sampled in Germany) belongs to the genotype Euro A, which is spread in freshwater ecosystems in continental Europe (unpublished results). The snails are kept at 15°C in aerated 10-L aquaria in artificial freshwater (0.5 g $NaHCO_3$, 5 g $CaCO_3$, and 5 g mineral salt per 10 L demineralized water) and fed regularly with a mixture of Tetra Phyll® (Tetra, Melle, Germany) and Fish Tamin® (Sera, Heinsberg, Germany), stirred in artificial medium (as described above). Additional calcium carbonate is added regularly to improve shell growth. The stock population density should not exceed 150 snails per litre.

The freshwater ramshorn snail *Marisa cornuarietis*

The freshwater ramshorn snail *M. cornuarietis* (Linné 1767) belongs to the phylum Mollusca, class Gastropoda, sub-class Prosobranchia, order Mesogastropoda, family Ampullariidae, and inhabits standing and slowly flowing water bodies in South, Middle, and North America, preferrably hard water. The species is gonochoristic. Originally, the ramshorn snail was not common in the South of the United States, but since the 1950s, it could extend its distribution (Hunt 1958). Today, it is present and highly abundant especially in the state of Florida, due to the favorable climate conditions. More than 10 years ago, *M. cornuarietis* was attributed high importance in its original habitats because of its significance in view of the combat of bilharziosis, as this species was believed to feed extensively on spawn of the *Schistosoma* host *Biomphalaria glabrata* (Jobin and Laracuente 1979).

The specimens for the reported experiments came from a laboratory breeding stock which was maintained at a temperature of 22 ± 1°C. The stock was derived from Aquazoo Düsseldorf (Germany) in 1991 with regular crossbreeding of wild-caught animals from Florida to avoid inbreeding. The snails are kept in 60 or 250-L aquaria in tap water and fed daily with Tetra Min® (Tetra) ad libitum alone or supplemented with untreated lettuce.

The netted whelk *Nassarius reticulatus*

The marine netted whelk *N. reticulatus* belongs to the phylum Mollusca, class Gastropoda, sub-class Prosobranchia, order Neogastropoda, family Buccinidae. This gonochoristic species is frequently found at European coasts and reaches shell heights of up to 40 mm. It is a sediment-dwelling scavenger feeding on carrion and lives burrowed in coastal fine sediment, which is usually only left for feeding. Sandy sediments are preferred habitats, but mud, coarse sands, and scree are also tolerated, and the species is even abundantly found in sandy hollows at rocky shores exposed to tides (Fretter and Graham 1994). However, most individuals of a population live subtidally in a depth of 15 m. They are spread from the Canary Islands and the Azores in the South up to Trondheim in the North, and also British and Irish coasts are inhabited. In the Mediterranean Sea and the Black Sea, the species lives only subtidally. As the species can

cope with lower salinities, it was also common in the Baltic Sea up to the Kieler Bucht (16‰ salinity; Tallmark 1980; Fretter and Graham 1994). On the German coast of the North Sea, the netted whelk was common in former times (Kuckuck 1953). However, during the second half of the 20th century and due to pollution of the Atlantic and the Baltic Sea with various contaminants, the species is nowadays extinct in this area, with the exception of one protected population in the area between Germany and Denmark.

The specimens used for the tests were collected in Brittany, France, at Pléneuf Val André, and kept at 15°C in the laboratory in aerated 80-L aquaria in artificial seawater (Instant Ocean, Sera, Heinsberg, Germany). The netted whelks are fed with untreated meat (pork or beef heart) once a week.

Overview of test systems with the three snail species, their application and performance

Biotest with *Potamopyrgus antipodarum*

The experiments are conducted as static systems (without water renewal) in 1-L glass Erlenmeyer flasks or glass beakers. 1 L of artificial medium (see above) is added to the flasks, which are subsequently aerated through glass pipettes (compressed air), enabling manual adjustment of air supply. For sediment exposure, 50 g of artificial sediment (dry weight, 95% quartz sand, and 5% crushed untreated beech leaves), and 1 L medium is added to each flask. This sediment assures an optimal embryo production of *Potamopyrgus* compared to other artificial and even natural sediments (Duft 2004). The organic carbon content of the artificial sediment should be ca. 2.3%, the mean grain size about 180 µm. In case of substance testing, the chemical is spiked into the sediment (or water) by applying about 2 mL of the respective concentration (dissolved in 100% ethanol or equivalent) to each treatment and homogenising by stirring. Complete removal of the solvent in sediment should be guaranteed by allowing sufficient time for evaporation. Finally, 80 adult female *Potamopyrgus* specimens (shell height 3.7–4.3 mm, aged about 3–6 months) are added to each vessel. At least five concentrations with minimum two replicates should be tested in a geometric series with a factor between concentrations not exceeding 2.2.

All tests are performed under constant conditions in a climate chamber with a temperature of 15 ± 1°C and a light:dark rhythm of 16:8 h. Test duration is 28 or 56 days. A subgroup of 20 snails is analyzed individually after 0, 14, 28 and 56 days, respectively. Prior to analysis, the snails are narcotized in $MgCl_2$ (2.5% in distilled water) for 2 h. Shell and aperture heights are measured, after which shells are cracked by a small vice and shell parts are removed. After removal of the shell, embryos can easily be seen through the epithelia. The brood pouch is opened carefully and the number of ''grown-up'' embryos (with shells) and ''new'' embryos (without shells) are removed and counted using a dissecting microscope (Fig. 1). Additionally, occurrence of egg cells in the oviduct and the maturity of the ovary are noted for each individual. Adult mortality is recorded and dead snails are removed.

Data are analyzed statistically either by using a regression model (e.g. non-linear regression according to a reparameterized Weibull model; compare Duft et al. 2003a; Weltje et al. 2004) in order to estimate the concentration that would cause x% of maximum effects (e.g. EC_{10} and EC_{50}), or by testing a statistical hypothesis (e.g. ANOVA followed by Tukey's multiple comparison of the means or non-parametric analogues, depending on the data) to determine the No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) values. Replicates in a given treatment group were tested for statistical differences using a *t*-test or a *U*-test. In case the test did not identify a significant difference, the replicates were merged. The software package Prism®, Version 4.03 (GraphPad Software, San Diego, CA, USA) for Windows NT may be applied for the analyses. Following validity criteria should be fulfilled:

- mortality in the controls should not exceed 20% and
- the dissolved oxygen concentration should be at least 60% of the air saturation value throughout the test.

The biotest and its application and performance is described more detailed by Schulte-Oehlmann et al. (2001a), Duft (2004), and Duft et al. (2003a, b, 2005). Test protocols are available for culturing and for testing in water and in sediment (Schmitt et al. 2006) and are currently being processed in the OECD ''Ad hoc Expert Group on Invertebrate Testing'' for standardization.

Biotest with *Marisa cornuarietis*

The experiments with adult *M. cornuarietis* are conducted as 24 h (weekends 48 h) semi-static renewal systems in 60-L glass aquaria filled with charcoal-filtered tap water or fully reconstituted water and provided with an Eheim power filter. Tests are performed under constant conditions at a temperature

of 22 ± 1°C (if nothing else is specified in the results section) and at a light:dark rhythm adjusted to 12:12 h. Test duration varies from 3 to 6 months.

About 30 specimens are analyzed at the beginning of the experiment and in monthly intervals from each group. Additionally, a complete life-cycle test can be added with an exposure *ex ovo* over a period of 12 months until the hatched F1 specimens are 1-year-old (compare Oehlmann et al. 2000). During the experiments, the production of spawning masses, the number of eggs in each of the aquaria and the mortality are recorded in daily intervals.

All specimens are narcotized prior to analysis (2.5% $MgCl_2$ in distilled water). The individual shell and aperture height are measured to the nearest 0.1 mm before the shell is cracked. The presence, normal appearance and extension to the nearest 0.1 mm of all sex organs are checked, as well as the occurrence of oocytes and sperm in the genital system and of visible excrescences on genital and other organs with a dissection microscope. Additionally, imposex parameters like the VDSI (Vas Deferens Sequence Index = mean value of imposex stages in a sample with values from 0 to 3) are calculated (for details see Oehlmann et al. 1991; Stroben et al. 1992; Schulte-Oehlmann et al. 1995). Furthermore, a histopathological analysis of the gonads (six males and six females specimens) may be performed. Therefore, samples are fixed in Carnoy's and Bouin's fluid, respectively, and then preserved in ethanol. After embedding in Paraplast, serial sections (5–7 µm) are made and stained with haemalum-chromotrope. The sections are analyzed by microscope, preferrably using an image analysis system. Specimens found to be afflicted with parasites, e.g. trematode larvae, should be excluded from the evaluation.

Standard statistical analyses of the results, e.g., analyses of covariance (ANCOVA) and analyses of variance (ANOVA) with multiple comparison of samples according to Tukey (low n) or Student-Newman-Keuls (high n), EC_{10} and EC_{50} calculations (probit analysis, maximum likelihood method), x^2-test, and Weir test for classified values are performed, using the computer programme StatEasy for Windows NT.

For the *Marisa* test, a SOP on culturing is in preparation. The test and its application and performance is described more detailed by Oehlmann et al. (2000) and Schulte-Oehlmann et al. (1995, 2000, 2001b).

Biotest with *Nassarius reticulatus*

Adult netted whelks may be exposed to water only or water-sediment systems. For substance testing, spiked artificial sediments are used, consisting of 90% quartz sand and 10% peat (untreated). For each treatment, 750 g fresh sediment is weighed into a 10-L aquarium and covered with artificial sea water (Instant Ocean, Sera, Heinsberg, Germany). Tests are performed under constant conditions at a temperature of 14 ± 1°C and a light:dark rhythm of 12:12 h. Test duration is 1–3 months. A solvent control (glacial acetic acid or ethanol) should be included in the test, if appropriate. 30 specimens are analyzed at the beginning of the experiment and from each group in monthly intervals.

During the experiments the production of spawning masses with the number of eggs in each of the aquaria and the mortality are recorded twice a week. All specimens are narcotized prior to analysis (7% $MgCl_2$ in distilled water). The individual shell and aperture height are measured to the nearest 0.1 mm before the shell is cracked. The presence, normal appearance and extension to the nearest 0.1 mm of all sex organs is checked, as well as the occurrence of oocytes and sperm in the genital system and of visible excrescences on genital and other organs with a dissection microscope. Additionally, imposex parameters like the VDSI are calculated (with values from 0 to 4, see above *Marisa* test). Furthermore, a histopathological analysis of the gonads may be performed for all treatment groups, the analysis being similar to the above-mentioned *Marisa* test. Serial sections (3–7 µm) are made and stained with haemalum chromotrope, haematoxylin eosin, and periodic acid Schiff.

Statistical analyses are analogous to those described for the *Marisa* test. The test and its application and performance is described more detailed by Schulte-Oehlmann et al. (2000, 2001a) and Tillmann (2004).

Results

Laboratory experiments with the freshwater mudsnail *Potamopyrgus antipodarum*

The exposure to various xeno-androgens [triphenyltin (TPT), tributyltin (TBT), and methyltestosterone (MT)] resulted in a marked decrease in the number of embryos in the brood pouch of *P. antipodarum*. The number of unshelled embryos turned out to be the most sensitive parameter.

As an example, the response to TPT during sediment exposure is shown here (compare Duft et al. 2003a). A decline of the embryo numbers was observed already after 4 weeks of exposure (results not shown). The production of "new", unshelled embryos was significantly inhibited especially at

lower concentrations (10, 25, 50, and 75 µg-Sn/kg) resulting in a kind of *u*-shaped curve, which may indicate the disturbance of an endocrine function. After 8 weeks, the decline of the embryo production was most conspicuous (Fig. 2a): the number of unshelled embryos was significantly lower in all tested concentrations compared to the solvent control sediment, hence the LOEC was 10 µg TPT-Sn/kg, the corresponding NOEC below this value. The calculation of effect concentrations yielded even lower values: the calculated EC_{50} turned out to be 0.74 µg TPT-Sn/kg, the EC_{10} was 0.03 µg TPT-Sn/kg (Table 1). The TPT inhibited the embryo production down to 25% and less in most exposure groups. The solvent, ethanol, had no effect on embryo production. No significant mortality occurred during the experiments with TPT.

Exposure to TPT via water, conducted as experiments during the EU-project COMPRENDO (project code: EVK1-CT-2002-00129) resulted in an identical response pattern. The total embryo number and the

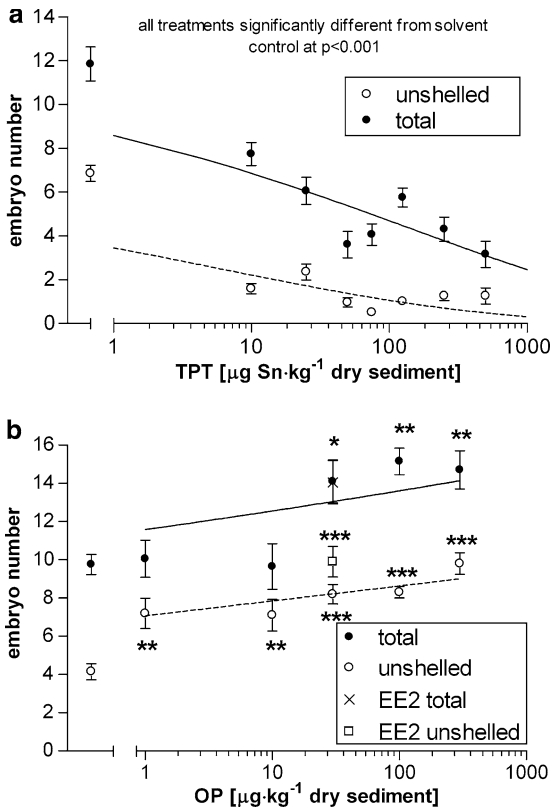

Fig. 2 Laboratory experiments with *Potamopyrgus antipodarum*. Effects of sediment concentrations (µg/kg d.w.) on embryo numbers (without shell and total) of *P. antipodarum* after 4 weeks of exposure. (**a**) Triphenyltin (*TPT*). (**b**) Octylphenol. Regression lines are added—*solid line* for the total embryo number, *dotted line* for the unshelled embryo number. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; for TPT, all treatments are significantly different from the solvent control with $p < 0.001$

number of unshelled embryos significantly decreased after 2, 4, and 8 weeks exposure at concentrations of 60, 125, and 250 ng as Sn/L, compared to the solvent control. An EC_{50} of 68 ng as Sn/L and an EC_{10} of 29 ng as Sn/L for unshelled embryo number was calculated after 8 weeks exposure (Table 1).

The same type of results, namely the decline in embryo production was recorded during experiments with TBT in spiked sediments (Duft et al. 2003a) and in spiked water (EU-project COMPRENDO; Schulte-Oehlmann 1997), resulting in an EC_{50} of 115 ng as Sn/L and an EC_{10} of 37.8 ng as Sn/L (see Table 1), and a LOEC of 50 to 100 ng as Sn/L after 8 weeks exposure (Schulte-Oehlmann 1997). Also, MT inhibited the reproductive output of the freshwater mudsnail in a similar way as described above (see Table 1), with an EC_{50} of 160 ng/L and an EC_{10} of 12 ng/L. Fenarimol (FEN) showed no significant effects on embryo production during the COMPRENDO experiments. Scholz (2003) observed a significant reduction of the embryo production after 4 weeks only at the lowest tested concentration of 13.2 ng/L, and after 8 weeks only at the highest concentration of 331 ng/L.

Contrarily, exposure to various xeno-estrogens (bisphenol A (BPA), octylphenol (OP), nonylphenol, ethinylestradiol) resulted in a marked stimulation of the number of embryos in the brood pouch of *P. antipodarum*. Again, one representative substance, OP, is presented here (compare Duft et al. 2003b).

For OP, a significant increase in the number of unshelled embryos was noticed already after 2 weeks for all treatments (except for 10 µg OP/kg). After 4 weeks, the effect was more evident: The number of unshelled embryos was significantly increased in all tested concentrations of OP (Fig. 2b) with up to 130% above the embryo number in the control sediment. The corresponding LOEC for the embryo production was 1 µg OP/kg (NOEC <1 µg/kg) and the estimated EC_{10} (10% stimulation) yielded a very low concentration of 4 ng/kg (EC_{50}, 50% stimulation: 0.07 µg/kg, Table 1). The total embryo number confirmed this tendency, with a corresponding LOEC of 30 µg OP/kg. Calculation of the EC_{10} resulted in a value of 2.11 µg/kg (EC_{50} 108.3 µg/kg, Table 1). After 8 weeks, a typical inverted *u*-shaped concentration-response curve was observed: at higher concentrations of 100 and 300 µg OP/kg, the number of unshelled embryos was only slightly elevated. All other treatments showed a highly significant stimulation of the embryo production with an increase of up to 150% more than in the control, thus the LOEC was again 1 µg OP/kg. The calculation of the EC_{10} and EC_{50} (<1 µg/kg) was not possible. The solvent control had no effect on embryo production.

Table 1 Overview of effect concentrations (EC_{10} and EC_{50}) as determined in biotests with the prosobranch snail species *Potamopyrgus antipodarum* (after 8 weeks), *Marisa cornuarietis* (after 5 months or life-cycle) and *Nassarius reticulatus* (after 4 weeks), in ng/L for water exposure and µg/kg d.w. for sediment exposure

	Potamopyrgus antipodarum		*Marisa cornuarietis*		*Nassarius reticulatus*	
Androgens						
Tributyltin	Water[a]:	EC_{10} 37.8 EC_{50} 115	Water[a]:	EC_{10} 3.42 EC_{50} 98.2	Water[i]:	NOEC <5
	Sediment[b]:	EC_{10} 2.98 EC_{50} 64.0			Sediment[c]:	EC_{10} 2.9 EC_{50} 16.9
Triphenyltin	Water[a]:	EC_{10} 29 EC_{50} 68	Water[a]:	EC_{10} 15.9 EC_{50} 38.8	Water:	–
	Sediment[b]:	EC_{10} 0.03 EC_{50} 0.74			Sediment:	no effect
Fenarimol	Water:	no effect	Water[a]:	EC_{10} 18.6	Water:	–
	Sediment:	–			Sediment[c]:	EC_{10} n.d. EC_{50} 0.19
Methyltestosterone	Water[a]:	EC_{10} 12.0 EC_{50} 160	Water[a]:	EC_{10} 35.9	Water:	–
	Sediment:	–			Sediment:	–
Estrogens						
Bisphenol A	Water[d]:	NOEC 1,000	Water[f]:	EC_{10} 13.9	Water:	–
	Sediment[e]:	EC_{10} 0.19 EC_{50} 5.67			Sediment[c]:	NOEC 100
Octylphenol	Water[d]:	NOEC 1,000	Water[g]:	NOEC <1,000	Water:	–
	Sediment[e]:	EC_{10} 0.004 EC_{50} 0.07			Sediment[c]:	EC_{10} n.d. EC_{50} 40.7
Ethinylestradiol	Water[d]:	NOEC 5,000	Water[h]:	NOEC 1	Water:	–
	Sediment:	–			Sediment[c]:	EC_{10} 2.2 EC_{50} 28.9

[a] COMPRENDO project, [b] Duft et al. 2003a, [c] Tillmann 2004, [d] Casey 2000, [e] Duft et al. 2003b, [f] Oehlmann et al. 2005, [g] Oehlmann et al. 2000, [h] Schulte-Oehlmann et al. 2001a, [i] Stroben 1994. *n.d.* not determined

No significant mortality occurred during the experiments with OP. Another study investigated exposure to OP via water and also showed a stimulation of the embryo production, with significant effects at a concentration of 5 µg/L (NOEC 1 µg/L) and clearly inverted *u*-shaped concentration-response curves after 3 and 9 weeks exposure (Casey 2000).

Also for the other tested xeno-estrogenic substances, the response pattern described above was observed. In the case of BPA, a similar massive stimulation of the reproductive output was noted, with an EC_{50} of 5.67 µg/kg after 4 weeks and an EC_{10} of 0.19 µg/kg (Duft et al. 2003b). Exposure to BPA via water was investigated in another study, again resulting in a stimulation of the embryo production, with significant effects at a concentration of 5 µg/L (NOEC 1 µg/L) and clearly inverted *u*-shaped concentration-response curves after 6 and 9 weeks exposure (Casey 2000).

In general, shelled and unshelled embryo numbers are not independent for each snail individual, but mostly do correlate. While the latest results (EU-project COMPRENDO) were obtained using the test protocol described before, other results were achieved applying slightly different protocols with regard to replicate numbers and statistical treatment of data, of which details are described in the respective publications.

Laboratory experiments with the freshwater ramshorn snail *Marisa cornuarietis*

The exposure to a number of xeno-androgens (TPT, TBT, MT, FEN) resulted in a marked increase in the VDSI (imposex development) and simultaneously, in a reduction of the egg production in *M. cornuarietis*. As before, one example, TBT, was chosen to be presented here (EU-project COMPRENDO; Schulte-Oehlmann 1997).

Figure 3a shows the development of the VDSI over the test period of 150 days. After 50 days, the highest concentration of 290 ng as Sn/L (measured) exhibited a significantly elevated VDSI in comparison to the control. After 100 and 150 days, all treatments yielded significantly increased VDSI levels in a concentration-dependent manner. Thus the LOEC in these experiments

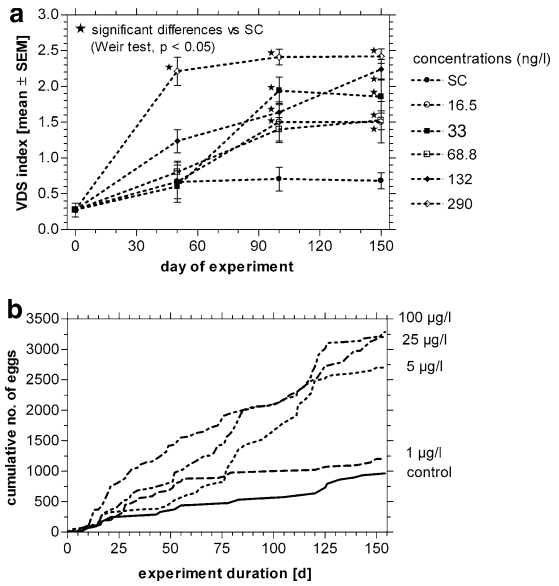

Fig. 3 Laboratory experiments with *Marisa cornuarietis*. Effects of water concentrations (ng/L) on (**a**) Vas Deferens Sequence Index VDSI (imposex development) and (**b**) egg production (cumulative egg numbers) over the test duration of 150 days. (**a**) Tributyltin (TBT). (**b**) Bisphenol A (BPA)

is equivalent to the lowest administered concentrations, namely 16.5 ng as Sn/L (measured), and calculations yielded an EC_{10} of 3.42 ng as Sn/L and an EC_{50} of 98.2 ng as Sn/L. The egg production (eggs per female), on the other hand, was significantly reduced in all tested concentrations, with an EC_{50} of 64.9 ng as Sn/L and an EC_{10} of 10.4 ng as Sn/L (results not shown).

TPT as well as MT and FEN exhibited similar responses during the *Marisa* tests, with even lower effect concentrations for TPT than for TBT: EC_{50} 38.8 ng as Sn/L (EC_{10} 2.48 ng as Sn/L) for imposex development and EC_{50} 28.4 ng as Sn/L (EC_{10} 15.9 ng as Sn/L) for reduction of egg production (based on measured concentrations). MT and FEN were comparably effective, with EC_{10} values of 35.9 ng/L (MT) and 18.6 ng/L (FEN) for imposex development and 1.73 ng/L (MT) and 6.89 ng/L (FEN) for egg production (nominal for MT, measured for FEN).

On the other hand, exposure to a variety of xeno-estrogens (BPA, OP, ethinylestradiol) resulted in a marked stimulation of the egg production. Again, one representative substance, BPA, is be presented here (see Oehlmann et al. 2000, 2005).

Marisa was exposed during 5 months using adult specimens and in a complete life-cycle test for 12 months. In both experiments, the xeno-estrogen induced a complex syndrome of alterations in female *Marisa* referred to as ''superfemales'' at the lowest concentrations. Affected specimens were characterized by the formation of additional female organs, an enlargement of the accessory pallial sex glands, gross malformations of the pallial oviduct section, and a massive stimulation of oocyte and spawning mass production (Fig. 3b), resulting in an increased female mortality. Statistically significant effects were observed at the lowest nominal test concentration of 1 µg/L. Another series of experiments in the low-dose range confirmed these findings and resulted in a NOEC of 7.9 ng/L and an EC_{10} of 13.9 ng/L (Oehlmann et al. 2005). For OP and ethinylestradiol, similar responses were observed (Oehlmann et al. 2000; Schulte-Oehlmann et al. 2001b), with a LOEC of 1 µg/L (lowest nominal test concentration; NOEC <1 µg/L) for OP and 10–25 ng/L being the estrogenic sensitivity window for ethinylestradiol.

Laboratory experiments with the netted whelk *Nassarius reticulatus*

The exposure to various xeno-androgens (TPT, TBT, FEN) resulted in a marked increase in the VDSI in *N. reticulatus*. TBT was chosen to be reviewed here (data from Tillmann 2004).

The concentration-response relationship between TBT concentrations in spiked artificial sediments and the increase of the VDSI within 1 months of exposure is shown in Fig. 4. Exposure to TBT showed that a maximum effect is reached if the VDSI increases by 1.0 during the test duration of 4 weeks (Tillmann 2004; Duft et al. 2005). The NOEC for the increase of the VDSI, thus imposex development, in TBT exposure experiments is equivalent to a concentration of 25 µg TBT as Sn/kg. An EC_{50} of 16.9 µg as Sn/kg was calculated, with a respective EC_{10} of 2.9 µg as Sn/kg.

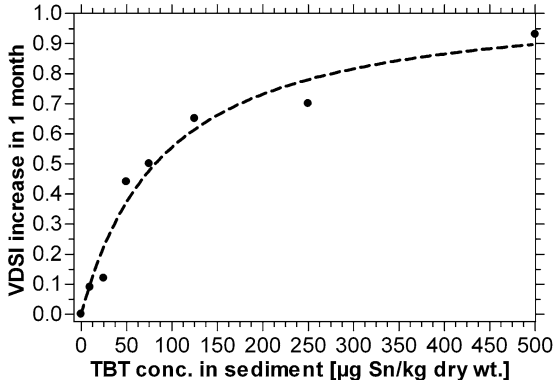

Fig. 4 Laboratory experiments with *Nassarius reticulatus*. Effects of tributyltin (TBT) sediment concentrations (µg/kg d.w.) on the Vas Deferens Sequence Index VDSI (imposex development) after 4 weeks of exposure

While exposure to TBT via sediments led to the expected statistically significant time-and concentration-dependent increase of imposex intensities, TPT exhibited no comparable androgenic activity at nominal concentrations ranging from 50 to 500 µg TPT as Sn/kg dry wt. The VDSI values in all TPT groups and in the control remained on the same level during the 3 months experiment. However, the gonads of *N. reticulatus* revealed marked disturbances in differentiation and maturation processes in both sexes (Schulte-Oehlmann et al. 2000).

Other investigations on effects of TBT during water exposure resulted in a LOEC of 50 ng as Sn/L (Stroben 1994). Exposure to FEN was simulated in two experiments with spiked sediments (Tillmann 2004). In the first experiment, FEN exhibited a significant increase of the VDSI after 4 weeks at all tested concentrations (10, 100, and 1,000 µg/kg). A second experiment was conducted in the low-concentration range (0.3, 3, and 30 µg/kg) and again, all tested concentrations resulted in a significantly elevated VDSI compared to the control, thus a NOEC of <0.3 µg/kg was derived. Calculation of effect concentrations yielded an EC_{50} of 0.19 µg/kg after 4 weeks (EC_{10} not calculable). Simultaneous application of FEN (0.3 µg/kg) and the antiandrogen cyproterone acetate (125 µg/kg) resulted in a clearly reduced increase of the VDSI.

Exposure to the xeno-estrogen OP via sediments induced an increase in the weight of the gland complex (Tillmann 2004) after 4 weeks with an EC_{50} of 40.7 µg/kg (EC_{10} 4.3 µg/kg).

Field effect monitoring with *Nassarius reticulatus*

During the last years, a field biomonitoring with *N. reticulatus* was conducted on sediments of the rivers Rhine, Main, Neisse, Weser, and Elbe in Germany (Schulte-Oehlmann et al. 2001a; Tillmann 2004). As an example, the Elbe results are shown here. Along the extension of the Elbe in Germany, 29 sampling sites have been analyzed. Site 1 is situated directly at the border between the Czech Republic and Germany, site 29 in the Elbe estuary and already influenced by the North Sea.

Figure 5 denotes the VDSI increase, hence the androgenic activity, and also the respective ecological status class of the sampling sites according to the new European Water Framework Directive, based on obtained test results with *Nassarius*. Obviously, there is only one sediment (site 1) which is categorized into class 1 and even in class 2, there are only eight sediments. Two hot spots can be identified: the most drastic effects—considering that the maximum possible

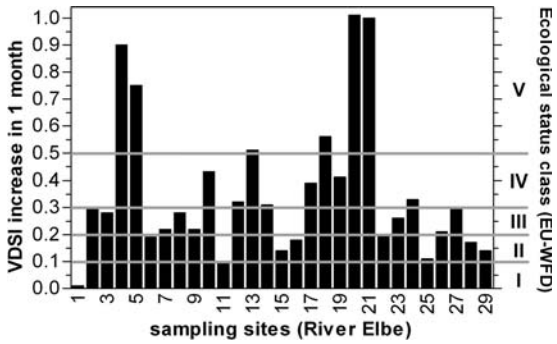

Fig. 5 Biomonitoring with the netted whelk *Nassarius reticulatus* in the river Elbe, Germany. Imposex development (increase of the VDSI vas deferens sequence index after 4 weeks exposure). Ecological status classes I–V according to the European Water Framework Directive

VDSI increase is 1.0 in 1 months—were found at station 4 and around station 20. At the first highly polluted sampling site, number 4, the river Mulde is joining the Elbe. In former times, before the German reunification, the Mulde received waste water from the greatest organotin producing plant in the former German Democratic Republic. The influence of this plant is still present today and can be found more than 50 km downstream the Elbe. The second hot spot is the Hamburg harbor, but even between the Mulde and Hamburg local point sources are visible.

Interestingly, the androgenic response in eight of the tested 29 sediments was so marked that it could not be explained by the analytically determined residues of TBT compounds in the sediments, so that additional and up to now unidentified androgenic compounds may have been present, contributing to the observed effects.

Further biological effect monitoring data with *N. reticulatus* are reported on samples from the rivers Danube, Main, Rhine, and Weser (Tillmann 2004) and on marine sediments (Stroben et al. 1992). Another effect monitoring survey of Polish sediments from the Gulf of Gdansk has been conducted during the EU-project COMPRENDO (unpublished results). Furthermore, the netted whelk was the most reliable indicator of TBT contaminations in sediments in the Ria de Aveiro (Portugal). As conclusion of this study, this snail is recommended for the monitoring of low to moderately contaminated sediments (Barroso et al. 2000).

Field effect monitoring with *Potamopyrgus antipodarum*

The same large German rivers mentioned before were also tested using the sediment biotest with the mudsnail

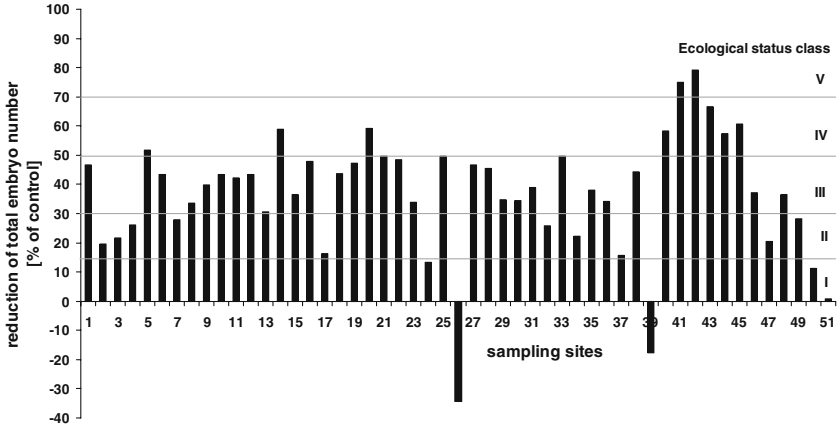

Fig. 6 Biomonitoring with the freshwater mudsnail *Potamopyrgus antipodarum* in the Neisse-Odra river system, Czech Republic/ Germany/Poland. Inhibition of the embryo production after 4 weeks exposure in comparison to the control sediment. Ecological status classes I–V according to the European Water Framework Directive

P. antipodarum (Schulte-Oehlmann et al., 2001a; Duft 2004). With this test, a total of 22 sampling sites from the river system Neisse-Odra along the German-Polish border were investigated. Site 1 is situated directly at the border triangle between the Czech Republic, Poland, and Germany, site 22 shortly before the Odra split, but not yet influenced by the Baltic Sea. Figure 6 shows the inhibition of the total embryo number in % of the control and also denotes the ecological status classes I–V of the stations, according to the new European Water Framework Directive, based on obtained test results with *Potamopyrgus*. In accordance with this five-stage ecotoxicologically based assessment concept (Duft 2004), there are only two sediments in class 1 and six in class 2. Strong inhibitions of the embryo production in the Neisse were observed near the Czech border and further on (N1, N6, and N9). At the junction of Neisse and Odra, the site was sampled before and after the junction of the two rivers, resulting in a clearly higher inhibition of the embryo production after the junction. Further hot spots were identified around Eisenhüttenstadt (O3-5), at Frankfurt (O7), Kietz (O11), and Groß-Neuendorf (O12), areas characterized by heavy industrial activity. Sediments taken from the Polish border mostly showed a greater response. Interestingly, there are two sites where a significant increase of the embryo number was observed. This might be due to estrogenic compounds in the sediment.

Further biological effect monitoring data with *P. antipodarum* on sediments from the rivers Elbe and Danube are reported by Duft (2004) and Schulte-Oehlmann et al. (2001a).

Also the influence of physical and chemical properties of sediments on the embryo production has been investigated (Duft 2004). As a result, it has been shown that *Potamopyrgus* is highly tolerant toward temperature (15–25°C), grain size, organic carbon content (1.2–9%), and salinity (0–5 %). Consequently the test result should not be influenced unless sediments with extreme values for these parameters are tested. In this case (e.g. high organic carbon content, high salinity) caution is required when interpreting the test result. However, for the majority of sediments, this should be no problem.

Discussion

The presented biotests with three prosobranch snail species show a high-sensitivity toward various xeno-androgens and xeno-estrogens in laboratory experiments. When comparing the calculated effect concentrations to those reported in the literature, it becomes clear that prosobranch snails are remarkably susceptible to endocrine disrupting compounds. For TPT, EC_{50} values of 38.8–68 ng as Sn/L and 0.74 µg as Sn/kg were calculated (respective EC_{10} values 15.9–29 ng as Sn/L and 0.03 µg as Sn/kg), whereas out of the few chronic studies on invertebrates, a lowest value of <68 ng/L (NOEC) is reported for reproduction in water fleas (Federoff et al. 1999). This holds true also for other investigated substances: with the exception of the marine copepod *Acartia tonsa* (larval development; Kusk and Petersen 1997), observed effects concentrations of TBT were clearly higher in most studies (e.g., NOEC for *Daphnia magna* 32 mg/L; Kühn and Pattard 1989). For xeno-estrogens, there is even greater difference in sensitivity (see also Oehlmann et al. 2000; Duft et al. 2003b): a lowest effect concentration of 20 µg/L was found in BPA exposed copepods (Andersen et al. 1999). Effects on the fiddler crab *Uca pugilator* were observed after exposure to 10 mg/L (Zou and Fingerman 1999).

In comparison to other invertebrate test systems, the proposed snail tests offer the advantage of an organism

with an endocrine system, which is at least partially comparable to that of vertebrates. With the exception of *N. reticulatus*, culturing of the organisms is possible and easy to achieve. Besides, snails undisputedly possess a high ecological relevance. Application of the various tests is possible for freshwater and marine samples, exposure via water and sediment, laboratory experiments and field effect monitoring, and further, effects on both sexes (except for *Potamopyrgus*) can be assessed. Mixture experiments, e.g. with androgens and anti-androgens or estrogens with anti-estrogens etc., revealed a reduction or suppression of the effects observed before in monosubstance experiments (Duft 2004; Tillmann 2004) and thus indicate that effects are endocrine-mediated.

Comparing effects of endocrine disruptors on vertebrates and invertebrates, a parallelism between effects on the embryo production in *P. antipodarum* and estrogenic responses in fish induced by sewage effluent and monosubstances was demonstrated by Jobling et al. (2004).

For the application of a biotest in a broader range and for an utilization as a standard test, it is fundamental to know about the reproducibility of experimental data. Due to availability of quite a number of recent studies conducted in the case of prosobranch snails, we are in the position to compare their outcomes. Several studies on *P. antipodarum* have investigated effects of TBT (Schulte-Oehlmann 1997; Duft et al. 2003b; EU-project COMPRENDO), TPT (Duft et al. 2003a; EU-project COMPRENDO) and FEN (Scholz 2003; EU-project COMPRENDO) as xeno-androgens, and effects of BPA and OP (Casey 2000; Duft et al. 2003b) as xeno-estrogens. Comparing these studies, mostly identical responses were obtained with corresponding effect concentrations (see also Table 1). For *M. cornuarietis*, two virtually identical studies were carried out on the effects of BPA (Oehlmann et al. 2000, 2005), which clearly demonstrated the reproducibility of the results. However, care should be taken when setting up an experiment, which is valid for all of the snail species, as there are seasonal fluctuations in the reproduction (egg or embryo production, cf. Schulte-Oehlmann 1997; Scholz 2003; Oehlmann et al., 2005; Schmitt et al. 2006). During phases of high embryo or egg production, estrogenic effects may be masked because the reproductive output is already at its maximum level. Thus, experiments testing potentially estrogenic active substance should be conducted preferrably in phases with normal or low-reproductive output, for which it is essential to have information on the respective annual reproductive cycle. Another substantial factor in the *Marisa* test is the temperature at which the experiments are conducted. Oehlmann et al. (2005) showed significant differences in test outcomes at different temperatures. Whereas at 20 and 22°C, the reproductive output was similarly increased compared to the control, egg production was already elevated in the control at 27°C and therefore showed a smaller increase during exposure to BPA.

At first, a stimulation of the egg or embryo production may not necessarily seem to be a negative effect, however, the ecological relevance of this effect is not to be underestimated. Snails exhibit annual maxima and minima in their reproductive cycle. Exposure to xeno-estrogens causes an enhanced production of female sexual products even during phases in the annual reproductive cycle with normally low reproduction rates. This means not only a limitation of the energy budget and causes the production of less embryos in the actual main reproductive phase, but also indicates that more juveniles are produced during a time period when environmental conditions are less favorable for growing up. The chances of survival of the juveniles are much lower off the actual reproductive phase. Therefore, population-relevant effects are to be expected from such an unnatural enhancement of reproduction. Besides, an extreme increase of egg production in *Marisa* can cause death of females due to oviduct ruptures.

A standard biotest should not only be sensitive toward model compounds, but also offer a variety of advantages for practical use. In this view, a comparison of the most relevant characteristics of the three presented tests is compiled in Table 2. Laboratory culturing has been proven to succeed for *P. antipodarum* and *M. cornuarietis*, whereas *N. reticulatus* cannot be cultured in the laboratory due to planktonic larval stages, but has to be collected at uncontaminated sites, e.g. at the Atlantic Coast of France. To start laboratory cultures, initial specimens have to be acquired; for *P. antipodarum*, specimens were collected at unpolluted sites, and for *M. cornuarietis*, a laboratory breeding stock was derived from Aquazoo Düsseldorf with regular crossbreeding of wild-caught animals from Florida to avoid inbreeding. Whereas *P. antipodarum* only exists in female form in Europe, reproducing parthenogenetically, both sexes are available for the other two species. It should be kept in mind that the presented results refer to clone Euro A. When applying the test with inviduals probably belonging to other genotypes (e.g. in UK, USA, Australia or New Zealand), it remains to be found out whether the results would be the same, as the among individual variation in response to any stress factor could be less

Table 2 Overview on the three prosobranch snail species *Potamopyrgus antipodarum*, *Marisa cornuarietis* and *Nassarius reticulatus* and characteristics of their biotests: culturing, sexes, habitats, exposure, test duration, endpoints, handling and sensitivity

	Potamopyrgus antipodarum	*Marisa cornuarietis*	*Nassarius reticulatus*
Culturing	Laboratory culture, to be initiated by collected specimens (e.g. in Germany)	Laboratory culture, to be initiated by collected or purchased specimens, e.g. from Florida	Laboratory culture not possible, sampling, e.g. at the Atlantic Coast
Sexes	Females only, parthenogenetic	Both sexes	Both sexes
Suitability for habitats	Freshwater and estuarine	Freshwater only	Marine and freshwater sediment, if articial seawater is added
Exposure	Water and sediment	Water	Water and sediment
Test duration	1–2 months	3–6 months	1–3 months
Endpoints	Mortality, embryo production	Mortality, VDSI (imposex), egg production	Mortality, VDSI (imposex), gland weight
Handling of analysis	Easy determination	Easy determination for egg production, advanced skills required for imposex	Advanced skills required
Sensitivity to EDCs	Estrogens (and androgens, but difficult to distinguish from general toxicity)	Estrogens and androgens	Androgens (and estrogens)

than in sexual species where individuals are genetically different. Freshwater and estuarine water and sediment can be tested with the *Potamopyrgus* test, but only freshwater with the *Marisa* test, and mainly marine water and sediment with the *Nassarius* test, however also freshwater sediment can be tested, if overlayed with artificial seawater. Duration is 1–2 months for the *Potamopyrgus* test, 3–6 months for the *Marisa* test and 1–3 months for the *Nassarius* test. The endpoints of the tests are mortality and embryo production (*Potamopyrgus*), imposex development (*Marisa* and *Nassarius*), and egg production (*Marisa*) or weight of glands (*Nassarius*). Most important for the application as routine test is the handling of the analysis for non-expert technical staff. In this view, the *Potamopyrgus* test is quite easy to handle and ''user-friendly'', whereas for the *Marisa* and the *Nassarius* test, advanced skills for the determination of imposex are required. Generally, the tests can be performed at low costs, as the required equipment should be available in each laboratory. As pointed out before, the tests slightly differ in sensitivity to substances or in the robustness of responses. *Potamopyrgus* displayed high sensitivity toward various estrogens and androgens, but in the case of androgens it is difficult to distinguish endocrine effects from general reproductive toxicity, especially when testing field sediments. It has to be considered that not only organotin or other xeno-androgenic compounds may be responsible for the observed effects, but also other substances with a general reproductive toxicity, like heavy metals or PAHs—the observed effect is so to say a ''net effect''. When testing field samples, which possibly contain a mixture of several contaminants, this should be kept in mind for the interpretation of the test result. An increase of embryo production is very likely to be caused by estrogenic compounds, whereas a decrease of embryo production can be caused by androgenic compounds, but also by other contaminants. At the presence of both androgens and estrogens in a hypothetical sediment, it is not very likely that embryo production will be unaffected of less affected. Rather, it can be expected that such an exposure equals double stress and will probably result in a decrease of the embryo production, as suggest results of mixture experiments (Duft 2004). With the *Marisa* test, both estrogenic and androgenic test compounds can be tested with adequate sensitivity, and the *Nassarius* test is mainly sensitive to androgens, but also to estrogens considering the weight of glands. In summary, it can be concluded that each of the tests is highly sensitive to compounds of relevant purpose, but each of them also includes its drawbacks. All in all, the test with the freshwater mudsnail *P. antipodarum* reveals most advantages of the three tests, especially with regard to practical aspects.

Conclusion

Three prosobranch snail species have been portraited and respective biotests have been presented. It can be concluded that all three biotests are highly sensitive toward tested model compounds at environmentally relevant concentrations in laboratory tests and also toward field sediment samples, thus representing promising test systems for the identification of endocrine disrupting substances. Due to its advantages

regarding practical implementation, the 28–56 d reproduction test with *P. antipodarum* was selected for standardization and has therefore been presented to the OECD ''Ad hoc Expert Group for Invertebrate Testing'' in Paris in November 2005. Respective SOPs (for culturing, water exposure, and sediment exposure) have been introduced to the expert group where there is continuous vivid interest in the test and broad support for a standardization procedure. The next step will be to submit the test to the guideline program of the standardization committee and to start the standardization protocol. This process is currently ongoing and the aim is to present the test to the OECD comittee in due course.

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