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Suitability of the marine prosobranch snail *Hydrobia ulvae* for sediment toxicity assessment: A case study with the anionic surfactant linear alkylbenzene sulphonate (LAS)☆

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ABSTRACT

Individuals of the mudsnail *Hydrobia ulvae* (Pennant) (Mollusca: Prosobranchia) were exposed to sediments spiked with increasing concentrations (1.59–123.13 mg kg^{-1} dry weight) of the anionic surfactant linear alkylbenzene sulphonate (LAS) which is employed in the formulation of laundry powders and liquids, as well as hand dishwashing products. The suitability of the selected organism, *H. ulvae* for routine sediment toxicity testing was evaluated by measuring acute toxicity recording survival. Sublethal toxicity was evaluated as total number of produced veliger larvae per treatment throughout the test (9 d). Mortality has shown to be a reliable and reproducible indicator of acute toxicity. LC_{50} values were comprised between 203.4 (48 h) and 94.3 mg kg^{-1} (9 d) dry weight. As sublethal endpoint, the total number of produced larvae showed to be a useful indicator of toxicity for this organism. The number of produced larvae increased at lower exposure concentrations, whereas at the highest LAS concentration, the number of produced larvae decreased. This is the first report of acute and sublethal toxicity of sediment associated LAS for this species.

1. Introduction

Estuarine and coastal bottom sediments accumulate anthropogenic chemicals and waste materials, constituting the final sink of many pollutants (US EPA, 1998). Sediments act both as reservoirs and as potential sources of these chemicals to the water column and can adversely affect sediment-dwelling organisms by causing direct toxicity or altering benthic invertebrate community structure (Martínez-Lladó et al., 2007; Roussiez et al., 2006). In order to protect benthic life, threshold levels for contaminants are determined below which benthic organisms are not likely to be affected. Sediment toxicity tests with spiked sediments are useful tools for the derivation of environmentally acceptable contaminant concentrations (US EPA, 1994a). Although in many cases, the ultimate disposal of chemicals is usually the marine environment, there is generally much more available data about freshwater than saltwater organisms (US EPA, 1994b; Canadian Sediment Quality Guidelines for the Protection of Aquatic Life, 1995). Even fewer information is available about marine sediment sorbed contaminants and their effects on sediment-dwelling organisms, which is also the case of the selected test compound, linear alkylbenzene sulphonate (LAS) (HERA-LAS, 2007; León et al., 2001).

A potential candidate for the performance of sediment toxicity tests is the hydrobiid mudsnail, *Hydrobia ulvae* (Pennant) (Mollusca: Prosobranchia). Hydrobiid snails belong to the most important deposit feeding invertebrates of European estuaries, forming populations with densities up to 300,000 individuals per square metre (Jackson, 2000). This species is found on mudflats, muddy sand, in estuaries (Fish and Fish, 1996) and in salt marshes (Jackson, 2000). It is most common on the middle and upper parts of the shore, although it has also been found at depths of 100 m (Jackson, 2000). These organisms ingest their substrate and assimilate the microorganisms attached to mineral and detritus particles. The reproductive cycle of this species depends on the latitudinal distribution, presenting in Southern Europe generally two reproductive peaks, one in late spring and a second one in autumn (UK Biodiversity Action Plan, 2002). The sexes are separate and fertilisation occurs internally. Egg masses of

☆ This work was carried out in the framework of the project: "Effects of Linear Alkylbenzene Sulfonate on benthic organisms" between the Instituto de Ciencias Marinas de Andalucía (ICMAN-CSIC) and Petroquímica Española, S.A. (PETRESA). Modest contaminant exposure trials with mudsnails were carried out in accordance with national and institutional guidelines for the protection of human subjects and animal welfare.

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4–8 eggs are usually cemented onto the shells of other laver spire snails or other substrates and become covered with a protective layer of sand grains (Fish and Fish, 1996). The duration of the *in ovo* development has been studied by several authors (Anderson, 1971; Pilkington, 1971; Rothschild, 1940) and is comprised between 8 and 24 d, depending on the latitude and, consequently, on the temperature. Size and development stage of the hatched veliger larvae depend on these parameters, too. Hydrobiid snails are ecologically important members of benthic communities and are a primary food resource for a number of marine invertebrate, fish and bird species, being an appropriate candidate for the standardisation of a sediment toxicity test protocol. Additionally, the possibility to induce breeding allows the evaluation of an important sublethal endpoint, an earlier indicator of toxicity than mortality.

LAS is an anionic surfactant with a global consumption of 350,000 metric tons in 2005 (HERA-LAS, 2007). It is, after soap, the most widely used surface active ingredient in washing powders and liquids and household cleaning agents and therefore present in urban and industrial wastewater effluents. Commercial LAS is a mixture of closely related isomers and homologues, each containing an aromatic ring sulphonated at the *para* position and attached to a linear alkyl chain. LAS is readily biodegradable in sludge amended soils with half-lives of 7–33 d (Ying, 2006). The average removal of LAS in wastewater treatment plants is high and comprised between 99.2% and 95.9%, depending on the facility (HERA-LAS, 2007). However, due to its widespread usage and inherent physical chemical characteristics, LAS can be found in wastewater effluents and adjacent estuarine and coastal environments (Temara et al., 2001; León et al., 2001). The LAS portion reaching marine environments tends to be sorbed on suspended solids and sediments due to its hydrophobic character (HERA-LAS, 2007). The toxicity of LAS to aquatic organisms is well documented (HERA-LAS, 2007; Hampel et al., 2001, 2004; Hampel and Blasco, 2002; Holmstrup and Krogh, 1996; Stalmans et al., 1990), but very few information exists on the effects of this compound on marine, sediment-dwelling organisms. Navas et al. (1999) have shown that the selected compound, LAS and its degradation products are not estrogenic.

Snails (Leung et al., 2004; De Vaufleury, 2000; Gomot, 1998) and specially hydrobiid snails (Duft et al., 2003a; Schulte-Oehlmann et al., 1997), have been used previously for the evaluation of toxic effects of contaminants. Recently, a new OECD test protocol using the freshwater mudsnail, *Potamopyrgus antipodarum*, has been proposed by Duft et al. (2007), stressing the suitability of these organisms for the evaluation of sediment associated contamination.

The aim of this study is to evaluate the suitability of the mudsnail, *H. ulvae* for its employment in marine and estuarine sediment toxicity tests and standardisation of the protocol for routine environmental risk assessment purposes. The tests were carried out with the anionic surfactant, LAS, which is a well studied and universally representative substance with high consumption rates.

2. Materials and methods

The test protocols were adapted to current sediment toxicity test guidelines (ASTM, 1997; US EPA, 1994a). Individuals of *H. ulvae* were collected from sites of the Bay of Cadiz (South–West Spain) which are known to be far from wastewater discharge points and were maintained for approximately two months in clean seawater. During this time, the individuals were fed lyophilised *Ulvae ulvae ad libitum*. After the acclimatising period, organisms were sexed (Schulte-Oehlmann et al., 1997) and maintained separately under controlled laboratory conditions for a further month until their employment in the exposure assays. Male and female organisms were distinguished by the penis at the dorsal part of the cephalic region (Fig. 1a and b). Although it has been shown that *H. ulvae* exhibits imposex and thus a penis in females may occur the sex ratio of 1:1 in the experiments shows that the occurrence of a penis is a suitable sexing characteristics for our samples.

Sediments were collected from a clean site of the Bay of Cadiz, far from any industrial sites or harbours and characterised in terms of grain size and organic carbon content previous to the experimentation. Once at the laboratory and no later than 48 h, sediments were spiked with LAS to obtain five increasing exposure concentrations comprised between 1.59 and 123.3 mg kg^{-1} dry weight. The selected concentration range covers both, environmentally relevant as well as higher LAS concentrations. We selected these concentrations to investigate the possible adverse effects on different lethal and sublethal responses by exposure to LAS spiked sediments, as well as to demonstrate the potential of the selected test organism for routine employment in toxicity testing. The spiking procedure was adapted to the protocol proposed by Casellato et al. (1992). The sediments were spiked under continuous agitation for 24 h. The employed surfactant was a commercial LAS mixture (CAS Nr. 68411-30-3, supplied by Petroquímica Española S.A., PETRESA) with an average alkyl chain length of 11.6 carbon atoms and homologue distribution of C_{10}–C_{13} of 10.9%; 35.3%; 30.4% and 21.2%, respectively. After spiking, the sediments were washed three times with distilled water in order to eliminate the LAS which was not associated to the sediment and dried at 70 °C until complete dryness.

For each trial, approximately 8 g of dry sediment was placed in a Petri dish and 16 mL of clean filtered seawater (<45 μm) were added. This volume was estimated adequate to moist the sediment but at the same time to guarantee the contact of the individuals with the spiked sediment avoiding the possibility to float at the water surface. A total of 30 individuals, 15 males and 15 females, corresponding to the sex distribution found in the natural populations during sexing, were placed in each Petri dish and exposed during 9 d in a thermostatic chamber at 21–22 °C and photoperiod corresponding to natural conditions of the month of June (15 h light: 9 h darkness). All assays were carried out in triplicates and control experiments

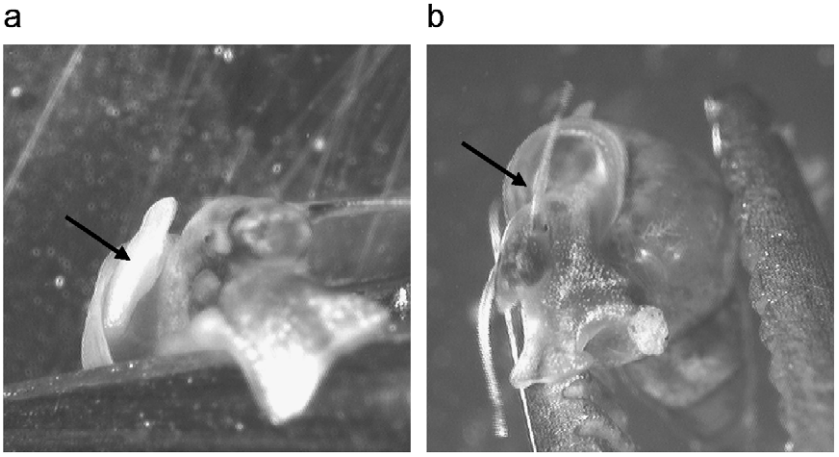

Fig. 1. Male (a) and female (b) specimen of the mudsnail, *H. ulvae*. The arrow indicates the male penis (a) and the absence of this organ in females (b).

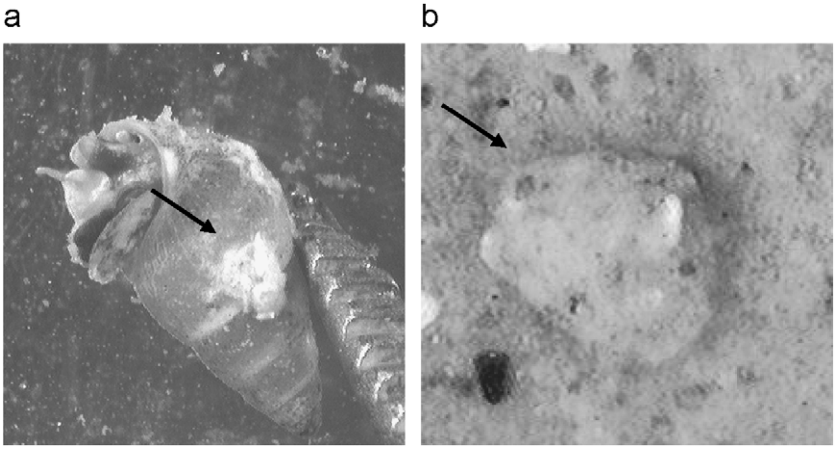

Fig. 2. Egg capsules laid on the shell of a snail (a) and in the sediment (b).

with untreated sediment were performed simultaneously. Mortality control was carried out daily by transferring the organisms into clean seawater and recording the number of dead and living individuals.

At the third day of exposure, temperature and light period were increased slightly for induction of reproduction. In those cases where reproduction occurred, egg masses were found on the shells of the individuals and on the sediment surface (Figs. 2a and b). The egg masses were transferred into multi-well plates containing 1 mL of filtered seawater and maintained under the same light and temperature conditions until hatching. Veliger larvae were counted at three successive days and the average number of produced larvae, as well as the ratio eggs per egg mass for each treatment calculated.

All solvents and reagents used for LAS analyses (water, methanol, HCl, formic acid) were of chromatographic quality. The analysis of exposure concentrations in the sediments was carried out once the experiment had finished. The surfactant was extracted from the sediment by sonication with methanol. After evaporation of the solvent and dissolution in 100 mL Milli-Q adjusted to pH = 3 with HCl, the extract was subjected to solid phase extraction passing it through a C_{18} column (Bond Elut, C18, Varian) and subsequently through an anionic exchange column SAX (Supelco). The resulting volume was evaporated, the precipitate dissolved in 1 mL MeOH:H_2O (80:20 v/v) and stored at −8 °C until LAS analysis by high performance liquid chromatography (HPLC, HP 1050) with fluorescence detector (λex = 225 nm, λem = 295 nm) as described by León et al. (2000).

Obtained mortality data was adjusted employing generalised linear models (GLM) for LC_{50} and LC_{10} calculation as described by Kerr and Meador (1996). One-way ANOVA was carried out with mean measured mortalities (SPSS Version 15.0). In order to obtain NOEC and LOEC values, post-hoc tests (Dunnett) were performed.

3. Results

Conventional parameters determined in the sediment are presented in Table 1. The organic carbon content (%OC) of the experimental sediments increased from 0.62% in non-spiked sediments up to 1.15% in the sediments spiked with the highest LAS concentration. Results are comprised within the normal values for littoral ecosystems and in agreement with previous studies conducted in the area (González-Mazo et al., 1997). The LAS concentrations in the experimental sediments measured by HPLC were: 1.59; 3.75; 24.53; 69.53 and 123.3 mg kg^{-1} dry weight.

No significant mortality was detected in control assays with reference sediment and survival in all the replicates matched the acceptance criteria for this kind of test (ASTM, 1995, 1998). This indicates a good resistance of this species to laboratory maintenance and handling. Mortality percentages within replicates of the same exposure concentration showed very low standard deviation values indicating the mortality with a similar response in all replicates to be a reliable and reproducible endpoint for routine toxicity testing.

Table 1
Organic carbon (%), grain size composition (-% fine sand-), elemental analysis (C, H, N) and pH (± SD) in the reference sediment employed for the bioassays

OC (%)	%<63 μm	C (%)	H (%)	N (%)	pH
0.618 ± 0.015	22	1.19 ± 0.42	1.06 ± 0.13	0.06 ± 0.01	7.5 ± 0.1

At the highest LAS exposure concentration, the test organisms withdrew into their shell closing the operculum and avoiding any contact with the substrate. Nevertheless, surviving organisms initially emerged from their shells during mortality control, when transferred into clean seawater. The influence of starving on mortality was not studied, as the final effect (mortality) is the same and a result of the presence of the contaminant. On the other hand, at lower exposure concentrations where no significant mortality was observed, the snails moved actively through the sediment showing a high tendency to aggregate. After increasing temperature and light period, egg masses (Figs. 2a and b) were found frequently on the shells of the organisms or in the sediment. The number of egg masses and hatched larvae (Fig. 3a and b) showed at sublethal exposure concentrations a clear tendency to increase with increasing LAS levels, whereas at the highest concentration, the number of hatched larvae decreased significantly (Fig. 4).

LC_{50} values and confidence intervals (CIs) for both concentrations and response (mortality) probability, obtained by GLM at consecutive days are presented in Table 2 and the dose-response curve for day 9 is shown in Fig. 5. However, LC_{50} estimations for 24, 48 and 72 h should be considered with caution as their value was higher than the highest exposure concentration in the tests and a result of an extrapolation of the dose-response curve. However, in all cases, the obtained LC_{50} values decrease with exposure time, being during the first 96 h one order of magnitude higher than at the end (9 d) of the experiment, indicating an increasing effect of continued exposure. The obtained LOEC and NOEC based on mortality were 79.27 and 68.1 mg kg^{-1}, respectively.

Fig. 4 shows the number of total larvae and Fig. 6 the ratio eggs per egg mass. The number of larvae represents the median number of veliger larvae per replicate produced during the experiment at a given exposure concentration. Values are comprised between 200 and 650 larvae. At concentrations where reproduction was observed and egg masses found, the number of offspring increased with the surfactant concentration until

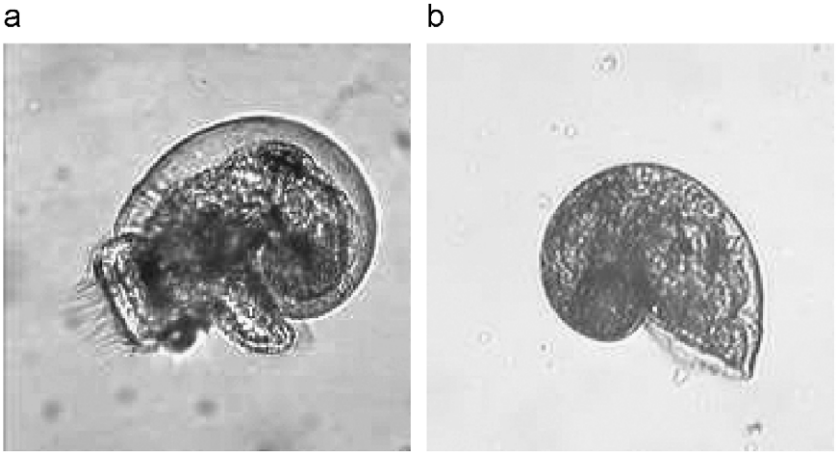

Fig. 3. Hatched veliger larvae at day 1(a) and 2(b) post hatching.

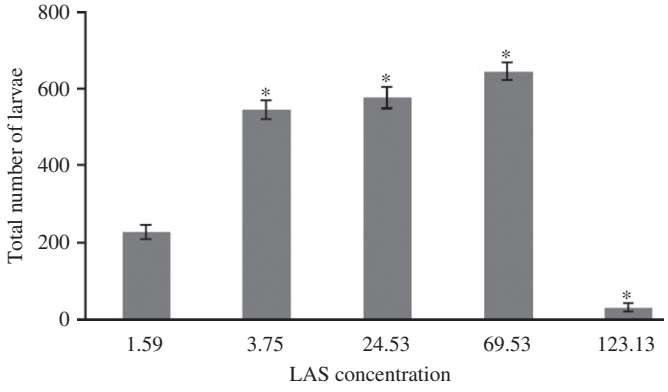

Fig. 4. Mean total number of hatched veliger larvae per replicate throughout the assay. LAS concentrations are in mg kg^{-1}. Error bars indicate standard deviations (*significant difference compared with controls $p<0.05$; one-way ANOVA, post-hoc Dunnett).

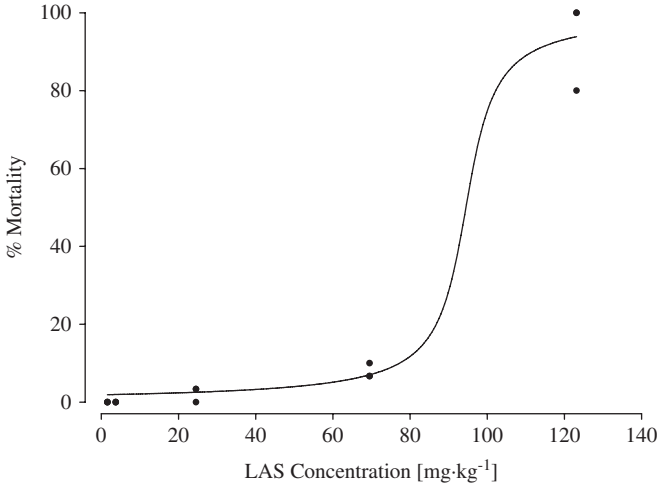

Fig. 5. Mortality curve at day 9 of exposure. LAS concentrations are in mg kg^{-1}.

Table 2
LC_{50} values (mg kg^{-1} dry weight) and confidence intervals (probability = 0.95) for concentration and response probability for the marine prosobranch *Hydrobia ulvae*

Time	LC_{50} (mg kg^{-1})	Concentration (mg kg^{-1})		Mortality (p)	
		Lower CI	Upper CI	Lower CI	Upper CI
24 h	203.42	161.71	370.59	0.12	0.88
48 h	183.85	151.07	400.67	0.09	0.91
72 h	127.37	120.44	136.94	0.39	0.61
96 h	101.77	95.69	107.54	0.38	0.62
5 d	98.08	92.55	103.47	0.38	0.62
6 d	97.28	91.27	103.28	0.37	0.63
7 d	94.99	89.10	101.11	0.38	0.62
8 d	94.30	88.51	100.35	0.38	0.62
9 d	94.30	88.51	100.35	0.38	0.62

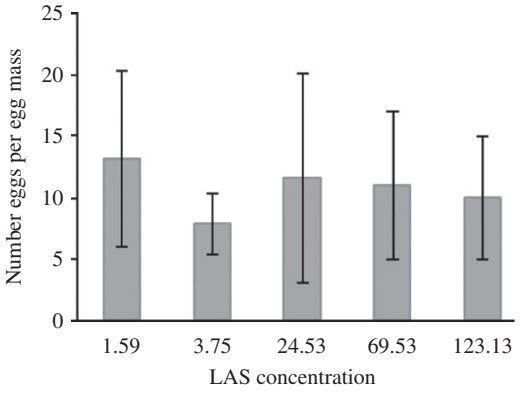

Fig. 6. Hatched larvae per egg capsule ratio. LAS concentrations are in mg kg^{-1}. Error bars indicate standard deviations ($p<0.05$; one-way ANOVA, post-hoc Dunnett).

reaching a threshold concentration above which the animals hardly moved over the contaminated sediment to remain isolated inside their shells. Under these circumstances, almost no reproduction could take place and, consequently, very few egg masses were found with the number of produced larvae about 20. The average number of larvae per egg mass was between 8 and 13 and seemed not to be affected by the surfactant concentration of the substrate (Fig. 6).

4. Discussion

Several effect concentrations have been reported for LAS by different authors in similar studies with sediment-dwelling organisms (Ying, 2006; Sanderson et al., 2006). For example, Bressan et al. (1989) derived effect concentrations of 200 mg kg^{-1} dry weight for the mussel, *Mytilus galloprovincialis* exposed to LAS contaminated sediments. However, this organism is actually

feeding by filtering nutrient particles from the overlaying water column and does not properly ingest sediment particles as in the case of *H. ulvae*. Generally, acute toxicity values (LC_{50}) differ between sediments and aqueous phase by 2–5 orders of magnitude, depending on the species used. In the case of LAS, Casellato et al. (1992) found this kind of difference in toxicity in a long-term experiment with the oligochaeta *Branchiura sowerbyi*. Thus, toxicity tests with filter feeding benthic organisms may provide an underestimation of the risk of a sediment associated contaminant, as these are often irreversible bound to sediment particles and the obtained toxicity parameters represent much more aquatic than sediment associated toxicity of a compound. In a study carried out with a sediment ingesting freshwater organism, the midge, *Chironomus riparius* (Pittinger et al., 1989), the obtained effect concentration in LAS spiked sediments was 319 mg kg^{-1}. Differences in effect concentrations observed by different authors are related to the increase of LAS toxicity in certain taxa from fresh to seawater environments. In this sense, Bressan et al. (1989) found an increase in sediment toxicity of LAS in salt water mussels by a factor of 2–4 compared to freshwater mussels. Comber et al. (2006) exposed two different worm species, *Lumbriculus variegates* and *Caenorhabditis elegans*, to LAS spiked sediments and obtained NOEC values of 81 and 100 mg kg^{-1}, respectively. These authors do not report the corresponding LC_{50} values which would be higher than the derived NOECs, being therefore higher than those obtained in our study (Table 2). However, the evidence of the toxicity measured in *H. ulvae* under LAS exposure and the similar toxicity parameters obtained in this study compared to other tests using different benthic species demonstrate the suitability of this organism to be recommended by different international environmental agencies for marine benthic environmental risk assessment.

The mortality data we obtained demonstrate clearly the effect of exposure time and concentration. A clear decreasing tendency of LC_{50} values with exposure time is observed as the effects in the organisms accumulate due to an integration of exposure level (concentration) and time. Therefore, the organisms die at lower exposure concentrations than earlier in the assay. An increased mucus production was also observed with increasing exposure concentration as indicator of defence mechanism to avoid the contact with the contaminated sediment. Even if at the end of the experiment (9 d) mortality approached 100% at the highest concentration, organisms survived by withdrawing into and closing the shell during the first days as observed when the organisms were transferred into clean filtered seawater for mortality control. After this time, mortality increased quickly within a narrow concentration range, indicating a uniform response towards the contaminant of the exposed population. The contribution of starving to total mortality should be studied by stomach content analysis. It is obvious that the impossibility to feed decreases the resistance of exposed organisms which, in combination with other environmental stress factors, is in a clear disadvantage in comparison with control organisms that feed normally.

With respect to the sublethal endpoint embryo production, a significant increase in the total number of hatched larvae was observed with increasing exposure concentration until an abrupt decline of the parameter at the highest concentration where the individuals did not emerge from their shell and did not copulate. A similar behaviour has been observed by Duft et al. (2003b) in the freshwater mudsnail *Potamopyrgus antipodarum* exposed to sediments spiked with the estrogenic compounds bisphenol A, octylphenol and nonylphenol. These authors reported a stimulated embryo production that increased along the exposure period of eight weeks reaching up to 170% above the solvent control value at the highest exposure. In a similar experiment with the freshwater ramshorn snail *Marisa cornuarietis* and the marine gastropod *Nucella lapillus*, Oehlmann et al. (2000) observed also a massive stimulation of oocyte and spawning mass production under bisphenol A exposure. However, enhanced reproduction as an adaptive response has so far only been described in studies with estrogenic compounds (Duft et al., 2003b; Oehlmann et al., 2000), but not, like in the present study, under exposure to non-estrogenic compounds such as LAS (Navas et al., 1999). In a study about *H. ulvae* in the Mondego river estuary, Lillebø et al. (1999) suggested an adaptation from "k" to "r" strategist with respect to the energetic investment on reproduction under adverse environmental conditions, producing a major number of planktonic veliger larvae that may float with the tides to different habitats where environmental conditions might be more favourable. Interruption of the reproductive activity over a longer period of time could have severe consequences for *H. ulvae* populations, as their life span is only 2 and 2.5 years (Fish and Fish, 1974; Lillebø et al., 1999). The stimulation of the embryo production may have significant ecological relevance which should be taken into account: *H. ulvae* has two annual peaks in its reproductive cycle. Exposure to LAS causes an enhanced production of larvae even during periods with normally low reproduction rates where the environmental conditions are less favourable for the larvae to survive. The reproductive effort in non-reproductive periods could additionally cause a limitation of the energy reserves and may cause a lower production of offspring in the actual main reproductive phase which may cause population-relevant effects. However, in a study exposing the freshwater snail *Lymnaea stagnalis* to increasing concentrations of cadmium, Gomot (1998) did not detect an increase in the number of produced embryos. Instead, the total number of laid eggs decreased slightly with the cadmium concentration, as well as the total number of hatchlings.

Mortality and number of offspring have shown to be reliable endpoints easy to detect, indicating unequivocally the threshold between no observed effect and adverse contaminant concentrations. The mortality is easily detected and the response of the exposed organisms is uniformly indicating small fluctuations in the resistance of the experimental population as indicated by the relatively low standard errors obtained in all assays. The effect on number of offspring under adverse conditions is a valuable proposal for routine sublethal effect evaluation and is therefore recommended for the assessment of sediments with different levels of contamination. Possible modifications in the evaluation of this parameter, as teasing gonads of mature females where mature and immature eggs may be distinguished (Fish and Fish, 1974) or staining of laid eggs or already hatched larvae for a better counting should be taken into consideration. In contrast to the endpoint number of produced veliger larvae, the criterion number of eggs per egg capsule remained constant for all those concentrations where reproduction took place and was comprised between 8 and 13 eggs per capsule, similar to the values obtained by Fish and Fish (1974). It seems that faced with adverse environmental conditions, a major quantity of egg capsules is laid by the organisms without changing the number of eggs per capsule making this latter effect criterion useless for environmental impact assessment.

5. Conclusions

Snails have previously shown to be suitable candidates for the evaluation of the effects of exposure to environmental contaminants (Schulte-Oehlmann et al., 1997; Gomot, 1998; Duft et al., 2003a, b; Snyman et al., 2005; Duft et al., 2007). The results obtained in the present study confirm that *H. ulvae* is an appropriate candidate for routine sediment toxicity testing in

both acute and sublethal toxicity tests with surfactants. Due to its feeding habit, ingesting sediment particles, toxicity tests with the mudsnail *H. ulvae,* can provide more reliable information about the effects of the bioavailable part of the sediment sorbed compound as they are directly exposed to the fraction which is adsorbed on the sediment particles.

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