

Using early life stages of marine animals to screen the toxicity of priority hazardous and noxious substances

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Abstract This study provides toxicity values for early life stages (ELS) of two phylogenetically distinct marine animal taxa, the sea urchin (*Paracentrotus lividus*), a deuterostome invertebrate, and the turbot (*Scophthalmus maximus*), a vertebrate (teleost), when challenged by six hazardous and noxious substances (HNS): aniline, butyl acrylate, m-cresol, cyclohexylbenzene, hexane and trichloroethylene. The aim of the study was to provide preliminary information on toxic effects of representative and relevant priority HNS to assess the risk posed by spills to marine habitats and therefore improve preparedness and the response at the operational level. Selection criteria to include each compound in the study were (1) inclusion in

the HASREP (2005) list; (2) presence on the priority list established by Neuparth et al. (2011); (3) paucity of toxicological data (TOXnet and ECOTOX) for marine organisms; (4) behaviour in the water according to the categories defined by the European Behaviour classification system (GESAMP 2002), by selecting compounds with different behaviours in water; and (5) physicochemical and toxicological properties, where available, in order to anticipate the most toxic compounds. Aniline and m-cresol were the most toxic compounds with no observed apical effect concentration (NOAEC) values for sea urchin ranging between 0.01 and 0.1 mg/L, followed by butyl acrylate and cyclohexylbenzene with NOAECs ranging between 0.1 and 1.0 mg/L and trichloroethylene with NOAEC values that were in the range between 1 and 10 mg/L, reflecting their behaviour in water, mostly vapour pressure, but also solubility and log Kow. Hexane was toxic only for turbot embryos, due to its neurotoxic effects, and not for sea urchin larvae, at concentrations in the range between 1 and 10 mg/L. The concentrations tested were of the same order of magnitude for both species, and it was observed that sea urchin embryos (length of the longest arm) are more sensitive than turbot eggs larvae (hatching and cumulative mortality rates) to the HNS tested (except hexane). For this specific compound, concentrations up to 70 mg/L were tested in sea urchin larvae and no effects were observed on the length of the larvae. Both tests were found to be complementary depending on behaviour in water and toxicity target of the compounds analysed.

Keywords Marine spill · Hazardous and noxious substances (HNS) · *Paracentrotus lividus* · *Scophthalmus maximus* · Early life stage (ELS) bioassays · Standard European Behaviour Classification (SEBC)

Introduction

Hazardous and noxious substances (HNS) are defined as any substances other than oil, which if introduced into the marine environment are likely to create hazards to human health, living resources and other marine life. Furthermore, they can cause damage to amenities and/or interfere with other legitimate uses of the sea (IMO 2000). Whereas there is a good level of knowledge available for the toxicity, behaviour and risk associated with marine oil spills, information for HNS is more limited (Solé et al. 2008; Cunha et al. 2015, 2016; Neuparth et al. 2011). Most oils float on the surface and are immiscible with water, but HNS chemicals exhibit a wider range of behaviours (i.e. either sinking, floating on or readily dissolving in water or forming a gas or evaporating) and may therefore impact marine organisms in different environmental compartments (CEFAS 2009). There is a current paucity of knowledge about the effects of HNS on a wide range of marine biota that might be representative of the different compartments that may be affected by different HNS spills, and the few data that are available on HNS ecotoxicology are mostly from experiments conducted with freshwater organisms (Mamaca et al. 2005; Purnell 2009), making it difficult to predict the effects on marine organisms and to prepare contingency plans for these substances (Neuparth et al. 2011).

One fundamental requirement of bioassays to assess pollution in marine systems is the ecological relevance of the species, a requirement that is met by *Paracentrotus lividus* and *Scophthalmus maximus* for East Atlantic areas. Both are native European species of ecological and economic importance. Early life stages (ELS) of these two species were used to enhance the sensitivity to the tests, since ELS are frequently less tolerant to toxicants than adults (Ringwood 1991; His et al. 1999; Mhadhbi et al. 2010). During early ontogenesis, critical development of tissues and organs takes place, a process which can easily be disrupted by unfavourable environmental conditions including exposure to toxic compounds (Foekema et al. 2008).

The sea urchin embryo-larval toxicity test (SET) is frequently used in environmental quality assessment for several reasons: the species is of ecological relevance; is easy to collect, handle and maintain in the laboratory; and has a high survival rate under control conditions and low sensitivity to natural variables (Salamanca et al. 2009). Sea urchin is also considered an ideal tool for marine ecotoxicological tests because it is sensitive to several kinds of organic and inorganic micropollutants (Bellas et al. 2005; Salamanca et al. 2009), including metals (Fernández and Beiras 2001; Salamanca et al. 2009) and emerging pollutants (Ribeiro et al. 2015; Nacci et al. 1986) that may

affect the survival of larvae and adult sea urchins, providing an indication of the toxicological effects of contaminants to the whole ecosystem. Furthermore, *in vitro* fertilization and gamete generation of sea urchin are relatively easy, and test results can be obtained in a short period of time (48 h) (Salamanca et al. 2009). Therefore, SET was considered a rapid, sensitive and cost-effective biological tool for marine monitoring worldwide (Saco-Álvarez et al. 2010; Ribeiro et al. 2015).

The vertebrate teleost fish, *S. maximus*, is considered a good biological model for toxicological studies (Mhadhbi et al. 2010) due to diverse characteristics, namely its rapid growth rate, efficiency in adapting to diverse food sources, great resistance to diseases, easy reproduction and, finally, good tolerance to a wide range of environmental conditions (Mhadhbi et al. 2012a, b). The early development of turbot from fertilization to hatching was thoroughly described by Tong et al. (2013), being a fundamental tool for teratogenic effect determination. Mhadhbi et al. (2010) reported that considering the short duration of the early life stage (ELS) turbot test and its high sensitivity, it is suitable for use as a standard test for marine fish. Mhadhbi et al. (2012a) also proved that the ELS turbot test is an excellent model for the study of ecotoxicity of contaminants in seawater.

To respond and to assess the impact of incidents involving HNS, the compilation of a comprehensive ecotoxicological dataset for marine organisms is a priority issue. Due to the high number and diversity of HNS transported by sea (Cunha et al. 2015), it is unrealistic to consider a full scientific ecotoxicological data survey for all such chemicals (Neuparth et al. 2011). In the context of the European funded project ARCOPOL, Neuparth et al. (2011) defined a priority list of 23 HNS, which were a subset from a previous list of the 100 most transported HNS in European Atlantic waters as described by HASREP (2005). Prioritization was based on available data from the literature considering the toxicity, carcinogenicity, bioaccumulation, biodegradation, physicochemical properties, incidence of previous spills and likelihood of future spillage at sea considering the transport volume of each HNS. The list produced by Neuparth et al. (2011) highlighted the gaps in ecotoxicological data for many priority HNS with some chemicals having little data or only data available for freshwater fauna, concluding that they would benefit from further investigation.

The aim of the current work was to build key toxicological knowledge on priority HNS to the marine environment (Neuparth et al. 2011) to support risk assessment studies, by testing in the laboratory their effects on two ecologically relevant and phylogenetically different marine animals. Produced data may also be used for regulatory and conservation purposes in contexts other than for HNS spills.

Materials and methods

Selection of HNS used

Chemicals for ecotoxicological work were chosen taking into consideration (1) the inclusion in the HASREP (2005) list; (2) presence on the priority list established by Neuparth et al. (2011); (3) paucity of marine toxicological data (TOXnet); (4) behaviour in water according to the categories defined by the European Behaviour classification system (GESAMP 2002), which catalogues the behaviour of chemicals based on combinations of physical and chemical properties (Bonn Agreement 2015), by selecting compounds with different behaviours in water; and (5) consideration of the following parameters, when available: bioaccumulation, bioconcentration factor (BCF), biodegradation, acute and chronic toxicity, carcinogenesis (GESAMP and TOXnet), water solubility, partition coefficient, log Kow, organic matter adsorption constant and log Koc, in order to anticipate the most toxic compounds (Cunha et al. 2016). The chemicals (HNS) selected and some of the characteristics that most determine their behaviour in seawater are presented (Table 1).

Chemicals were tested for sublethal and lethal endpoints in one representative species each of a marine vertebrate and a marine invertebrate to better understand their acute toxicity during a spill at sea.

Stock solutions of aniline (CAS number: 62-53-3), butyl acrylate (CAS number: 141-32-2), m-cresol (CAS number: 108-39-4), cyclohexylbenzene (CAS number: 827-52-1), hexane (CAS number: 110-54-3) and trichloroethylene (CAS number: 79-01-6) at different concentrations were prepared in glass vials. Stock solutions were made by measuring appropriate volumes of the test chemical, considering the density and dissolving them in a solvent. The highest test chemical concentration was used to produce the appropriate stock dilution series, using pure dimethyl sulfoxide (DMSO) or acetone in the case of hexane, as solvents. The stock solutions were stored in the dark at 4 °C. The experimental concentrations were obtained by diluting the DMSO/acetone-stock solutions in artificial seawater (ASW), making working solutions (dilution of 1:10,000). Therefore, subsamples of the primary stocks were used to make the definitive test dilution series. For all treatments, the final DMSO concentration was 0.01% (v/v) in the working solutions. This concentration was found to be non-toxic in other studies using turbot early life stages (ELS) (Mhadhbi et al. 2012a) and sea urchin (Bellas et al. 2005; Ribeiro et al. 2015). The experimental concentrations tested are shown in the supplementary materials (S1). Test concentrations were chosen on the basis of literature toxicological data (S2) and preliminary trials. Compounds were chosen based on their high probability of being spilt at sea and causing damage to the environment, according to HASREP (2005) and Neuparth et al. (2011). A dilution factor of 5 was

Table 1 Description of the solubility, vapour pressure, density, partition coefficient (log Kow), Standard European Behaviour Category (SEBC), traffic ranking and HNS priority values for aniline, butyl acrylate, m-cresol, cyclohexylbenzene, hexane and trichloroethylene, used in the bioassays

HNS	Solubility (g/L) ^a	Vapour pressure (Pascal; 25 °C) ^a	Density (kg/L) ^a	Log Kow ^a	Behaviour category ^b	Traffic ranking ^c	HNS priority value ^d
Aniline	36.0	88.9	1.022	0.90	FD	19	7
Butyl acrylate	1.4	727	0.890	2.36	FED	57	15
m-Cresol	22.7	14.7	1.034	1.96	SD	96	21
Cyclohexylbenzene	0.005	5.32	0.938	4.81	F	43	11
Hexane	0.0095	20,130	0.655	3.90	E	74	18
Trichloroethylene	1.28	9200	1.464	2.42	SD	73	17

The values of the table are not only from GESAMP 2002. They are from many sources as explained

E evaporator, F floater, SD sinker/dissolver, FD floater/dissolver, FE floater/evaporator, FED floater/evaporator/dissolver

^a Values collected from the bibliography and public databases e.g. PubChem

^b According to GESAMP (2014, 2016)

^c According to the list of the 100 HNS most transported in European Atlantic waters elaborated by HASREP (2005)

^d According to the list of 23 priority HNS that pose major risk in the marine environment in the European context (Neuparth et al. 2011)

used in a preliminary range finding assay with sea urchin and then 1.5 with sea urchins and 2.5 dilution factors with turbot in the definitive assays. Following the range finder studies, more definitive tests were conducted to refine the estimates of end-point concentration.

Bioassay methodology

Two toxicity bioassays were established using embryos of a deuterostome invertebrate, the sea urchin (*P. lividus*) and a vertebrate teleost fish (*S. maximus*).

Sea urchin

Adult sea urchins were collected from the intertidal rocky shores of northern Portugal during low spring tides. The animals were transported to the laboratory under controlled temperature and then were acclimated to laboratory conditions until the start of the assay. Within 30 min after fertilization, fertilized eggs of sea urchin were placed in 24-well plates with 3 mL of fresh test solution at a concentration of 50 eggs/mL to perform a sea urchin embryo test (SET). The 24-well plates were isolated with parafilm, and embryos were incubated at 20 °C in the dark for 48 h (Fernández and Beiras 2001; Ribeiro et al. 2015). Neither food nor aeration was provided during the bioassay. At the end of exposure time, embryos were fixed by adding two drops of 37% buffered formaldehyde until further observation. Embryogenesis success was recorded by applying two toxicity criteria: mean larval length and percentage ($n = 15 \times 8$) of fully developed four-arm pluteus larvae ($n = 20 \times 8$). The number of analysed individuals for the two criteria was based on Saco-Álvarez et al. (2010) and Ribeiro et al. (2015). The mean larvae length was defined as the distance between the apex and the end of the longest post-oral arm (Fig. 1). Larvae were considered

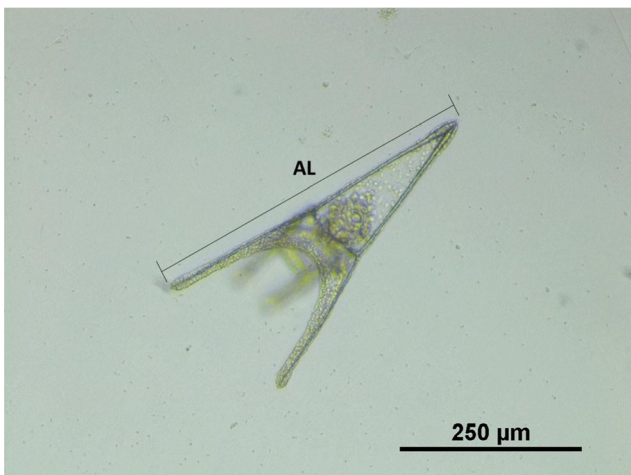


Fig. 1 Measure of maximum arm length dimension in sea urchin pluteus larvae. The four arms characteristic of this larval stage can be observed. AL arm length

normal by their pyramidal shape and the four fully separated arms (Saco-Álvarez et al. 2010). Larvae were measured and analysed directly in the 24-well plates using an inverted microscope (Nikon Eclipse TS100), equipped with a Nikon D5-Fi2 digital camera and software to perform measurements (Nikon Image Software). Changes to the normal development at 48 h post-fertilization (hpf) were recorded and presented as abnormal larvae.

For each toxicant, five and six concentrations plus a control (ASW) and a solvent control (pure DMSO or acetone in the sole case of hexane) were tested, in the preliminary and definitive assays, respectively (S1). Each treatment was replicated eight times, with each replicate test well containing ~150 larvae.

Turbot

Fertilized turbot eggs were provided by a commercial hatchery (Insuiña S.L., Mougás, Galicia, Spain), obtained from their own broodstock. Toxicity tests were also performed in 24-well plates, using 15–20 eggs in each well. Eggs were placed in the 24-well plates at the hatchery already immersed in the test solutions, immediately after fertilization, and transported to the laboratory inside styrofoam boxes at controlled temperature. Immediately after their arrival at the laboratory, plates were observed carefully, unfertilized eggs were discarded, and the density was adjusted to ten eggs/well. The bioassay was performed in an incubator, in the dark, at 14 ± 0.5 °C and salinity of 30‰. Medium was changed every day, following a semi-static technique, and dead individuals were removed and counted. A modification relative to the turbot ELS performed by Mhadhbi et al. (2010) was the exposure of the eggs immediately after fertilization (1 hpf), instead of initiating exposure at 72 hpf. Accordingly, they were exposed for 4 days (1–100 hpf) as embryos and for 6 days as lecithotrophic larvae (nurtured by the yolk reserves, mouth still closed), a total of 10 days (220 hpf). Neither food nor aeration was provided during the bioassay.

For each toxicant, five concentrations of each HNS (S1), plus a control (ASW—no chemical added) and a solvent control (DMSO or acetone in the sole case of hexane), were tested. Each treatment was replicated six times, each replicate consisting of a well with ten eggs or larvae.

During the exposure to different concentrations of the six HNS, endpoints were observed throughout the 10-day exposure, at four selected developmental points (Table 2), when specific developmental events occur: 75% epiboly (53 hpf), 50% hatching (100 hpf), 100% hatching (124 hpf) and mouth opening (220 hpf).

When the outer enveloping layer of the egg further flattens and differentiates into the epidermis, protecting the embryonic body at nearly 75%, was the moment when 75% epiboly was considered to occur. At this point, the neural rod derived from

Table 2 Developmental endpoints assessed in turbot embryos at the four sampling points established

	Time (hpf)			
	53	100	124	220
Endpoints assessed	Mortality 75% epiboly Abnormal cellular masses	Mortality Hatching Heart beat Embryo malformations–organogenesis	Mortality Hatching Embryo malformations Yolk sac volume Oil globule volume Oil globule position	Mortality Yolk sac absorption Oil globule volume Oil globule position Mouth opened Embryo malformations (others + jaw) Larvae length

hpf hours post-fertilization

ectoderm appears, the first somite also appears in the middle of the embryonic body, and the optic primordia are present. The different endpoints selected were observed using a stereomicroscope (LEICA). These developmental events have been well characterized and illustrated previously for *S. maximus* by Tong et al. (2013).

Hatching was defined as the rupture of the egg membrane, and partially as well as fully hatched larvae were counted as hatched (Mhadhbi et al. 2012a). At hatching, turbot lecithotrophic larvae possess a large yolk sac with a single oil globule, and the intestine is still closed at both ends. The frequency of heart beat gradually increases, there are clusters of pigment on the posterior to the fin-fold of newly hatched larva, and the larva remains motionless in the water (Tong et al. 2013).

Skeletal abnormalities seriously affect morphology of the fish, reduce growth and lower the market value of the product (Boglione et al. 2001). The skeletal deformities considered in the present work were abnormal dorsal curvature of trunk, abnormal dorsal curvature of tail, malformation of the jaw and malformation of the head.

Mortality was defined by coagulation of the embryos, missing heartbeat, failure to develop somites and a non-detached tail (Mhadhbi et al. 2012b).

Table 3 Effect concentration values (EC_{10}) and limits for the six compounds tested in *Paracentrotus lividus* upon the measure of the length of the longest post-oral arm at 48 hpf

	EC_{10} (mg/L)	[LCL; UCL] (mg/L)	Pearsons goodness-of-fit test			NOAEC (mg/L)	LOAEC (mg/L)
			χ^2	<i>df</i>	Slope sig.		
Aniline	0.807	[0.409; 1.907]	17.132	10	0.000	0.026	0.039
Butyl acrylate	0.358	[0.217; 0.527]	14.190	8	0.000	0.158	0.237
m-Cresol	0.345	[0.147; 3.523]	16.359	9	0.000	0.021	0.032
Cyclohexylbenzene	3.102	[2.289; 4.268]	22.122	10	0.000	0.658	0.988
Hexane	ND	ND	22.329	10	0.259	>70	>70
Trichloroethylene	ND	ND	81.456	10	0.000	1.19	1.78

Probit statistics were obtained through a Pearson goodness-of-fit test

LCL 95% lower concentration limit, UCL 95% upper concentration, limit, ND not determined

Statistics

One-way analysis of variance followed by the Dunnett post hoc test was used to investigate the effects of HNS on *P. lividus* and *S. maximus* endpoints, by comparison to the control group, with determination of no observed effect concentration (NOAEC) and lowest observed apical effect concentration (LOAEC). Non-parametric tests, Kruskal-Wallis and Mann-Whitney *U*, were used when data did not meet the requirements of ANOVA. Semi-maximum response concentrations, EC_{50} and LC_{50} , were determined using a Probit regression analysis. Pearson goodness-of-fit (chi-square) significance was considered at $p < 0.05$ for all analyses (Zar 1999), and 95% lower and upper confidence limits [95% LCL; UCL] were presented. Statistical analyses were performed on IBM SPSS Statistics v.20.

Results

Sea urchin bioassay

With the exception of hexane, all HNS resulted in significant effects on development of sea urchin embryos (Table 3).

m-Cresol had the lowest NOAEC and LOAEC 0.032 and 0.160 mg/L, respectively (preliminary test), 0.021 and 0.032 mg/L, respectively (definitive test), and also the lowest EC₁₀–0.345 mg/L, while hexane was the least toxic compound in both preliminary and definitive tests performed with *P. lividus* (Table 3). It was not possible to calculate an LOAEC or EC₁₀ for hexane because there was not a significant effect of any of the test concentrations on the length of the arm (Table 3). Therefore, at the concentrations tested, hexane did not affect the length of the longest arm of *P. lividus* larvae, and taking into account the LOAEC obtained on SET, the toxicity rank of the six chemicals tested was m-cresol > aniline > butyl acrylate > cyclohexylbenzene > trichloroethylene, while using EC₁₀, the toxicity rank was m-cresol > butyl acrylate > aniline > cyclohexylbenzene. It was not possible to determine EC₁₀ for trichloroethene because it falls beyond the maximal concentration tested (6 mg/L).

Turbot bioassay

Hexane was the only HNS that had a significant effect on turbot larvae. The endpoints affected were hatching rate at 124 hpf and cumulative mortality rate at the end of the experiment (220 hpf). NOAEC and LOAEC values were 2.8 and 7.0 mg/L, respectively (Table 4 and Fig. 2). EC₅₀ on hatching rate at 124 hpf was 11.64 mg/L [95% 4.12; 202.06], while the LC₅₀ at 220-hpf value was 1.53 mg/L [95% 0.67; 5.89] (Table 4).

None of the other five chemicals tested had significant effects on turbot eggs-larvae at the concentrations tested. Therefore, for these chemicals, the effective NOAECs and LOAECs were estimated to be greater than the highest nominal concentrations tested.

Discussion

The compounds tested here were carefully chosen as representative of a chemical that is frequently transported in bulk, pose risk to the environment, has little aquatic and in particular marine toxicity data available (S2), represents one of the Bonn Agreement behaviour categories and is anticipated to be toxic

based on its physicochemical properties. Aniline and m-cresol were the most toxic compounds with LOAEC values in the SET ranging between 0.01 and 0.1 mg/L, while trichloroethylene was the least toxic with SET's NOAEC values in the range between 1 and 10 mg/L. A similar rank was observed when analysing EC₁₀ data. Surprisingly, hexane was the only HNS of the six analysed that did not have an effect on the length of the longest arm of sea urchin larvae, at the range of concentrations tested and, at the same time, the only one that affected the egg hatching rate and cumulative mortality rate of turbot larvae, although only at the highest concentration tested (7 mg/L, Fig. 2).

Toxicity tests using ELS play an important part in risk assessment as they are generally considered more sensitive to toxic agents. The sensitivity of ELS tests is probably due to a larger body surface-to-volume ratio of eggs, embryos and larvae; their generally undifferentiated epithelia; and the vulnerability of the developmental processes (Oberemm 2000). The different permeability and composition of the eggshell of both organisms may be responsible for the disparity in the response to the compounds when comparing embryos of both species. Turbot eggs have a much thicker chorion, which protects the developing embryo for nearly 4 days before hatching. In contrast, in sea urchin eggs, the envelope lasts only up to 24 h. The lipid content of the egg/chorion of both species does not explain the different effects of the chemicals, considering that hexane is a very good lipid solvent since they have a similar total lipid content. Lipids are about 12.1% of *P. lividus* egg dry weight (Pais et al. 2011), while in turbot eggs, lipids are 13–14% dry weight (Silversand et al. 1996), and so, similar partition coefficients may be expected regarding this parameter, for the same chemical in both species.

The results of SET largely reflect the physicochemical properties of the test chemical and its behaviour in water (Bonn Agreement 2015) of the six chemicals tested (Table 1). The movement of a chemical between the air and water is affected by its volatilization and its water solubility. The high vapour pressure of hexane and trichloroethylene means that it is very likely that the concentration of these chemicals in solution will rapidly decrease during a test or when present in the sea following a spill. Hexane also shows one of the lowest potential solubilities (Table 1). Although all

Table 4 Hexane concentration values and limits of EC₅₀ and LC₅₀ in *Scophthalmus maximus*, for the parameters hatching rate at 124 hpf and cumulative mortality rate at 220 hpf, respectively

	Semi-maximum response concentration (mg/L)	LCL	UCL	Chi-square	df	Slope sig.
Hatching rate (124 hpf)	11.639	4.123	202.062	798.0	34	0.000
Cumul. mortality rate (220 hpf)	1.513	0.673	5.889	1164.2	34	0.000

Probit statistics were obtained following Pearson goodness-of-fit test, on the obtained values at six hexane concentrations. LCL 95% lower concentration limit, UCL 95% upper concentration limit

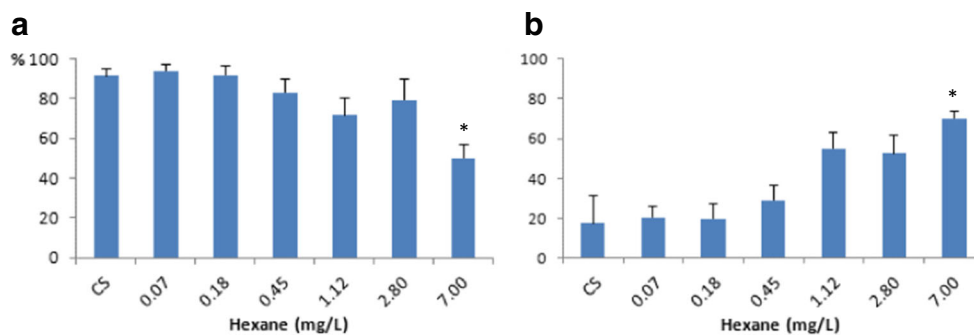


Fig. 2 a Hatching rate (%) at 124 hpf and b cumulative mortality rate (%) at 220 hpf of turbot larvae for six hexane concentrations. Differences were analysed as compared to solvent control (Cs) through the post hoc

Dunnett test. Results represent average values \pm standard error. Asterisk indicates significant differences to control ($p < 0.05$)

the six tested chemicals have a maximum solubility, under the conditions described, well within the test concentration range, the high vapour pressure of hexane and trichloroethylene possibly led to reduced exposure concentrations and hence lower toxicity (except in the case of hatching rate of turbot larvae at 124 hpf and cumulative mortality rate at 220 hpf). m-Cresol and aniline were the most toxic of the chemicals tested, which is probably related to the physicochemical properties like high solubility in water. Also, m-cresol is a sinker-dissolver (SD), and although sea urchin larvae swim in the water column, they rest at the bottom, where possibly the concentration of cresol was the highest.

In the turbot ELS bioassay with hexane, the most sensitive endpoint was cumulative mortality at 220 hpf (1.5 mg/L), as compared to egg hatching rate (11.6 mg/L). A similar result was observed for previous turbot ELS bioassays using polycyclic aromatic hydrocarbons (PAHs), metals (Mhadhbi et al. 2010), pesticides (Mhadhbi et al. 2012b) and polybrominated diphenyl ethers (PBDEs) (Mhadhbi et al. 2012a) where it was found that embryos are more tolerant than hatched larvae. Embryo's tolerance was nearly two times higher in the case of PAH, metals and some PBDEs. In the present work, regarding hexane, tolerance was seven times higher in embryos as compared to hatched larvae. The higher tolerance of embryos has been attributed to the protection that the chorion confers to embryos, acting as a barrier for some toxicants (Hallare et al. 2006; Barry et al. 1995; Humphrey and Klumpp 2003).

Since after hatching turbot larvae were still exposed to HNS for further 5–6 days and despite the renewal of media every day, they did not respond to any of the tested compounds, except to hexane. Hence, it seems that SET is more sensitive to the priority HNS tested than turbot larvae. However, Mhadhbi et al. (2010) have reported that considering the short duration of the turbot ELS and its high sensitivity as compared to other fish species, it is suitable for use as a standard test for marine fish. Despite differences in sensitivity, both tests are complementary, and when e.g. neurotoxic compounds are being tested, vertebrates or crustaceans are usually more appropriate because of the higher complexity of their

nervous system, which confers upon them a higher sensitivity to these types of compounds. This is the case for hexane, for which the nervous system is the primary target of toxicity (US EPA 2005). Accordingly, comparing turbot LC_{50} for hexane to those available for other aquatic species (S2), rotifer (*Brachionus plicatilis*) neonates (Snell et al. 1991; Ferrando and Andreu-Moliner 1992) and *Artemia salina* nauplii (Abernethy et al. 1986), the test with turbot embryos was confirmed to be more sensitive (Table 4). It was 100 \times more sensitive than for rotifers and 2 \times more sensitive than *A. salina*. Contrarily, hatching rate EC_{50} was much less sensitive than all the other EC_{50} values described in the bibliography, namely on *Skeletonema costatum* photosynthesis (Brooks et al. 1977) and *A. salina* immobilization (Foster and Tullis 1995).

Regarding the other endpoints analysed in the sea urchin SET (malformations) and turbot ELS (Table 3), none of the various HNS analysed had a significant effect on any endpoint. No significant abnormalities were observed after exposure, in any of the organisms, as compared to the control, showing that these HNS are not teratogenic at the concentrations tested.

Comparing the SET NOAEC results to the other species data from the literature indicates that SET is in a similar order of magnitude of sensitivity to aniline, trichloroethylene and hexane toxicity but potentially more sensitive to m-cresol. Regarding NOAEC values in chronic assays for aniline with the freshwater crustacean, *Daphnia magna*, 21-day reproduction test of 0.004 mg/L (Kühn et al. 1989) and fathead minnow fish, *Pimephales promelas*, ELS growth test of 0.422 mg/L (Russom 1993), the values obtained in the present study in SET for aniline are between that range of values (Table 3). However, the LOAEC values observed in SET (0.2 and 0.039 mg/L, for preliminary and definitive tests, respectively) indicate that it is more sensitive to aniline than *P. promelas* ELS assay (Russom 1993), corroborating also the low sensitivity observed in the 10-day turbot ELS (LOAEC >0.320 mg/L). Recently, de los Santos et al. (2015), using a 25-day partial-life cycle test with *Gammarus locusta*, reported that a

concentration as low as 0.0005 mg/L led to a reduction of newborn production, a concentration below that recorded here to affect sea urchin embryo development. These results provide preliminary information that is of value when assessing the risk of aniline spills to different marine species and habitats, as well as the choice of target organisms to test.

For butyl acrylate (Bringmann and Kuhn 1977; Paulet and Vidal 1975) and cyclohexylbenzene (Passino-Reader et al. 1997), only data reporting acute response, mostly LC₅₀ values of freshwater fauna are available, which makes the results reported here valuable in filling gaps of knowledge of the effects of such chemicals in the marine environment (Table 3) but makes comparisons to other seawater species data difficult (S2). A good literature review of benchmarks and description of test conditions for these six HNS is presented by Neuparth et al. (2011).

Regarding m-cresol, only EC₅₀ values were available for other species comparisons again and the EC₁₀ values obtained with SET for the length of the arm revealed were around 100× lower than the EC₅₀ values obtained for another sea urchin (*Strongylocentrotus droebachiensis*) and cod (*Gadus morhua*) embryos (Falk-Petersen et al. 1985), where multiple effects are reported as one result (death, pathology, inhibition of cleavage and differentiation and pigment defects).

Finally, regarding data reporting acute toxicity of hexane and trichloroethylene to seawater fauna, a few LC₅₀ values only are available from the literature. For trichloroethylene, LC₅₀ values of 48–96-h acute assays ranged from 2 mg/L in the crustacean *Mysidopsis bahia* (Borthwick 1977) to 99 mg/L in the fish *Cyprinodon variegatus* (Pearsons and McConnell 1975), while in the present study, NOAEC and LOAEC values observed for trichloroethylene in the SET were 1.19 and 1.78 mg/L, respectively. These data suggest that SET is probably of a similar order of magnitude of sensitivity to those other species.

Since there is a paucity of knowledge on HNS effects on marine biota and relatively few data are available on the HNS ecotoxicology, those data originating mostly from assays performed with freshwater organisms, additional species toxicity data obtained here will be of value in developing post-spill risk assessments for these chemicals and are essential to better anticipate the impact of HNS in marine species, therefore improving preparedness and response at the operational level.

Acknowledgements The present work was developed within the framework of the ARCOPOLplus project—Improving maritime safety and Atlantic Regions’ coastal pollution response through technology transfer, training & innovation (contract nr. 2011-1/150)—funded by the Atlantic Area Transnational Programme (Priority 2: Marine Environment and Renewable Energy) with support of the European Regional Development Fund (ERDF) and ARCOPOL Platform. We also acknowledge the Portuguese Foundation for Science and Technology (FCT) through the strategic project “UID/Multi/04423/2013”.

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