ATLANTIC AREA
Transnational Programme
INVESTING IN OUR COMMON FUTURE

European Union
European Regional Development Fund

ARCOPOL PLUS

Activity 2

Task 2.2.1; Determination of acute and chronic toxicity of priority HNS upon representatives of different marine plant and animal taxa.

ARCOPOLplus

Improving maritime safety and Atlantic Regions' coastal pollution response through technology transfer, training and innovation

Version:	1
Last updated on:	15/10/2013
Author: (CEFAS)	Thomas McGowan, Dave Sheahan
(CIIMAR)	Isabel Cunha, Helena Oliveira, Miguel Santos
Responsible partner:	CEFAS/CIIMAR

INDEX Pag.

1. Introduction

Hazardous and Noxious Substance (HNS) are defined as any substance other than oil, which if introduced into the marine environment is likely to create hazards to human health, to harm living resources and other marine life as well as to damage amenities and/or to interfere with other legitimate uses of the sea (IMO, 2000). Whereas there is a certain level of knowledge available for the toxicity, behaviour and risk associated with marine oil spills, there is not the same level of knowledge available for HNS. Most oils float on the sea and are immiscible with water, but HNS chemicals exhibit a wider range of behaviours (*i.e.* sinking, floating, gassing, evaporating, and dissolution) in the environmental compartments and toxicities to marine organisms (CEFAS, 2009).

Therefore, there is a current paucity of knowledge about the effects of HNS on marine biota and the relatively few data available on the 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).

In order to respond to incidents involving HNS, the systematic classification of scientific ecotoxicological data for marine organisms should be a priority issue. Due to the high number and diversity of HNS transported by sea, it is, in practice, unrealistic to consider a full scientific ecotoxicological data survey for all such chemicals (Neuparth et al., 2011). In the context of the ARCOPOL project, Neuparth et al. (2011) produced a priority list of 23 HNS that had several gaps and would benefit from further ecotoxicological data. This list took into account known toxicity and carcinogenity based on available data from the literature as well as other parameters like 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 and very little data available for algal taxa.

The aim of the current work is to create key knowledge on HNS, providing more ecotoxicological data for some of the HNS identified as those of major interest by Neuparth et al. (2011). Thus, this study aims at improving toxicological risk assessment of HNS by testing at laboratory the hazard for marine life of the HNS selected. Chemicals for further ecotoxicological work were chosen taking into consideration the following parameters:

- Bioaccumulation
- Bioconcentration factor (BCF)
- Biodegradation
- Acute and chronic toxicity
- Carcinogenesis (GESAMP and TOXNET)

- Lack of marine toxicological data (TOXnet)
- Behaviour (selecting compounds with different behaviours in water)
- Water solubility
- Partition coefficient; Log Kow
- Organic matter adsorption constant; Koc
- Traffic volume

The chemicals (HNS) selected were:

- Aniline (Floater/Dissolver (FD))
- Butyl acrylate (Floater/Evaporator/Dissolver (FED))
- m-cresol (Sinker/Dissolver (SD))
- Cyclohexylbenzene (Floater(F))
- Hexane (Evaporator (E))
- Trichloroethylene (Sinker/Dissolver (SD))

These chemicals were tested for lethal and sub-lethal/chronic endpoints in representatives of marine animal and algal species in order to develop understanding of their toxicity (acute and chronic) in the marine environment considering an ecosystem scale.

2. Methodology

2.1 Preparation of test solutions

Toxicity tests carried out at CEFAS

Test solutions of aniline (CAS number: 612-008-00-7, ACS grade ≥99.5 %), butyl acrylate (CAS number: 141-32-2, analytical standard), hexane (CAS number: 110-54-3, anhydrous 95 %) and trichloroethylene (CAS number: 79-01-6, analytical standard) were prepared in filtered (0.2 µm) seawater collected from the Cefas Lowestoft laboratory. All chemicals were purchased from Sigma-Aldrich UK. Stock solutions were made first by weighing appropriate quantities of the test chemical and adding to filtered seawater (unfiltered seawater was used for the *Pomatoceros* study). Subsamples of this primary stock were used to make the definitive test dilution series. Both butyl acrylate and trichloroethylene were readily dissolved in the primary stock solution but for aniline, which is classified as a floater/dissolver, stock solutions were spun (200 rpm) until no more aniline was visible as globules in solution (approximately 20 mins). Hexane is insoluble in water and to avoid using additional solvents, a Water Accommodated Fraction (WAF) was produced for tests using hexane, by adding a known weight of hexane to a known volume of filtered seawater, and spinning at 550 rpm (a vortex was present in the top third of the solution) for approximately 20 hours in the dark. This solution was then separated for 1 hour before approximately 200 ml of the WAF were removed from the bottom of the settled solution and used as the highest test

concentration to produce the test dilution series (i.e. representative of likely maximum quantity that readily dissolves and that is subsequently diluted during natural dispersion and mixing).

Toxicity tests performed at CIIMAR

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 in the CIIMAR laboratory (fig. 1A). To this end, the stock solutions were made by measuring appropriate volumes of the test chemical taking into account the density and dissolving it in a solvent. The highest test chemical concentration was used to produce the appropriate stock dilution series. Solvent used was pure dimethyl sulphoxide (DMSO) or acetone in the sole case of hexane. The stock solutions were prepared in sufficient volumes and stored in the dark at refrigeration temperature. For sea urchin, a dilution factor of 5 was used in a preliminary assay and 1.5 in a definitive assay. The dilution factor used for turbot was 2.5.

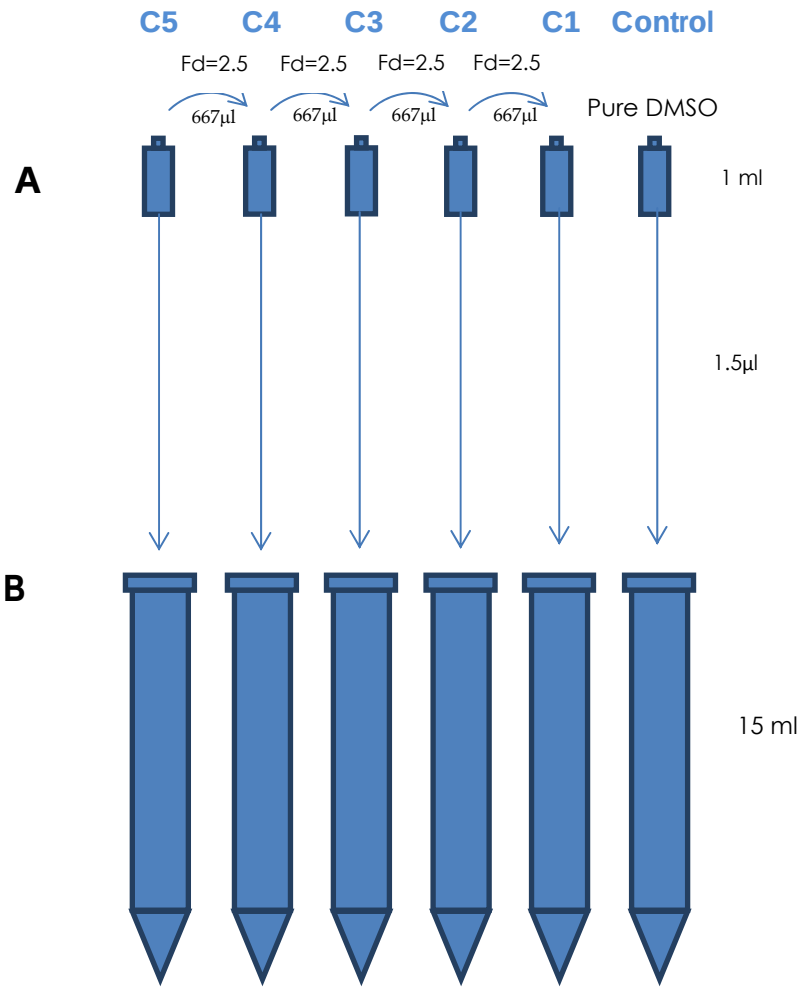

Figure 1. Experimental design of the preparation of 5 stock (A) and working (B) aniline solutions (C1, C2, C3, C4 and C5) for assays with turbot.

The experimental concentrations were obtained by diluting the DMSO/acetone-stock solutions in artificial seawater (ASW), making working solutions (dilution of 1:10000) (fig. 1B). Therefore, subsamples of the primary stock 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.

The experimental concentrations tested are shown in Table 1. These concentrations were chosen on the basis of toxicological data from the literature of the chemicals selected and other trials already performed with sea urchin that showed significant effects in such animals.

Table 1. Tested concentrations in preliminary and definitive trials (sea urchin and turbot) for aniline, butyl acrylate, m-Cresol, cyclohexylbenzene, hexane and trichloroethylene.

	Tested concentration (mg/L)		
Chemical	**Preliminary trial (sea urchin)**	**Definitive trial (sea urchin)**	**Definitive trial (turbot)**
Aniline	0.008	0.018	0.008
	0.040	0.026	0.020
	0.200	0.039	0.051
	1.000	0.059	0.128
	5.000	0.089	0.320
		0.133	
Butyl acrylate	0.010	0.105	0.031
	0.048	0.158	0.077
	0.240	0.237	0.192
	1.200	0.356	0.480
	6.000	0.533	1.200
		0.800	
m-Cresol	0.006	0.014	0.004
	0.032	0.021	0.010
	0.160	0.032	0.026
	0.800	0.048	0.064
	4.000	0.071	0.160
		0.107	
Cyclohexylbenzene	0.008	0.439	0.128
	0.040	0.658	0.320
	0.200	0.988	0.800
	1.000	1.481	2.000
	5.000	2.222	5.000
		3.333	
Hexane	0.011	9.2	0.179
	0.056	13.8	0.448

	0.280	20.7	1.120
	1.400	31.1	2.800
	7.000	46.7	7.000
		70.0	
Trichloroethylene	0.014	0.790	0.230
	0.072	1.185	0.576
	0.360	1.778	1.440
	1.800	2.667	3.600
	9.000	4.000	9.000
		6.000	

The artificial seawater was prepared as follows: 24.6 g of NaCl, 0.67 g of KCl, 1.36 g of $CaCl_2$, 0.39 g of $NaHCO_3$, 2.04 g of $MgSO_4$, 4.66 g of $MgCL_2.6H_2O$ and 1 L of distilled water. This mixture was stirred overnight in the dark and stored at 4 °C. The pH and the salinity were measured in artificial seawater to confirm that they remained within an acceptable range (8.2-8.3 and 30-1000, respectively), ensuring the acceptability of the tests. If necessary, NaCl was added to artificial seawater to adjust the pH. All chemicals were purchased from Sigma-Aldrich UK.

2.2 Chemical analysis - CEFAS

Samples of the test dilution series were analysed to determine the concentrations of aniline, butyl acrylate, hexane and trichloroethylene in freshly prepared and aged (either 24 or 48 hours) solutions in some tests.

Analysis of aniline, butyl acrylate, hexane and trichloroethylene samples was conducted by Environmental Scientifics Group (ESG). Samples for aniline analysis were extracted with dichloromethane (DCM) through a separating funnel, followed by acidification and a further extraction with DCM. These two samples were then dried using anhydrous sodium sulphate before being combined and concentrated to 1 ml by Kurdina Danish evaporation. This extract was then analysed by Gas Chromatography / Mass Spectrometry (GCMS). This method has an assured detection limit of 2 µg/l.

Butyl acrylate, hexane and trichloroethylene were analysed by GCMS headspace sample. This headspace sample was produced from a 5 ml sub-sample, which had been added to a vial, shaken and heated for a fixed period of time. This extract was then analysed by GCMS with an assured 1 µg/l detection limit using this method.

Samples were sent for analysis in amber glass bottles, filled with no headspace remaining, within cool boxes with frozen ice-packs. The volume of samples was 40 ml for aniline analysis and 200 ml for butyl acrylate, hexane and trichloroethylene analyses. Samples were transported on a next day delivery service.

The Environment Agency National Laboratory Service (NLS) (Leeds) conducted the analysis for some of the latter aniline toxicity tests using similar methods to those described above.

2.3 Bioassays methodologies

Four toxicity bioassays were established at CEFAS and two at CIIMAR. The organisms tested in CEFAS were *Tisbe battagliai* (copepod), *Fucus vesiculosus* (brown macro algae), *Ceramium tenuicorne* (red macro algae) and *Pomatoceros triqueter* (polychaete). The bioassays performed at CIIMAR were carried out using the sea urchin (*Paracentrotus lividus*) and the turbot (*Scophthalmus maximus*).

2.3.1 *Tisbe battagliai*

Copepods are crustaceans that are frequently dominant secondary producers in marine zooplankton (Hart, 1990) and are accordingly important to marine foodwebs. Copepods belonging to the genus *Tisbe* are particularly useful for risk assessment due to their small size, relatively short lifecycle and the ease of continuous culture.

48 hour mortality bioassays were conducted on juvenile *Tisbe battagliai* for aniline, butyl acrylate, hexane and trichloroethylene. Experiments with aniline and butyl acrylate were repeated 3 times in total.

Animals were obtained from in-house cultures held at Cefas. These cultures are maintained at 18 to 22 °C, pH 7.7 to 8.3 and salinity of 25 to 35 ppt. Juvenile animals of 6-7 days age were selected by filtering stock cultures of animals through a 106 µm mesh sieve and then selecting animals of an appropriate and uniform size using a binocular microscope with dark-field illumination. Five animals were allocated into each well of a polystyrene well plate in 5 ml of the appropriate tests solution (4 replicate wells per test concentration prepared as described above), or in filtered seawater as a control group (eight replicate wells). Test solutions were replaced after 24 hours. Water quality in terms of temperature, pH and salinity was tested at the start and end of each test to ensure that they remained within an acceptable range, as described above for cultured animals. So as not to disturb the test species, water quality was measured in a parallel set of test vessels held at the same temperature and in the same light cycle as the polystyrene test plates. Animal mortality was observed and recorded at 24 (±4) hours and 48 (±4) hours. Results of 48 hour observations were used to derive lethal effect concentrations for 50 % of the test animals (LC_{50} values).

A reference test using zinc sulphate was run in parallel for all *Tisbe* tests using animals from the same culture and under the same conditions, to confirm the condition of animals used in the tests. LC_{50} results for all zinc reference tests fell within acceptable limits as set by Cefas' internal culture control charts.

2.3.2 *Fucus vesiculosus*

As primary producers macro algae are at the base of the marine foodweb but plants in the coastal ecosystem also play a key part in creating habitat for other species including important nursery areas for various fish species (Pihl et al., 1995). A number of studies have used macroalgae such as the seaweed *Fucus serratus* to look at chemical effects upon growth, photosynthesis and reproduction *e.g.* the movement of spermatozoa or percentage fertilisation success (Scanlan and Wilkinson, 1987), as well as the growth of *Fucus* germlings exposed to contaminants (Braithwaite and Fletcher, 2005, Brooks et al., 2008).

96 hour tests were conducted on sporlings of *Fucus vesiculosus* for aniline, butyl acrylate, hexane and trichloroethylene. Frond growth and successful germination were measured as sensitive and ecologically relevant endpoints to determine effect concentrations.

Approximately 100 receptacles of *Fucus vesiculosus* (fig. 2) were collected from the shore of Lowestoft beach on 4/12/2012. On return to the laboratory, receptacles were washed in filtered seawater and refrigerated overnight at 5±3 °C and kept damp. On 5/12/2012 receptacles were removed from the fridge and placed into filtered seawater (at approximately 20 °C) in direct sunlight and with a table lamp shining directly on the beaker. After approximately 6 hours, sporlings have been released. These sporlings were washed through a 90 µm mesh sieve and retained in one of 24 µm mesh, before being washed into approximately 50 ml of clean seawater. The concentration of the sporlings was estimated using a Sedgwick-Rafter cell to be 3200 sporlings/ml. This solution was then diluted to approximately 1600 sporlings/ml in 100 ml seawater and was divided equally into 4 plastic trays. 25 glass cover slips (22 mm x 22 mm) were then placed into each tray and left overnight for sporlings to attach.

Figure 2. Fronds of *Fucus vesiculosus* with swollen receptacles at their tips.

On 6/12/12 cover slips were placed into well plates containing the appropriate solution for the treatment, 8 replicates were treated for each test concentration, including the control.

Water qualities of each solution were taken before the addition of the slides. Plates containing the slides were kept in a constant temperature room set to 15 °C. Slides were moved to fresh solutions after 48 hours. Water qualities of all test solutions were measured when first made up and after 48 hours. These solutions were excess solution held in beakers at the same temperature and in the same light cycle as the sporlings plates. Coverslips were removed from solution after 96 (±4) hours and preserved in 5 ml of 4 % buffered formalin solution and refrigerated at 5±3 °C until analysis. Samples from 0 and 48 hrs were sent for analysis.

Coverslips holding sporlings were analysed for frond length and for germination success after 96 hours. Coverslips were observed using a stereo-microscope and camera while they were within well plates, in solution. Frond length was measured using image analysis software (Myrmica v2.2.2 © Pilkington Image Analysis and Cefas).

2.3.3 *Ceramium tenuicorne*

Ceramium tenuicorne is a filamentous red macro algal species that can grow up to 10 cm in length (fig. 3). It is widely distributed in temperate waters and is found in both brackish and marine waters (Eklund, 2005). Growth is an expression of the combined influence of environmental factors including chemical exposures, and has been used as an endpoint by several authors both on green (Fletcher, 1989), brown (Thompson and Burrows, 1984) and red algae (Boney and Corner, 1962; Bruno and Eklund, 2003).

7 day tests were conducted on growing tips of *Ceramium tenuicorne* for aniline, butyl acrylate, hexane and trichloroethylene. Growth was used as the measure of chemical effect.

Cultures of *Ceramium tenuicorne* are held at Cefas' Lowestoft laboratory in sterile seawater with added nutrients according to methods described by Eklund (2005), at 15±2 °C, salinity of 26 ppt and a light:dark cycle of 16:8 hours.

Growing tips of between 0.6 and 1.2 mm in length (from the point of forking to tip end) were cut from plants using a binocular microscope on the day before the start of the test. Measurements were taken using a graticule placed into the culture dish with the culture plant. These measurements were recorded. Two tips were placed into 6 well polystyrene culture plates with 5 ml of solution (with the addition of nutrients), as described above. Solutions were changed at 48, 96 and 144 hours. Water qualities were measured for fresh solutions, aged solutions and at the end of the test. Samples from 0 and 48 hrs all test solutions were analysed, as previously described.

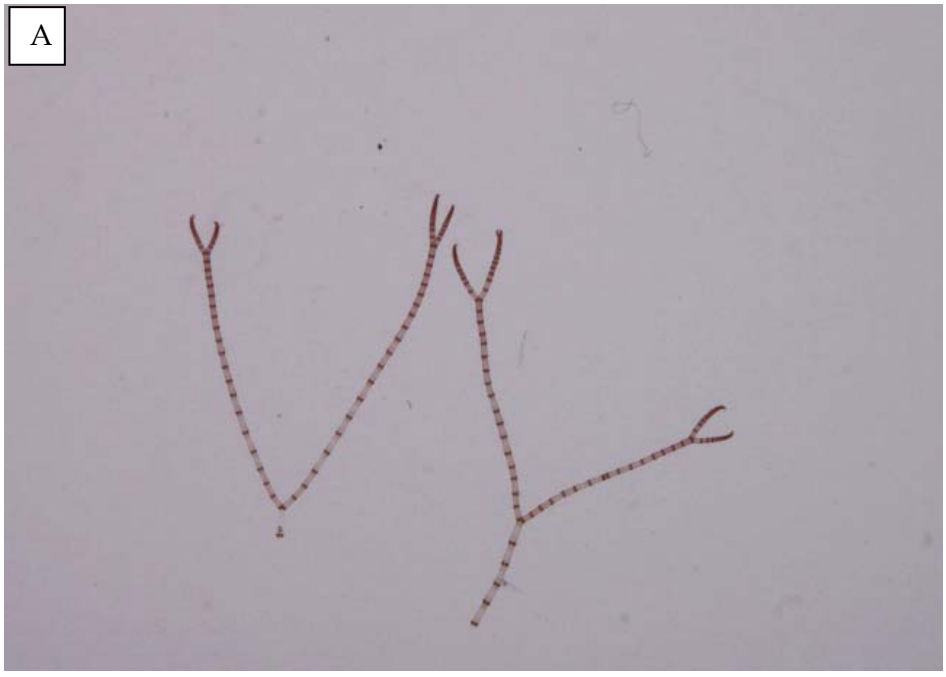

Figure 3. Tips of *Ceramium tenuicorne* at the beginning of a test (Top) and after seven days growth (Bottom).

At the end of the test, *Ceramium* tips were removed into well plates containing 5 ml of 4 % buffered formalin, and were stored at 5±3 °C until they were measured between 3 and 4 days after the test has finished. Tips were measured using a stereo-microscope and camera while they were within well plates, in solution, using image analysis software (Myrmica v2.2.2 © Pilkington Image Analysis and Cefas).

2.3.4 *Pomatoceros triqueter*

Pomatoceros triqueter is a tube dwelling, intertidal polychaete species (fig. 4) and represents a useful organism for environmental studies because of its small size and availability of sensitive life stages (Dixon et. al., 1999). Ripe gametes can be obtained from adult worms by removing them from their calcareous tubes using blunt forceps, which stimulates the release of sperm and eggs from males and females, respectively. Although the natural spawning period is between July and August they can be induced to spawn at most times of year with the exception of a few months immediately after their natural spawning period.

Two studies were carried out using *Pomatoceros triqueter*: firstly a range finding test was conducted in May for aniline and butyl acrylate. Following this test, definitive tests were carried out in June for aniline, butyl acrylate, hexane and trichloroethylene.
Pomatoceros triqueter were collected from Rottingdean, Sussex on the 16th May and 4th June. Once in the laboratory, *Pomatoceros* were stored in flowing, unfiltered seawater with gentle aeration.

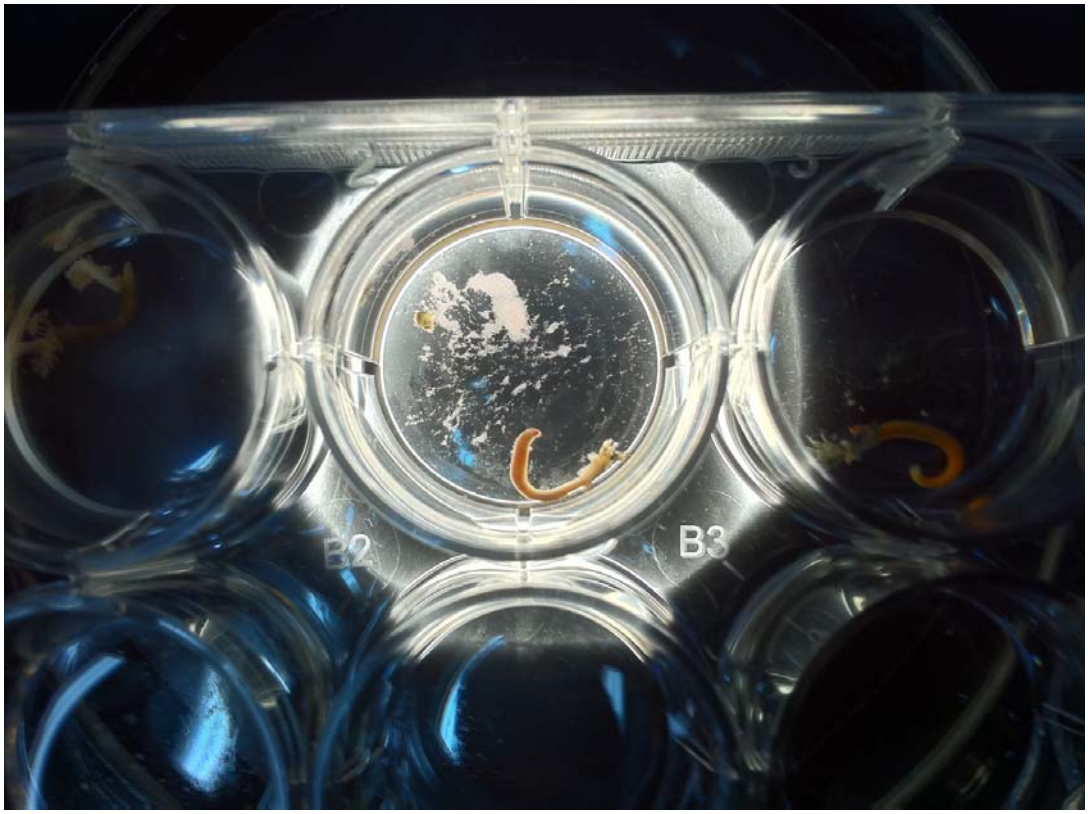

Figure 4. Adult female *Pomatoceros triqueter* in a well plate having released a mass of eggs.

Tests were conducted as soon as possible after animals' collection on the 22/5/13 and 12/6/13. *Pomatoceros* of a length greater than 2 cm (representing the largest size class) were preferentially selected for removal of their tubes. Once removed, these individuals were placed into separate well plates of unfiltered seawater and checked periodically to confirm release of gametes. Once enough worms had been obtained and had released eggs, they were combined into one group and filtered through a 90 µm mesh sieve to remove debris. At this stage, an estimate of numbers per ml was made using a Sedgwick Rafter counting cell. Sperm released into filtered seawater in individual cell wells was also combined and filtered through a 24 µm filter to remove debris and was checked under the microscope to confirm viability through movement. For both tests, approximately 1 ml of combined sperm solution was added per 25 ml of egg solution. This mixture was left for approximately 2 hours for the eggs to be fertilised and commence development. Once some of the eggs were observed to have reached the 4 cell stage, a count was made of fertilised/developing eggs per ml. Based on this count, a sufficient volume of fertilised eggs was added to each test well to achieve an approximate concentration of eggs/ml, making a total volume of 4.5 ml of the relevant test solution or control seawater. The tests were terminated after 48 hrs by the addition of 0.5 ml of buffered 4 % Formalin. Preserved material was stored at 5±3 °C. Temperature, pH and salinity were measured at the beginning and end of the tests (at 48 hours). Fresh (0 hrs) and aged (48 hrs) samples of the highest and lowest aniline concentrations (3 and 30 mg/l in the rangefinder and 10 and 300 mg/l in the definitive

tests) were sent for chemical analysis. Analysis of embryos involved counting the number of normally developed and abnormal or underdeveloped embryos (fig. 5). These values were used to calculate the Percentage Net Response (PNR) which is effectively the response adjusted for background levels of abnormality in the Control.

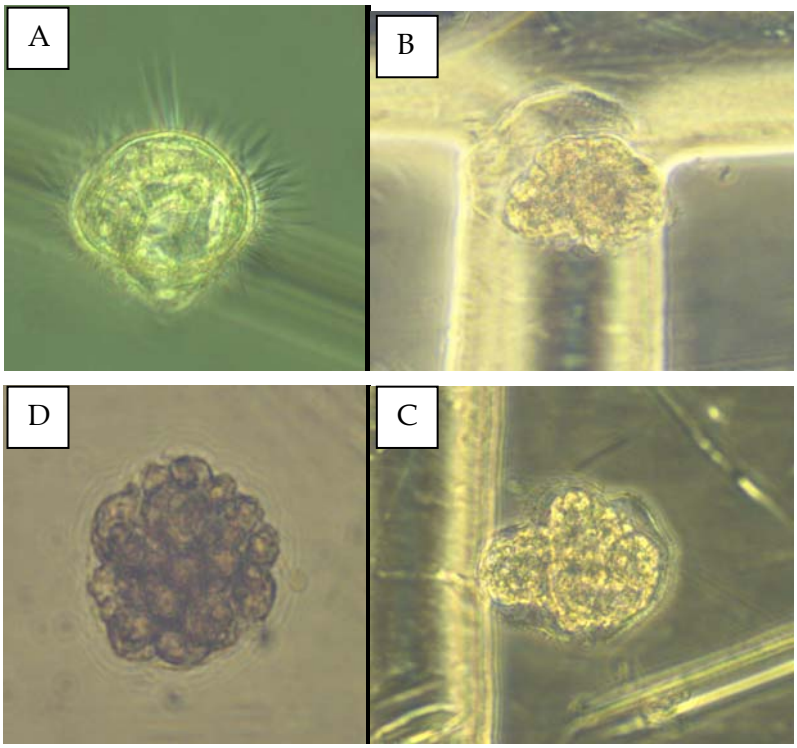

Figure 5. Clockwise from top left; A: live *Pomatoceros* embryo, B: preserved abnormally developed *Pomatoceros* embryo, C: preserved normally developed *Pomatoceros* embryo and D: undeveloped (fertilised) *Pomatoceros* egg.

2.3.5 Sea urchin (*Paracentrotus lividus*)

The edible *Paracentrotus lividus* (Lamarck, 1816) (fig. 6A) is an echinoid species distributed all along the Mediterranean and northeast Atlantic coasts, from Ireland to Morocco, including the Canary Islands and the Azores Islands (Boudouresque and Verlaque, 2007). The sea urchin is a herbivore but can also consume animal food (Fernandez and Boudouresque, 2000).

The sea urchin embryo-larval toxicity test is frequently used in environmental quality assessment due to several reasons, such as: is a species of ecological relevance, easy to catch, handle and maintain in the laboratory, 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 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). Nacci et al. (1986) also reported that many contaminants may affect the survival of larvae and adult sea urchins. Furthermore, it is well known that in vitro

fertilisation and gametes generation of sea urchin are relatively easy and such results can be obtained in a short-period of time (48 h) (Salamanca et al., 2009). Therefore, the sea urchin embryo test (SET) was considered a rapid, sensitive, and cost-effective biological tool for marine monitoring worldwide (Saco-Álvarez et al., 2010).

The organisms were collected in the intertidal rocky shore of the northern Portugal between 9-10 am with a tide of 0.6 cm and in another day between 7-8 am with a tide of 0.8 cm. The animals were transported at controlled temperature to the laboratory and then were acclimated to laboratory conditions in the bioterium of aquatic organisms (BOGA, CIIMAR) until the fertilization time. The experimental conditions were performed at 20 °C and with a salinity of 30 ‰.

The sea-urchins were dissected and the gametes (eggs and sperm) were obtained from a single female and male, respectively, in good conditions (fig. 6) by direct extraction with glass Pasteur pipettes. It was observed if the eggs had a round shape and the sperm good mobility, using an inverted microscope (Nikon Eclipse TS100).

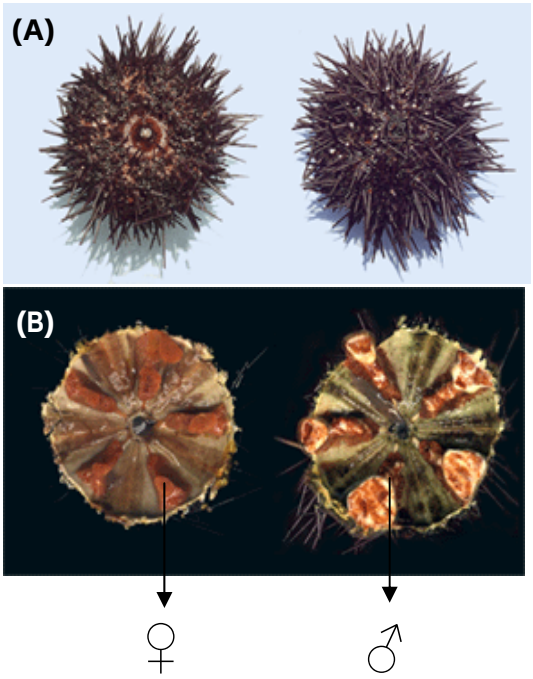

Figure 6. (A) Sea Urchin (*Paracentrotus lividus*); (B) Extraction of sea urchin gametes by dissecation of females and males.

The eggs were added to about 90-100 ml of artificial seawater (in a volumetric cylinder of 100 ml) until the suspension has been slightly orange. The cylinder was inverted to homogenize. Some µl of sperm directly pipetted (with a glass pipette) from the gonads were added to the egg suspension. Then, the suspension was stirred gently to allow the fertilisation. The fertilisation percentage was determined counting the total number of eggs and the number of fertilised eggs in aliquots of 10 µl. The fertilisation percentage was 100 %.

Preliminary tests (dilution factor of 5) were performed with sea urchin larvae in 24-well plates, being the volume of appropriate tests solutions placed in each well of 3 ml. The larvae were incubated in the test solution at an amount of 20 larvae/ml. On the other hand, the definitive tests (dilution factor of 1.5) were performed with polypropylene vials (fig. 7). In this case, the larvae were incubated in 12 ml of the test solution also at an amount of 20 larvae/ml. 15 larvae/well were measured in the preliminary tests and 30 larvae/vial in the definitive tests.
For each toxicant, 5 and 6 concentrations plus a control (artificial seawater) and a solvent control (pure DMSO or acetone in the sole case of hexane) were tested in the preliminary and definitive assays, respectively. Six replicates were prepared for each condition in the preliminary tests. However, 4 replicates for each concentration and 6 replicates for control and solvent control were done for each toxicant in the definitive tests (fig. 7).

Figure 7. One definitive test made with propylene vials. HNS tested was Trichloroethylene.

For *Paracentrotus lividus*, an incubation temperature of 20 ºC for 48 h is recommended (Fernández and Beiras, 2001). Hence, the samples were incubated at 20 ºC for 48 hours in the dark. After incubation, the samples were fixed with 2 or 8 drops of 37-40 % formaldehyde (with a Pasteur pipette), respectively, per well or vial.

The endpoint assessed for NOEC and LOEC determinations was the length of the longest arm of each larva at 48 h. Such endpoint was observed while the larvae were within the wells or vials, in solution, using an inverted microscope (Nikon Eclipse TS 100) with an image capture system associated (NIS Elements; version 4-13; Nikon Image Software) (fig. 8). Therefore, the length of the longest arm of each larva was measured as a sensitive and ecologically relevant endpoint to determine the chemical concentration effect.

Figure 8. (A) (B) Inverted microscope (Nikon Eclipse TS 100) with an image capture system associated (NIS Elements; version 4-13; Nikon Image Software); (C) Computer open in the programme of image analysis to observe one Pluteus larva (has four well developed arms) at 48 h.

2.3.6 Turbot (*Scophthalmus maximus*)

The turbot (*Scophthalmus maximus* (Linnaeus, 1758)) (fig. 9) is an important fishery and aquaculture species with high commercial value because of rapid growth and quality of flesh (Tong et al., 2013).

Figure 9. Turbot (*Scophthalmus maximus*).

This species is constantly at risk of being exposed to contaminants and is considered a good biological model for toxicological studies (Mhadhbi et al., 2010) due to diverse characteristics, namely their high growth rates, efficiency in adapting to diverse foods, great resistance to diseases, easy reproduction and finally, good tolerance to a wide range of environmental conditions (Mhadhbi et al., 2012b).

The early life stages (embryo, larval, and early juvenile stages) of fish are generally regarded as the most sensitive life-history stages to toxic agents (Mhadhbi et al., 2012b), being ideal for determining responses to environmental contaminants. 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 early development of turbot from fertilization to hatching was described by Tong et al. (2013). Mhadhbi et al. (2010) reported that, considering the short duration of the 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 turbot fish (ELS) is an excellent model for the study of ecotoxicity of contaminants in seawater.

One aliquot of fertilized turbot eggs was provided by a Spanish commercial hatchery (Insuiña S.L., Mougás, Galicia, Spain). The fertilized eggs of *Scophthalmus maximus* were thus obtained from spawns of broodstock mantained at Mougás under controlled temperature and photoperiod. The toxicity tests were performed with turbot (*Scophthalmus maximus*) eggs in 24-well plates. The volume (media) of working solutions placed in each well was 2 ml. The eggs were placed in petri dishes and about 15-20 eggs were pipetted in each well. The plates were transported back to the laboratory in styrofoam boxes at controlled temperature. Immediately after their arrival at the

laboratory (Porto), the unfertilised eggs were discarded and the density was adjusted to 10 eggs/well. The experimental conditions were performed in the dark at 14±0.5 °C and with a salinity of 30 ‰. The medium was changed every day.

For each toxicant, 5 concentrations plus one control (artificial seawater – no chemical added) and one solvent control (pure DMSO or acetone in the sole case of hexane) were tested, using six replicates for each condition.

Recorded endpoints included: embryo malformations – developmental delay, abnormal cellular masses, yolk sac alterations, oil globule fragmentation, oil globule position, no rupture of the egg membrane, pericardial edema, heart rate, eyes pigmentation and skeletal deformities (head, tail, vertebral, column) – and 75 % epiboly, mortality rate, hatching success, larvae length, mouth opening and jaw deformities. Hatching is defined as the rupture of the egg membrane and, partially as well as fully hatched larvae are counted as hatched (Mhadhbi et al., 2012a). The hatching happens when the larvae/embryo began to hatch with a closed gut and large yolk-sac, the frequency of heart beat gradually increases and there are clusters of pigment on the fin-fold posterior to the body of newly hatched larva and the larva remains motionless in 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 this work were: abnormal dorsal curvature of trunk, abnormal dorsal curvature of tail, malformation of the jaw and malformation of the head.

The effects of the different concentrations of the HNS selected on turbot embryos and larvae were observed throughout the 9 days exposure period, in four sampling points: 53 h post-fertilization (hpf) (day 2) – 75 % epiboly; 100 hpf (day 4) - Before hatching (108hpf) - after heart start beating (92hpf); 124hpf (day 5) – After hatching (108hpf); and 220hpf (day 9) – After mouth opening (204hpf). Some of the endpoints evaluated in the four sampling points are shown in Table 2.

Table 2. Endpoints assessed in the four sampling points.

	Time (hpf)			
	53	**100**	**124**	**220**
Endpoints	Mortality 75 % epiboly Abnormal cellular masses	Mortality Eclosion rate Heartbeat Embryo malformations – organogenesis	Mortality Hatching success Embryo malformations Yolk sac volume Oil globe volume Oil globe position	Mortality Yolk sac absorption Oil globe volume Oil globe position Mouth opened Embryo malformations (others + jaw) Larvae length

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

75 % epiboly happens when the outer enveloping layer (EVL) further flattens and differentiates into the periderm, protecting the embryonic body. The neural rod (NR) derived from ectoderm appears (fig. 10), the first somite appears in the middle of the embryonic body and the optic primordia (OP) is present.

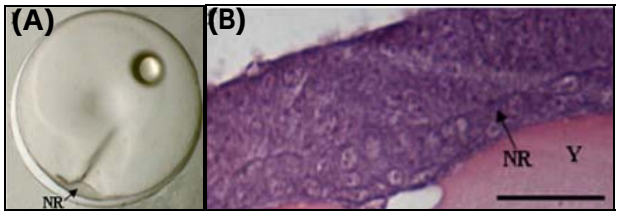

Figure 10. (A) The formation and (B) appearance of neural rod (NR) at 75 % of Epiboly (Y - yolk). (Tong et al., 2013).

The different endpoints selected were observed using a stereo-microscope (LEICA).

2.4 Statistical analysis made with bioassays results

Statistical analysis was conducted at CEFAS using CETIS software (v1.8.0.9 Tidepool Scientific Ltd) to calculate NOEC and LOEC values by either Fisher's Exact Test, Wilcoxon/Bonferroni Adj Test or Bonferroni Adj t Test according to the USEPA decision tree embedded within the program. EC values were calculated by Spearmann-Karber estimates (in preference) or by Linear Interpolation.

Statistical analysis was performed at CIIMAR with the computer program STATISTICA version 7.0 (StatSoft. Inc. Tulsa, OK, USA), in order to investigate the effects of the HNS selected in two marine species (*Paracentrotus lividus* and *Scophthalmus maximus*). Analysis of variance (one-way ANOVA) was used to analyze the data. Statistical significance was considered at $p<0.05$ for all analyses (Zar, 1999).

3. Results

3.1 Chemistry results

The results of the chemical analyses for preliminary trials are shown below in Table 3.

Table 3. Measured concentrations in preliminary trials for aniline, butyl acrylate, hexane and trichloroethylene at 0 and 48 hrs. Entries marked with – denote a result below the detection level for the method. Entries marked with * denote unidentified/non target peaks recorded during analysis.

		Measured concentration 0 hrs mg/l		Measured concentration 48 hrs mg/l	
Chemical	**Nominal Concentration (mg/l)**	**mg/l**	**% of nominal concentration**	**mg/l**	**% change from 0 hrs**
Aniline	0.03	-	-	-	-
	0.1	-	-	-	-
	0.3	-	-	-	-
	1	-	-	-	-
	3	-	-	-	-
	10	-	-	-	-
	30	-	-	-	-
Butyl acrylate	3	-	-	-	-
	6	*	-	-	-
	10	0.40	3.95	-	-
	30	3.18	10.59	-	-
	60	*	-	-	-
Hexane	1	-	-	-	-
	10	0.01	0.12	-	-
	30	0.01	0.03	-	-
	100	0.03	0.03	-	-
	300	-	-	-	-
Trichloroethylene	10	1.17	11.70	0.02	-98.63
	30	3.97	13.23	0.18	-95.59
	60	5.27	8.78	0.30	-94.35
	100	5.86	5.86	0.11	-98.09
	300	41.70	13.90	11.00	-73.62

It can be seen from Table 3 that aniline was below the detection level in preliminary trials at the beginning and end of the test. Only two samples analysed for butyl acrylate showed detectable concentrations at 0 hrs, these corresponded to approximately 4 and 11 % of the target concentration. No butyl acrylate was detected in the sample representing the highest experimental concentration, but other unidentified (non-target) peaks were detected, possibly these were breakdown products derived from butyl acrylate. Butyl acrylate concentration was below detection after 48 hours.

Samples from hexane test solutions also showed very low levels with no clear concentration series (*e.g.* hexane was below detection in the highest test concentration). Hexane was below detection in all samples at 48 hrs.

Measured concentrations of trichloroethylene showed a weak concentration series with concentrations measured in samples from 0 hrs approximately 10 % of the target concentration dropping to <1 – 3 % of nominal after 48 hours.

Following the results of the initial trials (Table 3), further studies focussed on aniline and butyl acrylate were conducted because these chemicals provided clear responses/effect in the first round of *Tisbe* mortality tests (see Section 3.2 below). For the second set of tests, aniline analysis was conducted by NLS. Such results are shown below in Table 4. As for the first trial, butyl acrylate was analysed by ESG, results shown in Table 5.

Table 4. Measured concentration of aniline at 0, 24 hrs and 48 hrs from two *Tisbe* and two *Pomatoceros* tests. 0 hrs values for 0.1, 30 and 300 mg/l n=3, all 48 hr values n=1. All other values n=2.

	Recorded concentration 0 hrs mg/l		Measured concentration 24 hrs mg/l		Measured concentration 48 hrs mg/l	
Nominal Aniline concentration mg/l	**Reported value**	**% of nominal concentration**	**Average (n=2)**	**% change from 0 hrs**	**Reported value (n=1)**	**% change from 0 hrs**
0.1	0.07	88.0	0.1	-29.0	0.04	-42.9
0.3	0.2	72.0	0.3	41.0	-	-
1	0.9	89.0	1.1	28.0	-	-
3	2.6	87.0	1.7	-35.0	-	-
10	9.8	98.0	7.8	-36.0	4.65	-52.5
30	29.5	113.0	25.6	-13.2	23.3	-21.0
300	343	114.0	-	-	450	31.2

The measured concentrations of aniline in the second trial are much closer to the nominal concentrations, the minimum average value was 72 % of the target. For the two highest concentrations, 10 and 30 mg/l, the observed values were actually higher on average than the target, the maximum being 123 % of the intended concentration. After 24 hrs, the top 3 concentrations reduced in value by 24 – 36 %. However the lowest 3 concentrations were still very close to nominal concentrations, showing an increase relative to values recorded at 0 hours.

Table 5. Measured concentrations after 0 and 24 hours for butyl acrylate from trial two.

	Measured concentration 0 hrs mg/l		Measured concentration 24 hrs mg/l	
Nominal Butyl acrylate concentration mg/l	**mg/l**	**% of nominal concentration**	**mg/l**	**% change from 0 hrs**
1	0.088	8.8	0.053	-40
3	0.337	11.2	0.183	-46
10	1.209	12.1	0.576	-52
30	0.050	0.2	1.887	+3674
60	0.050	0.1	3.226	+6352

Table 5 showing measured concentration for butyl acrylate in the second trial indicates that there was no clear initial concentration series at least for the two highest test concentrations for which very low initial concentrations were recorded. After 24 hours, the measured values show a

well-defined dilution series but at approximately 5 % of nominal values. Nevertheless, butyl acrylate concentration data appear unreliable particularly as analysis of an additional test concentration series showed all concentrations of butyl acrylate below the detection level.

Both sets of results show a difference in behaviour between the lower and higher concentration ranges tested over the 24 hour period. For aniline, the lower concentrations increase over time, while the higher concentrations decrease and the opposite is true for butyl acrylate.

The variable results of the chemical analysis highlight the difficulty of maintaining nominal concentrations for all of the selected test chemicals. Due to the unreliability of measured concentrations (possibly due to incomplete mixing and loss during storage and prior to analysis) the preliminary assessment of bioassays results uses only nominal test concentrations but the following discussion considers the measured concentration data.

3.2 Bioassays results

Toxicity tests results (NOEC, LOEC and E(L)C_{50} (95 % LCL - UCL)) performed with marine species (*Tisbe battagliai*, *Pomatoceros triqueter*, *Ceramium tenuicorne*, *Fucus vesiculosus*, *Paracentrotus lividus* and *Scophthalmus maximus*) from different phyla are presented in Table 6.

Table 6. Lethal and effect concentrations for 50 % of the test organisms together with 95 % confidence intervals for *Tisbe battagliai*, *Pomatoceros triqueter*, *Ceramium teunicorne*, *Fucus vesiculosus*, *Paracentrotus lividus* and *Scophthalmus maximus* following exposure to aniline, butyl acrylate, m-Cresol, cyclohexylbenzene, hexane and trichloroethylene. The *Tisbe* mortality bioassay was repeated 3 times for aniline and butyl acrylate. Two endpoints, growth and germination were investigated for *Fucus vesiculosus*.

Species (Endpoint)	Phylum	Chemical	NOEC mg/l	LOEC mg/l	E(L)C_{50} (95 % LCL - UCL) mg/l
Tisbe (mortality)	Arthropoda	Aniline	0.1	0.3	1.2 (0.77-1.88)
		Aniline	3	10	8.32 (4.1 - 16.82)
		Aniline	0.3	1	1.96 (1.01 - 3.77)
		Butyl acrylate	6	10	15.7 (12.3-20.1)
		Butyl acrylate	<3	3	2.53 (1.19 - 4.00)
		Butyl acrylate	3	10	4.2 (1.01 - 3.77)
		Hexane	10	30	>300mg/l
		Trichloroethylene	100	300	>300mg/l
Pomatoceros (embryo development)	Annelida	Aniline	<10	10	34.4 (25.23 - 42.03)
		Butyl acrylate	<10	10	10.6 (7.49 - 13.89)
		Hexane	<10	10	172 (109.1 - 311.6)
		Trichloroethylene	100	300	>300
Ceramium (growth)	Rhodophyta	Aniline	0.3	1	2.01 (0.37 - 5.37)
		Butyl acrylate	positive growth response		

		Hexane	positive growth response		
		Trichloroethylene	positive growth response		
Fucus (growth)	Heterokontophyta	Aniline	>30		
		Butyl acrylate	>60		
		Hexane	>300		
		Trichloroethylene	>300		
Fucus (germination)	Heterokontophyta	Aniline	>30		
		Butyl acrylate	>60		
		Hexane	>300		
		Trichloroethylene	>300		
Paracentrotus lividus (lenght of the longest arm) Preliminary test	Echinodermata	Aniline	0.040	0.2	
		Butyl acrylate	0.240	1.2	
		m-Cresol	0.032	0.160	
		Cyclohexylbenzene	1.0	5.0	
		Hexane	>7.0	>7.0	
		Trichloroethylene	1.8	9.0	
Paracentrotus lividus (lenght of the longest arm) Definitive test	Echinodermata	Aniline	0.026	0.039	
		Butyl acrylate	0.158	0.237	
		m-Cresol	0.021	0.032	
		Cyclohexylbenzene	0.658	0.988	
		Hexane	>70.0	>70.0	
		Trichloroethylene	1.2	1.8	
Scophthalmus maximus (eclosion rate at 124 hpf)	Chordata	Aniline	>0.320		
		Butyl acrylate	>1.2		
		m-Cresol	>0.160		
		Cyclohexylbenzene	>5.0		
		Hexane	2.8	7.0	
		Cyclohexylbenzene	>9.0		

It can be seen from the data presented in Table 6 that, at the concentrations tested, none of the chemicals affected the growth or the germination of *Fucus vesiculosus*. Therefore, the highest nominal concentrations tested are the effective NOECs for each test substance and the LOECs are greater than these values.

Butyl acrylate, hexane and trichloroethylene did not reduce the growth of *Ceramium tenuicorne*, in fact there is evidence of a positive effect on growth of *Ceramium* for these chemicals (see fig. 11). *Ceramium* also showed a positive growth response at low concentrations of aniline, but at increasing concentrations a significant growth reduction relative to the control ($p<0.001$) was observed. The 7 day EC_{50} for the growth of *Ceramium* exposed to aniline was 2.01 mg/l and the NOEC and LOEC were 0.3 and 1 mg/l, respectively (Table 6).

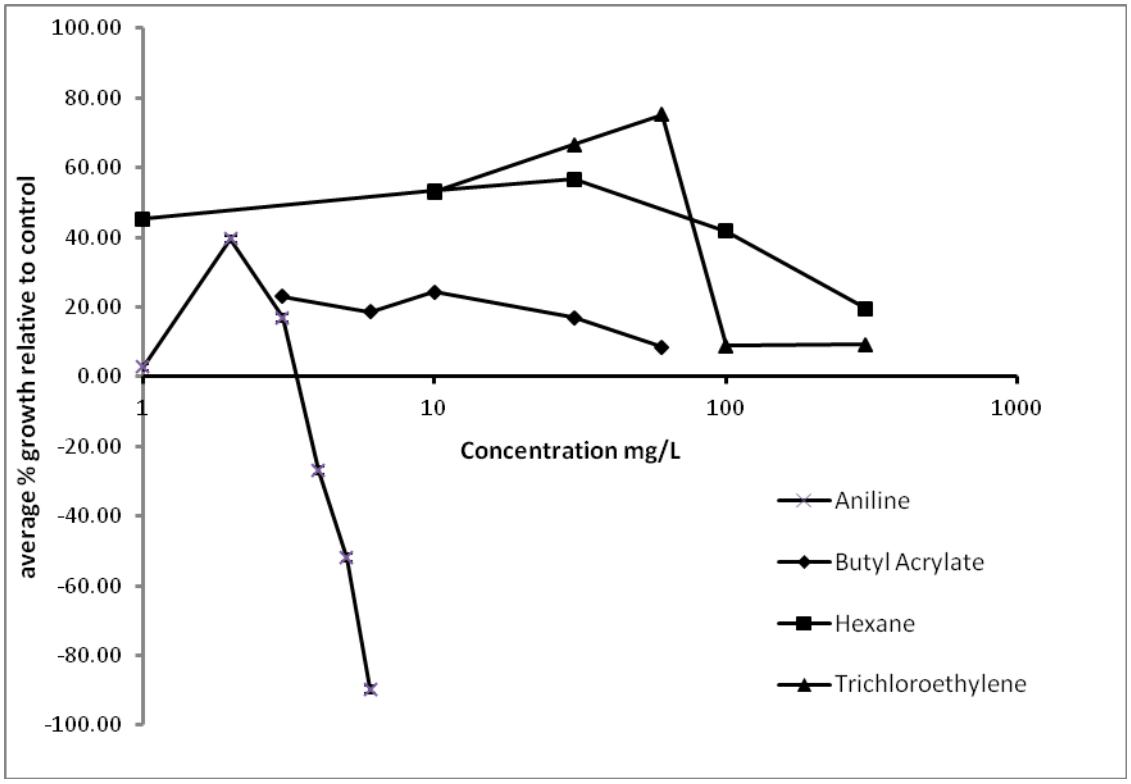

Figure 11. Average percent growth (or growth inhibition), relative to average control growth for *Ceramium* tips exposed to aniline, butyl acrylate, hexane and trichloroethylene for a 7 day exposure period.

Aniline also showed the greatest effect of the 4 chemicals tested using the *Tisbe* mortality bioassays, this test was run 3 times. Across the three *Tisbe* mortality tests conducted with aniline, the NOEC and LOEC ranged from 0.1 to 3 mg/l and 0.3 to 10 mg/l, respectively and the three 48 hour EC_{50} values obtained in repeat tests were 1.20, 8.32, and 1.96 mg/l. The second test in the series had quite a low toxicity compared to the first and third tests (Table 6). Three mortality bioassays were also conducted on *Tisbe* with butyl acrylate. The NOEC and LOEC ranged from < 3 to 6 mg/l and 3 to 10 mg/l, respectively and the 3 EC_{50} values were 15.7, 2.53 and 4.20 mg/l, as observed with the aniline tests one of the three assays showed lower toxicity (Table 6).

There were also measurable effects on *Pomatoceros* embryo development shown by all test chemicals. *Pomatoceros* embryo development showed the greatest response to butyl acrylate followed by aniline, hexane and trichloroethylene, respectively. The EC_{50} for butyl acrylate was 10.6 mg/l and the EC_{50} for aniline was 34.4 mg/l. An EC_{50} of 172 mg/l was calculated for hexane but this had associated large 95 % confidence limits (109.1 - 311.6) and the NOEC and LOEC were <10mg/l and 10 mg/l, respectively. Trichloroethylene had the least effect on *Pomatoceros* development, no EC_{50} could be calculated but a NOEC and LOEC of 100 and 300mg/l were observed.

According to the Table 6, m-Cresol presented the lowest NOEC and LOEC - 0.032 and 0.160 mg/l, respectively (preliminary test); 0.021 and 0.032 mg/l, respectively (definitive test) - while hexane was the least toxic compound in both preliminary and definitive tests performed with *Paracentrotus lividus*. Therefore, at the concentrations tested, hexane did not affect the length of the longest arm of *Paracentrotus lividus* larvae and m-Cresol showed the greatest effect of the 6 chemicals tested using the same endpoint, followed by aniline, butyl acrylate, cyclohexylbenzene and finally, trichloroethylene.

The only endpoint affected by the highest hexane concentration selected for turbot (*Scophthalmus maximus*) was the larvae eclosion rate at 124 hpf. At this time, the eclosion rate of turbot larvae was significantly lower for the highest hexane concentration tested than that observed for the solvent control ($p = 0.0018$) (fig. 12). However, the chemicals concentration selected for turbot (*Scophthalmus maximus*) did not affect the other endpoints analysed in this species.

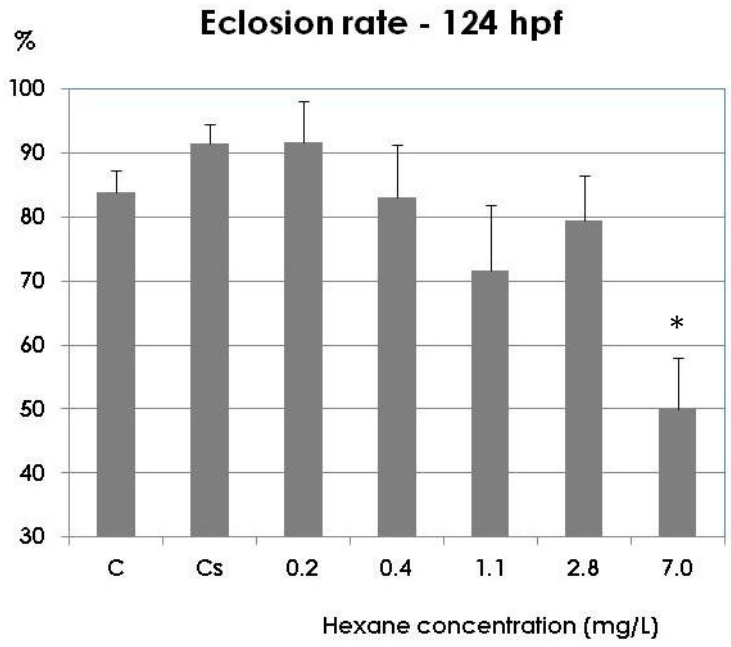

Figure 12. Eclosion rate (%) of turbot larvae at 124 hpf for 5 hexane concentrations (0.2, 0.4, 1.1, 2.8 and 7.0 mg/L) and two controls without hexane added (C and Cs). Results represent average values ± standard error. Asterisk (*) indicates significant differences ($p<0.05$) between concentrations and solvent control (Cs).

3.3 Water quality results during tests

As described in section 2.3, water qualities (temperature, pH, salinity and dissolved oxygen) were measured for all freshly prepared solutions. These parameters were measured again when solutions were changed or at the end of a test (Tables 7, 8, 9 and 10).

The water qualities recorded remained largely within the desired range with a slight deviation relative to standard test methods of pH in the *Tisbe* studies (Table 7) and of temperature during

the studies with *Ceramium* and *Fucus* (Tables 9 and 10). In neither case are these deviations considered to have a significant influence upon the test results.

3.3.1 *Tisbe*

Table 7. Observed water qualities for tests with *Tisbe battagliai*. The ranges described above apply to parameters from the 3 repeats of the *Tisbe* mortality bioassays.

Physicochemical parameter	Temperature (°C)	pH	Salinity (ppt)	Dissolved Oxygen (as percent of the ASV)
Threshold criteria	18 – 24	7.7 – 8.3	20 - 36	≥50
Observed minimum and maximum	19.5 – 22.5	7.71 – 8.45	29.3 - 32.8	97.7 – 118.1

3.3.2 *Pomatoceros*

Table 8. Observed water quality ranges for tests with *Pomatoceros triqueter*.

Physicochemical parameter	Temperature (°C)	pH	Salinity (ppt)	Dissolved Oxygen (as percent of the ASV)
Observed minimum and maximum	15 – 15.6	7.3 – 8	31.1 – 32.6	95.1 – 110.8

3.3.3 *Ceramium* and *Fucus*

Table 9. Observed water quality ranges for tests with *Ceramium tenuicorne*.

Physicochemical parameter	Temperature (°C)	pH	Salinity (ppt)	Dissolved Oxygen (as percent of the ASV)
Threshold criteria	13 - 17	6.5 – 9.5	25 – 35	None specified
Observed minimum and maximum	15.9 – 19.8	7.79 – 8.23	24.0 – 29.9	76.7 – 103.1

Table 10. Observed water quality ranges for tests with *Fucus vesiculosus*.

Physicochemical parameter	Temperature (°C)	pH	Salinity (ppt)	Dissolved Oxygen (as percent of the ASV)
Threshold criteria	13 - 17	6.0 – 8.5	22 – 36	≥60
Observed minimum and maximum	16.1 – 19.8	7.79 – 8.23	24.0 – 29.9	76.7 – 103.1

4. Discussion

This study provides results for aniline toxicity to a variety of marine taxa. Data from the literature has described the toxic effects of aniline on marine and freshwater species and these data are represented in Table 11. It can be seen that the mortality results reported here for *Tisbe batagliai* and the development of *Pomatoceros* embryos are in a comparable range (1 $x10^{-1}$ to $1x10^{1}$) to values reported in the literature for freshwater fauna. In addition, we include data on two taxa of algae previously untested (to the authors knowledge) with aniline, for the red macroalgae *Ceramium tenuicorne* and the brown macroalgae *Fucus vesiculosus.* Growth of *Ceramium tenuicorne* is more sensitive to aniline (EC_{50} 2.01 mg/l) than *Fucus vesiculosus* (NOEC >30 mg/l). These results provide preliminary information that is of value when assessing the risk of aniline spills to inter-tidal habitats with different macro-algal assemblages.
Furthermore, the NOEC results obtained in this study with the length of the longest arm of sea urchin (*Paracentrotus lividus*) larvae (Table 6) are within the NOEC values range found in the literature (0.004-0.422 mg/l) for freshwater species, as shown in Table 11. The literature also show a LOEC value of 0.735 ml/l (32 days) for aniline in *Pimephales promelas* (a fish species) but the results showed here reveal lower LOEC values for sea urchin (0.2 and 0.039 mg/l, for preliminary and definitive tests, respectively). These results indicate that sea urchin is more sensitive to aniline than *Pimephales promelas*.

Only data reporting acute toxicity of butyl acrylate, m-Cresol and Cyclohexylbenzene to freshwater fauna are available from the literature (Table 11), making the results reported here valuable in filling gaps in our knowledge of the effects of such chemicals in the marine environment. Results reported here for butyl acrylate provide a range of LC_{50}'s from 2.53 to 15.70 for *Tisbe battagliai* over 48 hours showing that this species is a lot more sensitive to butyl acrylate than *Daphnia magna* (a freshwater planktonic crustacean). *Pomatoceros* embryo development showed a similar level of sensitivity with an EC_{50} of 10.6 mg/l for exposure to butyl acrylate. As with aniline and the other chemicals tested here no result was found in the literature for any macro-algal species. The results reported here show that butyl acrylate had no effect on the growth or growth and germination of *Ceramium tenuicorne* and *Fucus vesiculosus* respectively, at the highest concentrations tested (60 mg/l). In fact, *Ceramium tenuicorne* showed an increased growth, which could be interpreted as a hormetic response as this positive growth effect was reduced as concentration increased (*e.g.* fig. 11). This apparent lack of effect for *Fucus* and positive/homeostatic response for *Ceramium* was also apparent for hexane and trichloroethylene which were both tested to a maximum concentration of 300 mg/l.

Table 11. Summary of toxicity data available for target HNS from the literature adapted from Neuparth et al. (2011).

HNS	Test Species	Test medium	mg/l (effect)	References
Aniline	Crustacea *Crangon septemspinosa*	Seawater	29.4 (96h LC50)	McLeese and Zitko (1979)
	Crustacea *Daphnia magna*	Freshwater	0.004 (21 days NOEC)	Kühn et al. (1989a,b)
	Fish *Pimephales promelas*	Freshwater	0.735 (32 days LOEC), 0.422 (32 days NOEC)	Russom (1993)
Butyl acrylate	Crustacea *Daphnia magna*	Freshwater	230 (24h LC50)	Bringmann and Kühn (1977)
	Fish *Osteichthyes* sp.	Freshwater	5 (72h LC50)	Paulet and Vidal (1975)
m-Cresol	Crustacea *Daphnia magna*	Freshwater	18.8 (96h LC50)	Parkhurst et al. (1979)
	Fish *Danio rerio*	Freshwater	15.9 (96h LC50)	Wellens (1982)
	Fish *Oncorhynchus mykiss*	Freshwater	3.88 (96h LC50)	Saglam and Ural (2005)
Cyclohexylbenzene	Crustacea *Daphnia pulex*	Freshwater	0.55 (48h LC50)	Passino-Reader et al. (1997)
Hexane	Crustacea *Artemia salina*	Seawater	1.51 (24h EC50)	Foster and Tullis (1985)
Trichloroethylene	Crustacea *Mysidopsis bahia*	Seawater	14 (96h LC50)	Ward et al. (1986)
	Crustacea *Palaemonetes pugio*	Seawater	2 (96h LC50)	Borthwick (1977)
	Mollusc *Eliminius modestus*	Seawater	20 (48h LC50)	Pearson and McConnell (1975)
	Fish *Cyprinodon variegates*	Seawater	52 (96h LC50)	Ward et al. (1986)
	Fish *Limanda limanda*	Seawater	16 (96h LC50)	Pearson and McConnell (1975)

Additionally, only data reporting acute toxicity of hexane and trichloroethylene to seawater fauna are available from the literature (Table 11), making also the results shown here important to fill gaps in our knowledge of the effects of such chemicals in the marine environment. Hexane and trichloroethylene showed some effect in the *Tisbe* 48 hour mortality assay and *Pomatoceros* 48 hour development assay. Trichloroethylene had a NOEC and LOEC of 100 and 300 mg/l, respectively in both the *Tisbe* and *Pomatoceros* bioassays. These results indicate much lower sensitivity for these species than for other species including crustacea, reported in the literature (see Table 11). The low toxicity for these test substances reported here may be a result of different methodology. In this study, bioassays were carried out in well plates with loose fitting lids whereas in the work reported by Pearson and McConnell (1975), for example, tests were carried out in closed vessels. While tests carried out in (semi) open vessels may result in chemical concentrations that are lower than the nominal target tests carried out in closed vessels, they do reflect the more dynamic exposure conditions in a marine spill. *Pomatoceros* development was affected by hexane since a LOEC of 10 mg/l and a EC_{50} of 172 mg/l were derived (Table 6). Hexane also showed an effect in the *Tisbe* bioassay, a NOEC and LOEC of 10 and 30 mg/l, respectively. Foster and Tullis (1985) reported a 24 h EC_{50} of 1.51 mg/l for the intoxication of *Artemia* nauplii exposed to hexane in an acetone solution (*i.e.* not produced as a WAF as in the work described here). The differences in effect concentrations mentioned by Foster and Tullis (1985) and those reported here for *Tisbe* are presumably due to differences in methodology.

The results reported here show that the hexane was the only one of the 6 chemicals analysed that had no effect on length of the longest arm of sea urchin larvae at tested concentrations and the only one that affected the eclosion rate of turbot larvae at 124 hpf, at the highest concentration tested. These results are probably due to the physicochemical properties of hexane as well as its behaviour in the water (evaporator) (Table 12). For the other endpoints analysed in the turbot ELS, the different chemicals selected had no effect in the range of concentrations tested (Table 6). The chemicals concentrations tested with turbot were in the same order of magnitude of those tested in the definitive tests using sea urchin. Therefore, turbot is less sensitive to the chemicals selected (except hexane) than sea urchin and consequently, is less suitable for determining responses to environmental HNS contaminants at low concentrations. These results provide also preliminary information that is of value when assessing the risk of the HNS spills to marine habitats.

The current study gathered information on the effects of priority HNS on an ecosystem scale since it presents data from different phyla. Thus, studies with organisms of different phyla are important because they allow a better framing of the risks of contamination by HNS since there is a paucity of knowledge on HNS effects on marine biota and the relatively few data available on the HNS ecotoxicology are mostly from assays performed with freshwater organisms.

The test solution analysis results largely reflect the physicochemical properties of the 6 chemicals tested. The European Behaviour classification system categorises the behaviour of chemicals based on combinations of physical and chemical properties (Bonn Agreement, 1994). Aniline is described as a Floater/Dissolver, butyl acrylate as a Floater/Evaporator/Dissolver, m-Cresol as a Sinker/Dissolver, cyclohexylbenzene as a Floater, hexane as an Evaporator and trichloroethylene as a Sinker/Dissolver.

Table 12. Description of the solubility, partition coefficient (log Kow), vapour pressure and GESAMP behaviour categories (GESAMP, 2002) (E: evaporator; F: Floater; SD: sinker/dissolver; FD: floater/dissolver; FE: floater/evaporator; FED: floater/evaporator/dissolver) of aniline, butyl acrylate, m-cresol, cyclohexylbenzene, hexane and trichloroethylene.

Chemical	Solubility (g/L) corrected for salinity (31.5ppt)	Log Kow	Vapour Pressure (Pascal)	Description of GESAMP behaviour categories
Aniline	25.200	0.9	40	FD
Butyl acrylate	0.980	2.4	727	FED
m-Cresol		2.0	14.7	SD
Cyclohexylbenzene	Insoluble in water	4.8	6.1	F
Hexane	0.007 (insoluble)	3.9	20398	E
Trichloroethylene	0.896	2.6	8600	SD

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 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 lowest potential solubility in seawater as calculated for the salinity under-which these bioassays were conducted. Although all six test chemicals have a maximum solubility (under the conditions described) well within the test range, the high vapour pressure of hexane and trichloroethylene led to reduced exposure concentrations and hence lower toxicity (except in the case of eclosion rate of turbot larvae at 124 hpf). Generally, aniline was the most toxic of the chemicals tested and this is comparable to data from the literature. However, for sea urchin, m-Cresol was the most toxic compound followed closely by aniline (Table 6) probably due to the physicochemical properties like high solubility of m-cresol and aniline as well as the behaviour of these compounds in the water (aniline is a floater/dissolver (FD) and m-cresol a sinker dissolver (SD), as referred above) (Table 12). Additional species toxicity data for aniline, butyl acrylate, m-cresol and hexane obtained here will be of value in developing post spill risk assessments for these chemicals. Therefore, the data produced here will be essential to better anticipate the impact of HNS in marine species, and therefore improve preparedness and the response at operational level.

5. References

Bellas J., Beiras, R., Mariño, J.C., Fernández, N., 2005. Toxicity of organic compounds to marine invertebrates' embryos and larvae: a comparison between sea urchin embryogenesis bioassay and alternative test species. Ecotoxicology 14, 337–353.

Boglione, C., Gagliardi, G., Scardi, M., Cataudella, S., 2001. Skeletal descriptors and quality assessment in larvae and post-larvae of wild-caught and hatchery-reared gilthead sea bream (*Sparus aurata* L. 1758). Aquaculture 192, 1–22.

Boney, A.D., Corner, E.D.S., 1962. On the effects of some carcinogenic hydrocarbons on the growth of sporelings of marine red algae. Journal of the Marine Biological Association of the UK 42, 579–585.

Bonn Agreement, 1994. European classification system, "Bonn Agreement: Counter-Pollution Manual", Bonn Agreement, London, chap. 25.

Borthwick, P.W., 1977. Summarized Results of Toxicity Tests with Sheepshead Minnows (*Cyprinodon varlesatus*) and Grass Shrimp (*Palaemonetes pugio*). US Environmental Protection Agency, Environmental Research Laboratory, Gulf Breeze, Florida.

Boudouresque, C.F., Verlaque, M., 2007. Ecology of *Paracentrotus lividus*. In: Lawrence, J.M. (Ed.), Edible sea urchins: biology and ecology, 2nd ed.: Dev Aquac Fish Sci, 37, pp. 243–285.

Braithwaite, R.A., Fletcher, T.R.L., 2005. The toxicity of Irgarol 1051 and Sea-Nine 211 to the non-target macroalga *Fucus serratus* Linnaeus, with the aid of an image capture and analysis system. Journal of Experimental Marine Biology and Ecology 322, 111–121.

Bringmann, G., Kühn, R., 1977. Results of the damaging effect of water pollutants on *Daphnia magna*. Zeitschrift fur Wasser – und Abwasserforschung 10, 161–166.

Brooks, S.J., Bolam T., Tolhurst, L., Bassett, J., Roche, J.L., Waldock, M., Barry, J., Thomas, K.V., 2008. Dissolved organic carbon reduces the toxicity of copper to germlings of the macroalgae, *Fucus vesiculosus*. Ecotoxicology and Environmental Safety 70, 88-98.

Bruno, E., Eklund, B., 2003. Two new growth inhibition tests with the filamentous algae *Ceramium strictum* and *C. tenuicorne* (Rhodophyta). Environmental Pollution 125, 287–293.

CEFAS, 2009. UK Risk Assessment for Hazardous and Noxious Substances – RP 593.

Dixon, D.R., Wilson, J.T., Pascoe, P.L., Parry, P.L. 1999. Anaphase aberrations in the embryos of marine tubeworm *Pomatoceros lamarckii* (Polychaeta: Serpulidae): a new in vivo test assay for detecting aneugens and clastogens in the marine environment. Mutagenesis 14 (4), 375-383.

Eklund, B., 2005. Development of a growth inhibition test with the marine and brackish water red alga *Ceramium tenuicorne*. Marine Pollution Bulletin 50, 921–930.

Fernandez, C., Boudouresque, C-F., 2000. Nutrition of the sea urchin *Paracentrotus lividus* (Echinodermata: Echinoidea) fed different artificial food. Marine Ecology Progress series 204, 131-141.

Fernández, N., Beiras, R., 2001. Combined toxicity of dissolved mercury with copper, lead and cadmium on embryogenesis and early larval growth of the *Paracentrotus lividus* sea-urchin. Ecotoxicology 10, 263–271.

Fletcher, R.L., 1989. A bioassay technique using the marine fouling green alga *Enteromorpha*. International Biodetermination 25, 407–422.

Foekema, E.M., Deerenberg, C.M., Murk, A.J., 2008. Prolonged ELS test with the marine flatfish sole (*Solea solea*) shows delayed toxic effects of previous exposure to PCB 126. Aquatic Toxicology 90, 197–203.

Foster, G.D., Tullis, R.E., 1985. Quantitative structure toxicity relationships with osmotically stresses *Artemia salina* nauplii. Environmental Pollution 38, 273–281.

GESAMP, 2002. The Revised GESAMP Hazard Evaluation Procedure for Chemical Substances Carried by Ships. GESAMP Reports and Studies No. 64, Publication Number 463/02, p. 137.

Hart, R.C., 1990. Copepod post-embryonic durations: pattern, conformity, and predictability. The realities of isochronal and equiproportional development, and trends in the copepodid-naupliar duration ratio. Hydrobiologia 206, 175-206.

IMO – International Maritime Organisation, 2000. Protocol on Preparedness, Response and Co-operation to Pollution Incidents by Hazardous and Noxious Substances (OPRC-HNS Protocol).

Kühn, R., Pattard, M., Pernak, K.D., Winter, A., 1989a. Results of the harmful effects of selected water pollutants (anilines, phenols, aliphatic compounds) to *Daphnia magna*. Water Research 23 (4), 495-499.

Kühn, R., Pattard, M., Pernak, K.D., Winter, A., 1989b. Results of the harmful effects of water pollutants to *Daphnia magna* in the 21 day reproduction test. Water Research 23, 501–510.

Mamaca, E., Bechmann, R.K., Torgrimsen, S., Endre Aas, E., Bjørnstad, A., Baussant, T., Le Floch, S., 2005. The neutral red lysosomal retention assay and Comet assay on haemolymph cells from mussels (*Mytilus edulis*) and fish (*Symphodus melops*) exposed to styrene. Aquatic Toxicology 75, 191–201.

McLeese, D.W., Zitko, V., 1979. Structure–lethality relationships for phenols, anilines and other aromatic compounds in shrimp and clams. Chemosphere 8, 53–57.

Mhadhbi, L., Boumaiza, M., Beiras, R., 2010. A standard ecotoxicological bioassay using early life stages of the marine fish *Psetta maxima*. Aquatic Living Resources 23, 209–216

Mhadhbi, L., Fumega, J., Boumaiza, M., Beiras, R., 2012a. Acute toxicity of polybrominated diphenyl ethers (PBDEs) for turbot (*Psetta maxima*) early life stages (ELS). Environmental Science and Pollution Research 19, 708–717.

Mhadhbi, L., Toumi, H., Boumaiza, M., Aloui, N., 2012b. Toxicity of three selected pesticides (Alachlor, Atrazine and Diuron) to the marine fish (turbot *Psetta maxima*). African Journal of Biotechnology 11, 11321-11328.

Nacci, D.E., Jackim, E., Walsh, R., 1986. Comparative evaluation of three marine toxicity tests: Sea urchin embryo growth test, sea urchin sperm cell toxicity test and microtox. Environmental Toxicology and Chemistry 5, 521-529.

Neuparth, T., Moreira, S., Santos, M.M., Reis-Henriques, M.A., 2011. Hazardous and Noxious Substances (HNS) in the marine environment: prioritizing HNS that pose major risk in a European context. Marine Pollution Bulletin 62, 21–28.

Parkhurst, B.R., Bradshaw, A.S., Forte, J.L., Wright, G.P., 1979. An evaluation of the acute toxicity to aquatic biota of a coal conversion effluent and its major components. Bulletin of Environmental Contamination and Toxicology 23, 349–356.

Passino-Reader, D.R., Hickey, J.P., Ogilvie, L.M., 1997. Toxicity to *Daphnia pulex* and QSAR predictions for polycyclic hydrocarbons representative for great lakes contaminants. Bulletin Environmental Contamination and Toxicology 59, 834–840.

Paulet, G., Vidal, M., 1975. Toxicity of some acrylic and methacrylic esters of acrylamide and polyacrylamides. Archives des Maladies Professionnelles de Medecine du Travail et de Securite Sociale 36, 58–60.

Pearson, C.R., McConnell, G., 1975. Chlorinated C1 and C2 hydrocarbons in the marine environment. Proceedings of the Royal Society of London Series B 189, 305–332.

Pihl, L., Isaksson, I., Wennhage, H., Moksnes, P.-O., 1995. Recent increase of filamentous algae in shallow Swedish bays: effects on the community structure of epibenthic fauna and fish. Netherlands Journal of Aquatic Ecology 29, 249–358.

Purnell, K., 2009. Are HNS Spills more dangerous than oil spills? Conference Proceedings, Interspill, Marseille, 12 - 14 May 2009.

Russom, C.L., 1993. Acute and Early Life Stage Toxicity Data. June 21 Memo from US EPA, Duluth, MN to R. Spehar US EPA, Duluth, MN, p. 41.

Saco-Álvarez, L., Durán, I., Lorenzo, J.I., Beiras, R., 2010. Methodological basis for the optimization of a marine sea-urchin embryo test (SET) for the ecological assessment of coastal water quality. Ecotoxicology and Environmental Safety 73, 491-499.

Saglam, N., Ural, M.S., 2005. Acute toxicity of m-cresol on the fry rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792). Fresenius Environment bulletin 14, 536–538.

Salamanca, M.J., Fernández, N., Cesar, A., Antón, R., Lopez, P., Delvalls, Á., 2009. Improved sea-urchin embryo bioassay for in situ evaluation of dredged material. Ecotoxicology 18, 1051–1057.

Scanlan, C. M., Wilkinson, M., 1987. The Use of Seaweeds in Bioeide Toxicity Testing. Part 1. The Sensitivity of Different Stages in the Life-history of *Fucus*, and of Other Algae, to Certain Biocides. Marine Environmental Research 21, 11-29.

Thompson, R.S., Burrows, E.M. 1984. The toxicity of copper, zinc, and mercury to the brown macroalga *Laminaria saccharina*. In: Persoone, G., Jaspers, E., Claus C. (Eds), Ecotoxicological Testing for the Marine Environment vol. 2, State Univ. Ghent and Inst. Mar. Scient. Res., Bredene, Belgium, pp. 259–268.

Tong, X.H., Xu, S.H., Liu, Q.H., Li, J., Xiao, Z.Z., Ma, D.Y., 2013. Stages of embryonic development and changes in enzyme activities in embryogenesis of turbot (*Scophthalmus maximus* L.). Aquaculture International 21, 129–142.

Ward, G.S., Tolmsoff, A.J., Petrocelli, S.R., 1986. Acute toxicity of trichloroethylene to saltwater organisms. Bulletin of Environmental Contamination and Toxicology 37, 830–836.

Wellens, H., 1982. Comparison of the sensitivity of *Brachydanio rerio* and *Leuciscus idus* by testing the fish toxicity of chemicals and wastewaters. Journal for Water and Waste Water Research 51, 49–52.

Zar, J.H., 1999. Biostatistical Analysis, fourth edition. New Jersey: Prentice Hall. 663 p.