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Simulation of a Hazardous and Noxious Substances (HNS) spill in the marine environment: Lethal and sublethal effects of acrylonitrile to the European seabass

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HIGHLIGHTS

- Additional marine toxicological data is needed for most priority HNS.
- Important toxic effects were observed in seabass exposed to acrylonitrile.
- Fish were able to manage the acrylonitrile toxicity by increasing the activity of CAT and SOD.
- After 7 d of a recovery period, almost all acrylonitrile toxic effects returned to control levels.
- The results obtained are potentially useful for acrylonitrile spills preparedness.

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ABSTRACT

Despite the extensive maritime transportation of Hazardous and Noxious Substances (HNS), there is a current lack of knowledge on the effects posed by HNS spills on the marine biota. Among the HNS identified as priority, acrylonitrile was selected to conduct ecotoxicological assays. We assessed the acute and subletal effects of acrylonitrile in seabass, followed by a recovery phase to simulate the conditions of a spill incident. The work aimed at testing a broad range of biological responses induced by acrylonitrile. Sublethal exposure to the highest two doses increased the fish mortality rate (8.3% and 25% mortality in 0.75 and 2 mg L^{-1} acrylonitrile concentrations), whereas no mortality were observed in control and 0.15 mg L^{-1} treatments. Additionally, important alterations at sub-individual level were observed. Acrylonitrile significantly induced the activities of Catalase– CAT and Glutathione S-Transferase – GST; and the levels of DNA damage were significantly increased. Conversely, Superoxide Dismutase– SOD – activity was found to be significantly inhibited and no effects were found on Lipid Peroxidation– LPO and ethoxyresorufin O-deethylase – EROD – activity. Following a 7 d recovery period, the levels of CAT, GST and EROD fell to levels at or below those in the control. In the 2 mg L^{-1} group, SOD remained at the levels found during exposure phase. This study has gathered essential information on the acute and subletal toxicity of acrylonitrile to seabass. It also demonstrated that 7 d recovery allowed a return of most endpoints to background levels. These data will be useful to assist relevant bodies in preparedness and response to HNS spills.

1. Introduction

The maritime transport of chemicals substances has increased in the last decades due to the continuous development of the chemical industry and the need to transport high volume of products from the industries to the customers (HASREP, 2005). This high chemical traffic has raised concern regarding the ecological risks of hazardous material spills. The volume of chemicals, or Hazardous and Noxious Substances (HNS), transported by sea is still significantly lower than the seaborne oil trade; and consequently chemical spills occur at a much lower frequency than oil spills (ITOPF, 2010). Nevertheless, while the environmental impacts of oil spills can be identified from the response experiences and the lessons learnt from past major oil spills, the potential ecological hazards and risks posed by HNS spills are much less recognized and understood (Kirby and Law, 2010; Neuparth et al., 2011, 2012). The information available on HNS spills illustrates the poor documentation or mistreatment of many HNS incidents (IMO, 2010). The wide variety of chemicals transported, their varying physical and chemical properties, the wider range of behaviours in the environment (i.e. gas, dissolves, evaporates,

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floats, sinks) and toxicities to marine organisms mean that response to HNS spills is not as straightforward as for oil (ITOPF, 2010). Attempts to better understand the risk of HNS spills in a meaningful way is not a simple issue, considering the lack of reliable information available. In comparison with oil spills, there is a lower investment in research and development dealing with the environmental impact of HNS pollution (IMO, 2010).

In the context of the ARCOPOL project, Neuparth et al. (2011) developed a weight-of-evidence approach aimed at prioritizing HNS that pose major environmental risks to European waters. The approach took into consideration the occurrence probability of HNS spills in European Atlantic waters, HNS physico-chemical properties and their toxicities to marine organisms. Furthermore, the work included a collection of marine toxicological data available for the 23 HNS identified as priority. Neuparth et al. (2011) concluded that marine chronic toxicity data was lacking for most of the priority HNS and in some cases, only fresh water acute toxicity data was available supporting the need to conduct research to collect additional toxicological data.

Among the 23 HNS identified by Neuparth et al. (2011) as priority, acrylonitrile was selected in this study based on the indication of being highly transported in European waters and its involvement in previous accidental spills. Several acrylonitrile spills have been reported worldwide e.g. the Anna Broere which sank in the Netherlands in 1988, released 200 tones of acrylonitrile, and the Alessandro Primo which carried 550 tones of acrylonitrile sank in the Adriatic Sea near the coast of Italy (Mamaca et al., 2009). More recently, in 2010, an unknown amount of acrylonitrile was released into the Mississippi River. Acrylonitrile, a chemical used extensively in the production of plastics, resins, synthetic fibres and rubbers (IARC, 1999; EU RAR, 2004), has obtained worldwide attention in recent years based on its moderate toxicity to aquatic organisms, evidence of carcinogenicity in rats and a potential human carcinogen (Tong, 1999; NTP, 2001; Johanssen and Levinskas, 2002; EU RAR, 2004). In fact, acrylonitrile is recognized as a priority pollutant in United States, Canada, Netherlands, Germany and China, (Keith and Telliard, 1979; Tong, 1999). Furthermore, this HNS ranks in the position 25 in the traffic ranking of the 100 most transported HNS in Atlantic European waters (HASREP, 2005); and few chronic toxicity data for marine organisms is available. To the best of our knowledge, only two chronic acrylonitrile studies with the fish *Oryzias latipes* and the alga *Skeletonema costatum* are available (Hawkins et al., 1991; AN group 1997 (b), cited in EU RAR (2004)).

In this study, a marine fish (the European seabass – *Dicentrarchus labrax*) was experimentally exposed to acrylonitrile followed by a recovery observation period to simulate the conditions of a spill incident. The European seabass was selected as test organism due to its ecological and economic relevance. *D. labrax* is a key species in European estuaries and coastal areas having an important function in pelagic food webs and therefore it is a vulnerable species to anthropogenic contamination (Almeida et al., 2012). Here, we aimed at testing a broad range of biological responses induced by acrylonitrile during exposure and recovery phase that will provide important information about the general health status of the European seabass. The work included a battery of endpoints indicative of lethal effects (survival-determination of LC_{50}), and sublethal parameters: DNA damage (comet assay as a measure of the general DNA integrity) oxidative stress (Catalase – CAT, Glutathione S-Transferase – GST, Superoxide Dismutase – SOD – activities, and Lipid Peroxidation – LPO – levels to evaluate the antioxidant defenses) and metabolic responses (hepatic ethoxyresorufin-O-deethylase – EROD – activity and GST activity involved in the biotransformation of xenobiotics). The data generated from this study will be useful to gather information on the ecological hazards and consequences of acrylonitrile spills in marine environment and to assess the detoxification capability of the European seabass after an acrylonitrile spill.

2. Material and methods

2.1. Fish maintenance

Acute and sublethal toxicity assays were carried out with juvenile seabass (*D. labrax*), obtained from the fish farm "Maresa" in Huelva, Spain, all from the same cohort (30–34 g weight). Three hundred animals were acclimated to controlled laboratory conditions for 5 weeks, in 1000 L tanks with clean, filtered and aerated seawater (33–35‰). During this period, fish were kept at 18 ± 1 °C, photoperiod at 8 h light:16 h dark and fed three times per week with commercial fish pellets (Sorgal, Ovar, Portugal). The seawater used in the holding and test periods was collected from the coastal area near Porto, Portugal, in a site free of direct exposition to contaminated effluents. The seawater passed through sand and carbon filters before being used. The 16 EPA priority PAHs analysis performed in the seawater shown that the PAHs levels are bellow the detection limit of 20 $\mu g\ L^{-1}$, and acrylonitrile was also below the detection limit of 25 $\mu g\ L^{-1}$.

2.2. Acute bioassay

Fish were exposed to six treatments: control (natural filtered seawater at 33–35‰) and five levels of acrylonitrile nominal concentrations (2.5, 4.5, 8, 8.5 and 10 $mg\ L^{-1}$), in a semi-static daily replacement regime for 96 h. The experiment was performed in 30 L glass tanks covered with appropriate lids sealed with parafilm to minimize the acrylonitrile volatilization. Two replicates per treatment were considered with 3 animals per aquaria. Test solutions of acrylonitrile were prepared directly in each aquarium, by dilution of the respective stock solution, made in ultra-pure bi-deionised water, with filtered seawater. The solutions were then properly stirred. Seventy-five per cent of the test media of each aquarium was replaced every day and the animals were not fed during the assay. Salinity and temperature were maintained at 33–35‰ and 18 ± 1 °C, photoperiod at 8 h light:16 h dark and aeration was provided continuously. Toxicity data generated in this assay were statistically analyzed by probit analysis using the Sat-Plus Portable (AnalystSoft Inc.) software and the 96-h LC_{50} was determined.

2.3. Sublethal bioassay (exposure and recovery phase)

The assay was carried out in 30 L glass aquaria with three juvenile animals each during 22 d (15 d of exposure and 7 d of recovery) at 18 ± 1 °C, under a photoperiod of 8 h light:16 h dark and aeration was provided continuously. The aquaria were covered with appropriate lids sealed with parafilm. In the exposure phase, the fish were allocated to one of the four treatments, with four replicates each (control-natural filtered seawater at 33–35‰ salinity and three acrylonitrile nominal concentrations: 0.15, 0.75 and 2 $mg\ L^{-1}$). These concentrations were selected based on the acrylonitrile LC_{50} value obtained in this study. The test solutions were prepared under the conditions described in the acute bioassay. Seventy-five per cent of the test media of each aquarium was replaced every day and any uneaten food, faeces or detritus were siphoned out before de water change. Animals were fed with commercial fish pellets, 3 d per week. Test aquaria were inspected daily for aeration and dead animal were removed. Abiotic parameters (temperature, salinity, dissolved oxygen, pH ammonia, nitrates, and nitrites) were monitored twice a week, during the assay.

2.3.1. Tissues preparation and biomarkers determination

At the end of the 15 exposure days, six animals per treatment (two replicate aquaria per treatment) were immobilized in ice-cold water, a blood sample was collected from fish caudal peduncle for comet assay. Then fish were immediately decapitated, their length and body weight were recorded, samples of liver were frozen in liquid nitrogen and stored at −80 °C until further analysis of the following biomarkers: Catalase (CAT), Glutathione S-Transferase (GST), Hepatic ethoxyresorufin O-deethylase (EROD), Superoxide Dismutase (SOD), and Lipid Peroxidation (LPO).

After the 15 d of the exposure phase, the remaining two aquaria per treatment (6 animals) were cleaned and fish were placed in fresh seawater, during the 7 d (recovery phase) with the same physical conditions of the exposure phase described before. At the end of the recovery phase, the comet assay, the activity of CAT, GST, EROD, SOD and the LPO levels were determined according the methods described below.

The alkaline single-cell gel electrophoresis assay (comet assay) was used to determine the level of DNA damage (DNA strand breaks) in whole-blood according to Singh et al. (1988). Analysis was run immediately after blood collection to ensure maximum cell viability. The preparation of slides for the comet assay, subsequent electrophoresis and staining were carried out as described previously by Costa et al. (2011).

Liver samples were homogenized in 1 mL of ice-cold sodium phosphate buffer. Mitochondrial fractions were obtained after centrifugation at 15 000 rcf for 20 min, at 4 °C and the supernatant used for biochemical determinations of CAT, GST, SOD activities and LPO levels. CAT activity was determined by measuring the consumption of H_2O_2 at 240 nm as described previously by Ferreira et al. (2007). GST was determined according to the method of Habig et al. (1974) adapted to microplate. The GST activity, expressed in μmol min^{-1} mg^{-1} protein, was measured every 20 s, at 340 nm, during the first 5 min. The SOD activity was measured by an indirect method involving the inhibition of cytochrome *c* reduction at 550 nm as described in Ferreira et al. (2010). The activity was given in SOD units (1 SOD unit = 50% inhibition of the xanthine oxidase reaction) per mg of protein. The LPO levels were expressed as μmol of Malondialdehyde (MDA) equivalents per mg of protein. MDA was determined by the thiobarbituric acid method as described in Ferreira et al. (2008). The EROD activity was measured according to Cheah et al. (1995). Portions of the liver were homogenized separately in ice-cold Tris–KCl buffer. Homogenates were centrifuged at 12 000 g for 20 min and the resulting supernatant was further centrifuged at 100 000 g for 60 min to obtain the microsomal fraction. EROD activity, expressed in pmol min^{-1} mg^{-1} protein, was measured at λex = 530 nm and λem = 585 nm and determined by comparison to a resorufin standard curve.

2.3.2. Statistical analyses

Data of each biochemical marker were first checked for normality (Kolmogorov–Smirnov test) and homogeneity of variances (Levene's test); and subsequently analyzed by one-way ANOVA comparing different treatments for each endpoint. When significant differences were found by ANOVA, the Fisheŕs least significant difference test (LSD) was used. Each treatment was compared with the respective control; and the exposed-recovery treatments of each acrylonitrile concentration were also compared. The values of the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) of exposure phase were also determined. The Statistica 8.0 package (Statsoft Inc., 2007) was used for statistical analysis and significant differences were established at $p < 0.05$.

2.4. Acrylonitrile analytic quantification by high performance liquid chromatograhy (HPLC)

The actual concentration of acrylonitrile was determined at 24 h in the acute bioassay and three times during the sublethal bioassay (on days 1, 9 and 15 at 0, 6 and 24 h after the daily water change). Seawater samples, collected in duplicate, were directly analyzed by high performance liquid chromatograhy (HPLC) with a photodiode array detector (DAD) for acrylonitrile determination. The liquid chromatographic system used was a Hitachi LaChrom Elite HPLC, constituted with a L-2130 quaternary pump, an in line degasser, a L-2455 photodiode detector, and a L-2200 autosampler. Separations were performed with a 250 × 4.6 mm (length × i.d.), 5 μm particle, Purospher® STAR RP-18e analytical column (Merck) and a 4 × 4 Chmm i.d., 5 μm particle, guard column with the same packing material. The mobile phases used, acetonitrile (A) and water (B), were at a flow rate of 1 mL min^{-1} and the injection volume was set to 50 μL. The acrylonitrile elution was done in an isocratic mode (50% A and 50% B) maintaining the temperature of the column at 30 °C. Acrylonitrile was monitored at a wavelength of 195 nm and the detection and quantification limits (LOD and LOQ) were 25 and 75 μg L^{-1}, respectively. The acrylonitrile analytical quantification was adapted from the methods described by USA EPA (1994).

3. Results

3.1. Acute bioassay

The 96-h LC_{50} test resulted in an estimated LC_{50} of 8.1 mg L^{-1} with a 95% confidence interval of 7.8–8.4 mg L^{-1}. There were no mortality in control treatment and 100% mortality in the highest tested concentration of 10 mg L^{-1}. The NOEC value was the lowest concentration tested (2.5 mg L^{-1}) while the LOEC was 8 mg L^{-1}.

3.2. Sublethal bioassay

3.2.1. Survival

All the animals survived in control and 0.15 mg L^{-1} acrylonitrile concentration, but 8.3% and 25% mortality were registered in 0.75 and 2 mg L^{-1} acrylonitryle concentrations, respectively. During the exposure phase, in comparison with fish from the other acrylonitrile concentrations, animals from 2 mg L^{-1} acrylonitrile concentration had increased pigmentation, stopped feeding, and presented reduced swimming activity, with slow movements at the top of the aquaria. All the lethargic fish that survived in the 2 mg L^{-1} acrylonitrile exposure, recovered during the recovery phase.

3.2.2. Biochemical markers

The CAT, GST, SOD, and EROD activity and the LPO levels in seabass liver, for the exposure phase and subsequent recovery period, are displayed in Fig. 1. In the exposure phase, a significant induction of CAT and GST ($p < 0.01$) was recorded in the highest concentrations of acrylonitrile (2 mg L^{-1} for CAT; 0.75 mg L^{-1} and 2 mg L^{-1} for GST). The NOEC and LOEC for CAT were 0.75 mg L^{-1} and 2 mg L^{-1}, respectively; and for GST were 0.15 mg L^{-1} and 0.75 mg L^{-1}, respectively. An inhibition of SOD activity was observed for the two highest acrylonitrile concentrations ($p < 0.05$ for 0.75 mg L^{-1} and $p < 0.01$ for 2 mg L^{-1}) with a NOEC and LOEC values of 0.15 mg L^{-1} and 0.75 mg L^{-1}, respectively. For LPO and EROD, no significant differences were observed between exposed and control groups, although the LPO levels in the intermediate

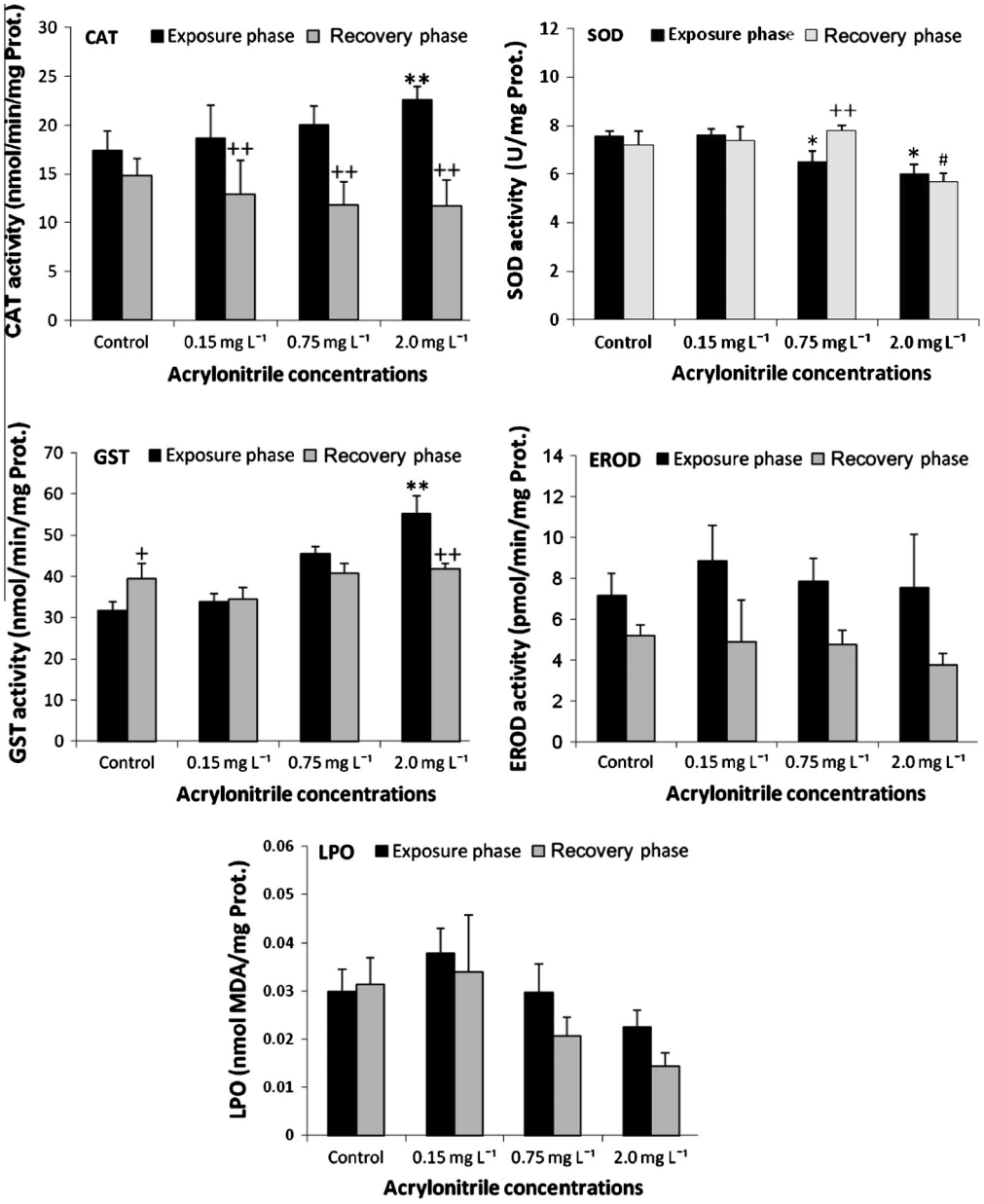

Fig. 1. Effects of acrylonitrile on liver Lipid Peroxidation (LPO), ethoxyresorufin-O-deethylase (EROD), Glutathione-S-Transferase (GST), Catalase (CAT), and glutathione peroxidase (GPx) of *Dicentrarchus labrax* after 15 d of exposure to acrylonytrile (black columns) plus 7 d of depuration (grey columns). Error bars indicate the standard errors; asterisks indicates significant differences from control in the exposure phase: $^{*}p < 0.05$ and $^{**}p < 0.01$; plus indicate differences between exposure and depuration phase for each acrylonitrile concentration: $^{+}p < 0.05$ and $^{++}p < 0.01$ and cardinal indicates significant differences from control in the depuration phase $^{\#}p < 0.05$.

and low concentrations were either about the same or above the control during the exposure phase (Fig. 1).

After 7 d in the recovery phase, no significant changes were detected between the acrylonitrile groups and control for most of the biochemical parameters analyzed. With the exception of SOD activity (2 mg L^{-1} acrylonitrile concentration) which continue to present a significant inhibition comparatively with the control, all the acrylonitrile groups were reduced to control levels in the others biochemical markers (Fig. 1). Considering CAT and GST activities, which were significantly induced in the exposure phase, a significant reduction was also recorded in some acrylonitrile groups after the recovery phase comparatively with the same acrytonitrile groups of the exposure phase (Fig. 1).

The comet assay results showed that the incidence of total-strand DNA breaks (% of DNA in the tail) was greater in acrylonitrile-exposed fish than in control group (Fig. 2). In the exposure phase, fish blood collected from all acrylonitrile concentrations displayed significant induction of total DNA strand breakage (TSB) when compared with the control fish. The increase in TSB ranged from1.6-fold (fish exposure to 0.15 mg L^{-1} acrylonitrile, $p < 0.05$) and 2.5-fold (fish exposure to 0.75 and 2 mg L^{-1} acrylonitrile, $p < 0.01$) with a LOEC value of 0.15 mg L^{-1}.

In the recovery phase, no statistical significant differences were observed between the acrylonitrile and control fish and, therefore, the TSB levels were reduced to control levels. Furthermore, significant reduction of TSB was recorded in the 0.75 and 2 mg L^{-1} acrylonitrile groups of the recovery phase comparatively with the same acrytonitrile groups of the exposure phase ($p < 0.01$) (Fig. 2).

3.3. Acrylonitrile analytic quantification by HPLC

Table 1 summarizes the actual concentrations of acrylonitrile measured during the acute and sublethal bioassays. No acrylonitrile was detected in the control group. The results presented here

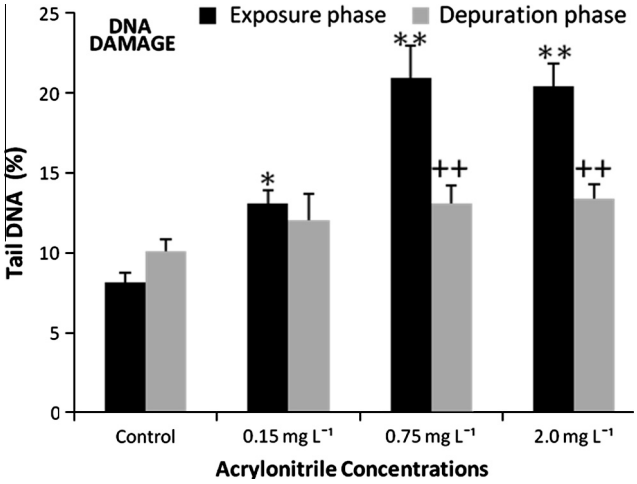

Fig. 2. Effects of acrylonitrile on whole blood DNA integrity (% of DNA in the tail) of *Dicentrarchus labrax* after 15 d of exposure to acrylonytrile (black columns) plus 7 d of depuration (grey columns). Error bars indicate the standard errors; asterisks indicates differences from control both for exposure phase and depuration phase: $^{*}p < 0.05$ and $^{**}p < 0.01$, and plus indicate differences between exposure and depuration phase for each acrylonitrile concentration: $^{+}p < 0.05$ and $^{++}p < 0.01$.

indicate that, in the acute bioassay, acrylonitrile was stable in water with a maximum loss of 11.1% in the 8.0 mg L^{-1} acrylonitrile concentration. In the sublethal bioassay, at the beginning of the experiment, acrylonitrile was also stable, with a maximum decay of 15.9% in the 0.15 mg L^{-1} acrylonitrile concentration at the end of day 1 (Table 1). Over time, some increase of acrylonitrile degradation was observed. To ensure the exposure of fish to an acrylonitrile concentration close to the nominal concentration throughout the assay, the water was changed daily.

4. Discussion

Acrylonitrile is present in the aquatic environment primarily due to industrial waste discharge (IARC, 1999; World Health Organization, 2002; EU RAR, 2004), but also from accidental spills. Because of the large amounts of acrylonitrile transported by sea and its high water solubility, the ecological risks associated with an accidental spillage are considerable. In fact, acrylonitrile has been classified as toxic to aquatic organisms with long lasting effects (based on 2010 acrylonitrile dossier for the EU Classification, Labeling and Packaging (CLP) regulation), besides being an EPA priority pollutant (Keith and Telliard, 1979).

There is a considerable body of work focusing on the toxicology of acrylonitrile in mammalians, mainly in rodents and humans (Jiang et al., 1998; Whysner et al., 1998; IARC, 1999; Kirman et al., 2005; Cole et al., 2008; Watcharasit et al., 2009). However, the current knowledge on the subletal effects of acrylonitrile to marine organisms is scarce; and the investigation on the mechanisms of acrylonitrile toxicity has seldom been addressed in aquatic organisms. Here, we addressed the lethal and sublethal effects of acrylonitrile in the marine fish – *D. labrax*; the detoxification capability of *D. labrax* and the potential toxicity mechanism(s) of acrylonitrile in fish are also discussed.

In the present study, acrylonitrile induced several toxic effects in seabass. In the acute toxicity assay, a 96 h LC_{50} of 8.1 mg L^{-1} was obtained. Prior to this work, other 96 h LC_{50} values have been reported for marine fish. A 96 h LC_{50} of 8.6 mg L^{-1} and 14 mg L^{-1} were reported for *Cyprinodon variegatus* (Adema, 1976, in EU RAR) and for *Gobius minutus* (AN group 1997 (a) in EU RAR), respectively. The 96 h LC_{50} obtained here for *D. labrax* are on the same range of the 96 h LC_{50} available for other seawater organisms and suggest that acrylonitrile has a moderate acute toxicity to marine fish. The sublethal exposure to the two highest doses (0.75 and 2 mg L^{-1}) increased the mortality rate in comparison with the other treatments. Fish from the 2 mg L^{-1} acrylonitrile exposure presented the skin extremely dark and impairment of swimming performance, characterized by low swimming activity with uncontrolled circular movements at the top of the aquarium, together with bumps with the aquarium walls. The swimming performance observed in fish at the highest acrylonitrile exposure dose, suggest an impact in the nervous system, which integrates well with the observations in mammals (Ghanayem et al., 1991; Gagnaire et al., 1998; Esmat et al., 2007). However, understanding the mechanisms underlying a potential effect on fish nervous system requires further investigation through the measurement of neurotoxic parameters such as cholinesterase activity in fish brain. Additionally, important alterations at sub-individual level (biochemical markers) were observed. In liver, acrylonitrile significantly induced the activities of CAT and GST. Conversely, SOD activity was found to be significantly inhibited. Moreover, no significant effects were found on LPO levels and EROD activity. After 7 d of recovery, almost all of the toxic effects of the acrylonitrile exposure were recovered.

Biotransformation enzymes are responsible for the biodegradation of several xenobiotics, converting them into more hydrophilic metabolites. Among these, the activity of cytochrome P450 system enzymes in general and the sub-family CYP1A particularly, have been widely used as environmental biomarkers in fish (Billiard et al., 2004; Ferreira et al., 2010; Santos et al., 2010; Almeida et al., 2012). CYP1A isoenzymes catalyze the conversion of O-dealkylation

Table 1
Nominal and measured concentrations of acrylonitrile (mg L^{-1}) in water samples collected in each treatment during the acute assay at day 1; and in sublethal assay (exposure phase) at days 1, 9 and 15. Data expressed as mean ± standard deviation.

Time (h)	Acute assay				
	2.5 mg L^{-1}[a]	4.5 mg L^{-1}[a]	8.0 mg L^{-1}[a]	8.5 mg L^{-1}[a]	10.0 mg L^{-1}[a]
0	2.7 ± 0.06	4.1 ± 0.1	8.1 ± 0.4	8.2 ± 0.2	9.5 ± 0.3
24	2.7 ± 0.08	3.6 ± 0.2	7.2 ± 0.2	7.8 ± 0.1	9.1 ± 0.1

	Sublethal assay								
	0.15 mg L^{-1}[a]			0.75 mg L^{-1}[a]			2 mg L^{-1}[a]		
	Day 1	Day 9	Day 15	Day 1	Day 9	Day 15	Day 1	Day 9	Day 15
0	0.17 ± 0.003	0.13 ± 0.01	0.12 ± 0.003	0.77 ± 0.002	0.74 ± 0.01	0.65 ± 0.02	1.95 ± 0.05	2.03 ± 0.10	1.58 ± 0.07
6	0.16 ± 0.001	0.06 ± 0.02	0.08 ± 0.02	0.73 ± 0.01	0.70 ± 0.02	0.44 ± 0.06	1.84 ± 0.05	1.89 ± 0.08	0.75 ± 0.02
24	0.14 ± 0.002	0.02 ± 0.0002	0.03 ± 0.001	0.68 ± 0.01	0.62 ± 0.02	0.14 ± 0.04	1.68 ± 0.009	1.74 ± 0.05	0.05 ± 0.02

[a] Nominal acrylonitrile concentration.

of 7-ethoxyresorufin to resorufin, a reaction that is commonly used to assess their activity. In this study, no significant effects were found on liver EROD activity of seabass exposed to acrylonitile suggesting that CYP1A is not involved in the biotransformation of acrylonitrile in this marine fish. Therefore, other P450 isoenzyme is most likely responsible for the metabolism of acrylonitrile. Several studies indicate that in mammalian, the metabolism of acrylonitrile is determinant for its mode of action and toxicity. In rats, mice and humans, the toxicokinetics of acrylonitrile is well understood (see Kirman et al., 2005 for review). One of the initial metabolic pathways is the epoxidation of acrylonitrile by the P450 isoenzyme P450 2E1 to form 2-cyanoethylene oxide (CEO), and further metabolism to generate cyanide (Sumner et al., 1999; Nerland et al., 2001; Kirman et al., 2005). In fact, P450 2E1 is referred as the major catalyst of acrylonitrile epoxidation and that other P450 isoenzymes play only a minor role in the metabolism of acrylonitrile in mammalian (Nerland et al., 2001). In fish, the potential metabolic detoxification pathway(s) of acrylonitrite has not been studied yet. The identification and quantification of acrylonitrile metabilites and the involvement of CYP 450 families in fish acrylonitrile metabolism should be addressed in futures studies to provide more insights into the mechanism(s) of acrylonitrile induced toxicity in fish. Another biotransformation enzyme screened in this study was the GST. In fish, this enzyme is involved in the detoxification of many contaminants by catalyzing the conjugation of chemicals or their metabolites with the endogenous glutathione, facilitating their elimination from the organism. Moreover, GST plays an important role in the protection against oxidative stress. GST have been described to be required for the cellular resistance to oxidative stress, protecting cells from oxidative stress by detoxifying some of the secondary reactive oxygen species (ROS) (Veal et al., 2002). In the present study, GST activity was found to be significantly increased in seabass exposed to acrylonitrile at the highest concentration of 2 mg L^{-1} (Fig. 1), suggesting the involvement of this enzyme in the biotransformation of acrylonitrile and/or in the antioxidant defense.

Together with GST, the antioxidant enzymes, CAT and SOD, have an important role in the detoxification of ROS, such as the superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) to non-reactive molecules (Winston and Di Giulio, 1991). Therefore, changes of these enzymatic activities should indirectly indicate toxic effects of acrylonitrile on seabass. SOD, one of the first lines of antioxidant defenses, is involved in the dismutation of $O_2^{\cdot-}$ into H_2O_2, which is further scavenged by CAT. The significant decreased of SOD activity at the two highest acrylonitrile concentrations (0.75 and 2 mg L^{-1}) observed in the present experiment, could be a part of acrylonitrile toxic response. SOD is a metallic protein, being the Cu-Zn SOD the most representative form in fish liver (Radovanović et al., 2010). In addition, copper and zinc play a key role in the SOD activity. Acrylonitrile is highly reactive with certain metals forming stable complexes with copper (Fredriksson et al., 1996), and in rats, it has been reported that acrylonitrile is able to covalent bind to tissue proteins and metallo-proteins (e.g. hemoglobin) (Benz et al., 1997; Whysner et al., 1998). Accordingly, it is likely that a decreased of the available copper in liver, and/or the potential acrylonitrile binding to SOD, may lead to the observed reduction in SOD activity. However, other antioxidants enzymes played an active role to overcome ROS toxicity resulting from the inhibition of SOD activity. The significant induction of CAT observed in the highest acrylonitrile concentration could indicate the presence of H_2O_2 and that the generation of H_2O_2 is still within its elimination capacity. Apparently, the CAT and GST induction were enough to cope with the oxidative stress induced by acrylonitrile, since no significant differences in LPO levels were detected between exposed and control fish. This means that the production of ROS associated with exposure to acrylonitrile did not overwhelm the antioxidant defenses of seabass liver and therefore oxidative damage of lipids (LPO levels enhancement) did not occur.

Thus, our results revealed the capacity of sea bass to cope with the stress induced by acrylonitrile exposure via enhancing the activity of the antioxidant enzymes CAT and GST. The organisms may have adapted to the acrylonitrile exposure, conferring an improved protection to the presence of ROS. These findings suggest that in seabass liver, the acrylonitrile was not implicated on an overall increase of intracellular ROS and oxidative damage. In mammals, several studies have indicated the role of oxidative stress in the toxicity of acrylonitrile (Jiang et al., 1998; Zhang et al., 2002; Kirman et al., 2005; Guangwei et al., 2010), and some authors have demonstrate that acrylonitrile induce tissue-specific toxicity effects in rodents. Jiang et al. (1998) showed that a chronic exposure to acrylonitrile increased the levels of ROS and LPO, accompanied by a significant reduction in CAT, SOD and GSH levels in rat brain; with no changes of these indicators of oxidative stress in the livers of acrylonitrile treated rats. These authors concluded that rat liver is not a target tissue for acrylonitrile toxicity, being the brain, stomach and zymbaĺs gland the target organs. In agreement with the findings obtained by Jiang et al. (1998) in rats, no oxidative stress was produced by acrylonitrile in seabass liver, which could be indicative that fish liver is not a target tissue of acrylonitrile toxicity or, alternatively, the defense mechanisms in liver is able to cope with acrylonitrile at the concentration range tested in the present study. The results obtained in this study suggested that the detoxification pathways used by fish to cope with acrylonitrile toxicity are similar to those used by mammals. However, additional studies should be performed to provide more insight into the mechanism(s) of acrylonitrile toxicity in fish.

Several studies have revealed that the comet assay is a rapid and sensitive technique to assess DNA damage in aquatic organisms produced by genotoxic chemicals (Xu et al., 2003; Dhawan et al., 2009). In this study, the comet assay results revealed a significant dose dependent increase of DNA strand breaks in blood cells of seabass exposed to acrylonitrile. These results indicate that acrylonitrile is able to causes DNA damage in seabass. In rats, several studies have demonstrated that acrylonitrile causes oxidative stress and oxidative DNA damage in rat brain and blood (Jiang et al., 1998; Esmat et al., 2007; Pu et al., 2009). Several observations suggested that the oxidative stress produced by acrylonitrile through the depletion of antioxidant defenses and increased production of ROS, may result in modifications to cellular DNA such as DNA damage in rat brain and blood (Pu et al., 2009). Other authors have reported that acrylonitrile induce *in vivo* DNA damage in human sperm cells (Xu et al., 2003). To the best of our knowledge, the present study is the first that provides evidences of DNA damage of acrylonitrile in fish, although additional studies should address the underlying mechanism(s).

In the recovery phase, when fish were transferred to clean seawater, the activity of antioxidant enzymes and the level of DNA strand breaks returned to the control level. This may be explained by: (1) acrylonitrile or its metabolites which may had been accumulated in fish tissues (liver and blood) were quickly biotransformed/eliminated; (2) when the acrylonitrile exposure stopped, the protection conferred by the antioxidant enzymes to acrylonitrile ROS production was reduced, which means that fish had adjusted itself the prooxidant/antioxidant balance and returned to the normal physiological conditions; (3) the decrease of DNA damage recorded in the recovery phase would probably be related with compensatory mechanisms that repaired the DNA strand breaks produced by the exposure to acrylonitrile. Although an apparent improvement in the biochemical endpoints assessed after the recovery phase was recorded, some caution should be taken in data

interpretation as long term effects cannot be ruled out. In the literature at least two long term acrylonitrile toxicity studies exist in fish, which showed significantly reduced growth rate of *Pimephales promelas* at concentrations >0.34 mg L^{-1} (Analytical BioChemistry Laboratories Inc., 1980); and no evidence of carcinogenicity in acrylonitrile-exposed *O. latipes* (Hawkins et al., 1991). However, future studies should focus on acrylonitrile effects in more ecological relevant endpoints such as reproduction and gamete viability.

In conclusion, the present study has gathered essential information on the acute and sublethal toxicity of acrylonitrile and the effects of a recovery period to seabass. Furthermore, first steps were taken to better understand the toxicity mechanisms of acrylonitrile in fish. Increasing the toxicity datasets of priority HNS is essential to assist relevant bodies in preparedness and response to HNS spills.

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