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ARCOPOL

Laboratory assays for the study of chronic toxicity and decontamination kinetics of acrylonitile in seabass (*Dicentrarchus labrax*)

Activity 6

Task 6.3.2

ARCOPOL

The Atlantic Regions' Coastal Pollution Response

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1. Introduction

Accidental HNS spills can pose a significant threat to fishing and aquaculture resources. Some of the major concerns after an accidental spill is the economic loss from fishery interruption and the contamination of commercial fish and shellfish. Severe contamination can give rise directly to concern human consumers, for example high concentrations of carcinogenic chemicals or tainting of edible tissues.

In the context of Arcopol project, CIIMAR have produced two reports within the activity 6.3, concerning the kinetic of decontamination from fish and shellfish after oil and HNS spills (Neuparth *et al.*, 2011a), and the definition of procedures for management of contaminated marine marketable resources affected by oil and HNS spills (Cunha *et al.,* 2011). The data produced in these two documents could be useful to better anticipate economic losses during the demand of compensation for damages and to improve accidental spill response, specifically the management of risks to fisheries and consumers. However, as was pointed in these studies, much more is needed to be done concerning the assessment of potential contamination and decontamination of marine marketable resources after an HNS spill. Therefore, experimental studies on the chronic toxicity and decontamination kinetics of HNS chemicals in selected fish and selfish should be performed in laboratory. In this context, the report here produced ensemble the main findings produced in a laboratory contamination/decontamination assay conducted with one of the priority HNS identified by Neuparth et al. (2011b), the Acrylonitrile, and the target marine organism, the European seabass (*Dicentrarchus labrax*).

2. Methodology

2.1. Selection of the target marine organism - the seabass (Dicentrarchus labrax)

The European seabass - *Dicentrarchus labrax*, was chosen due to its ecological and economic relevance. The seabass is a marine fish species widely distributed in the Mediterranean and European Atlantic coasts and one of the species mostly produced in aquaculture of Mediterranean areas (Ferreira *et al.*, 2010). Its biology is well known, they are easy to maintain in laboratory.

2.2. HNS tested

The selection of HNS to conduct the laboratory contamination/decontamination study was based on 23 HNS identified as priority by Neuparth et al. (2011b). From the dataset created in the Neuparth *et al*. (2011b), with the acute and chronic toxicity data for marine species, the priority HNS selected was the acrylinitrile. This HNS was chosen considering the limited ecotoxicological data available for marine organisms, its involvement in previous accidental spills, the indication to be carcinogenic to mammals and to be highly transported in European waters.

2.3. Contamination/decontamination assay with *D. labrax* exposed to Acrylonitrile

The experiment was carried out with juvenile Seabass (*D. labrax*), weighing 30-34g, from the fish farm "Maresa" in Huelva, Spain. Three hundred animals were acclimated to controlled laboratory conditions, in 1000L tanks with constant filtered seawater circulation, for ten weeks before the experiment began.

The assay was carried out in 30-L glass aquaria with 3 juvenile animals each during 22 days (15 day exposure phase and 7 days decontamination phase) at 16-17ºC under a photoperiod of 8h light:16 dark and aeration was provided continuously. In the exposure phase, the animals were divided in four treatments, with four replicates each (control - natural seawater at 33-35‰ salinity and three acrylonitrile nominal concentrations: 0.15, 0.75 and 2mg/l). 75% of the test media of each aquarium was replaced every day and animals were fed with commercial fish food, three day per week. Test aquaria were inspected daily for aeration and to remove dead animals (figure 2). The real concentration of acrylonitrile were determined once per week, immediately after the change of water and 6 and 24h later. The acrylonitrile in water of each treatment was measured by high performance liquid chromatograhy (HPLC) with a photodiode array detector (DAD). Detection and quantification limits (LOD and LOQ) were 25 and 75 µg/L, respectively.

Figure 2 - Representation of the experimental design used in acrylonitrile chronic toxicity assay with seabass (*Dicentrarchus labrax*),

At the end of the 15 days exposure, six animals per each treatment (two 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 weight were measured, and samples of liver were frozen and stored at -80ºC for later quantification of the following biomarkers: Catalase (CAT), Glutathione S-Transferase (GST), Hepatic ethoxyresorufin O-deethylase (EROD), Superoxide dismutase (SOD), and Lipid peroxidation (LPO). The study of acrylonitrile bioaccumulation was also performed in liver and muscle samples stored a -80ºC. See the methods description below.

After the 15 days exposure phase, the remaining two aquaria per treatment (6 animals) were cleaned and fish were placed in clean seawater, during the 7 days of depuration phase with the same physical conditions of the exposure phase described before. At the end of the depuration phase, the comet assay, the activity of CAT, GST, EROD, SOD and LPO; and the study of the acrylonitrile persistence tissues and were determined according the methods described below.

The comet assay was used to determine the level of DNA damage (DNA strand breaks) in blood cells of all fish. The preparation of slides for the comet assay, subsequent electrophoresis and staining were carried out as described previously by Liney *et al*. (2006).

The study of acrylonitrile bioaccumulation (uptake and depuration) in liver and muscle are being performed according the static head-space technique combined with

gas chromatography-mass spectrometry (GC/MS), as described by Ventura *et al.* (2004).

Catalase (CAT) activity was determined by measuring the consumption of H_2O_2 at 240 nm. The reaction volume was 1ml containing 67.5mM potassium phosphate buffer, pH 7.5, and 12.5mM H_2O_2. The reaction was started by the addition of the sample. CAT activity is expressed as ηmol/min/mg protein.

Glutathione S-transferase (GST) was determined according to the method of Habig *et al.* (1974) adapted to microplate, using glutathione (GSH) 10mM in phosphate buffer 0.1M, pH 6.5, and 1-chloro-2,4-dinitrobenzene (CDNB) 60mM in ethanol prepared just before the assay. The reaction mixture consisted of phosphate buffer, GSH solution and CDNB solution in a proportion of 4.95ml (phosphate buffer):0.9ml (GSH):0.15ml (CDNB). In the microplate, 0.2ml of the reaction mixture was added to 0.1ml of the sample, with final concentration 1mM GSH and 1mM CDNB in the assay. The GST activity was measured immediately every 20 s, at 340nm, during the first 5 min, and calculated in the period of linear change in absorbance. The GST activity was expressed in ηmol/min/mg protein.

The EROD activity was measured according to Ferreira *et al.* (2008). Briefly, liver and gills were homogenized separately in ice-cold buffer (50 mM Tris–HCl, pH 7.4, 0.15 M KCl). Microsomes were obtained by centrifugation of the 9000*g* supernatant at 36 000*g* for 90 min. The pellet was then resuspended in buffer (50 mM Tris–HCl, 1 mM Na_2EDTA pH 7.4, 1 mM dithiothreitol, 20% v/v glycerol) and spun down at 36 000 g for 120 min. Microsomes were suspended in EDTA-free resuspension buffer and stored at -80ºC until use. Microsomal suspension (50 ml) was incubated with ethoxyresorufin 0.5 mM for 1 min, and the enzymatic reaction was initiated by the addition of 45 mM NADPH. EROD activity was measured for 5 min at λ_{ex}=530 nm and λ_{em}=585 nm, and determined by comparison to a resorufin standard curve. The EROD activity in liver and gills was expressed in pmol/min/mg protein.

The SOD activity was determined by an indirect method involving the inhibition of cytochrome *c* reduction. In this method SOD competes with cytochrome *c* for the superoxide anion generated by the hypoxanthine and xanthine oxidase reaction. SOD activity was determined in the mitochondrial fraction as the degree of inhibition of cytochrome *c* reduction at 550 nm. The concentration of the reactives was potassium phosphate buffer 50 mM, pH 7.8, hypoxanthine 50 mM, xanthine oxidase 1.98 mU/ml

and cyto-chrome *c* 10 mM (Ferreira *et al*., 2010). The activity is given in SOD units (1 SOD unit=50% inhibition of the xanthine oxidase reaction) per mg of protein.

The peroxidative damage to lipids that occurs with free radical generation, and results in the production of malondialdehyde (MDA) was assessed by the determination of thiobarbituric acid reactive substances (TBARS). MDA was determined by the thiobarbituric acid method, the homogenates were incubated with trichloroacetic acid (TCA) 100%, after centrifugation the supernatant was incubated at 100°C, for 30 min, with thiobarbituric acid (TBA) 1%, NaOH 0.05 M and BHT 0.025%. The absorbance was measured at 532 nm. Lipid peroxidation (LPO) is expressed as ηmol of MDA equivalents per mg of protein.

2.4 Statistical analyses

A one-way analysis of variance (ANOVA) was carried out for each studied variable (Comet assay and enzymatic activities) to determine if there are differences in responses between treated - control organisms and between exposed - depurated animals. Significant differences were established at $p<0.05$. The Fisher´s least significant difference test (LSD) was used for multiple comparisons between pairs of means and all statistics were performed using the software Statistica 8.0 (Statsoft, Inc., 2007).

3. Results

3.1. Chemical analyses

Table 1 summarises the real acrylonitrile concentration measured during the exposure phase of each water samples (control 0.15, 0.75 and 2mg/l). The measurements were made once per week in three different times: immediately after the daily water change (initial time); 6 hours after the initial time (middle time) and at 24hours immediately before the daily water change (final time).

Table1. Acrylonitrile concentrations (mg/l) in water samples collected in each treatment during the exposure phase. Data expressed as mean ± standard deviation

Nominal concentration		**0.15mg/l**	**0.75mg/l**	**2mg/l**
Actual concentration	Initial	0.16±0.004	0.80±0.002	2.07±0.055
	Middle	0.14±0.002	0.76±0.013	1.95±0.066
	Final	0.13±0.002	0.70±0.013	1.78±0.010

Acrylonitrile proved to be a very stable chemical in water. The actual acrylonitrile concentrations were very close to the nominal concentrations at all sampling times with a small percentage of loss within 24 hours.

3.2. Acrylonitrile bioaccumulation (uptake and depuration)

The acrylonitrile bioaccumulation are under analysis at the time of this report.

3.3 Survival

All the animals survived in control and 0.15mg/l acrylonitrile concentration but one and two animals dead in 0.75 and 2 mg/l acrilonitryle concentrations respecively at the end of the exposure phase. Fish from 2mg/l acrylonitrile concentrations presented the skin extremely dark and low swimming capacity comparatively with fish from the other acrylonitrile concentrations.

3.4. Comet assay

In the exposure phase, the incidence of total-strand DNA breaks (% of DNA in the tail) was greater in acrylonitrile-exposed fish than in controls (Figure 2). Fish blood collected from fish from all the acrylonitrile concentrations presents significant induction of total DNA strand breakage (TSB) relatively to control fish. The increase in TSB, ranging between 1.6-fold (fish exposure to 0.15mg/l acrylonitrile) and 2.5-fold (fish exposure to 0.75 and 2mg/l acrylonitrile). In the depuration phase, no statistical differences were observed between the depurated acrylonitrile fish and control fish, and therefore the TSB levels of depuration acrylonitrile fish reduced to control levels. Also, significant reduction of TSB was recorded in the 0.75 and 2mg/l acrylonitrile groups of the depuration phase comparatively with the same acrytonitrile groups of the expousure phase (figure 2).

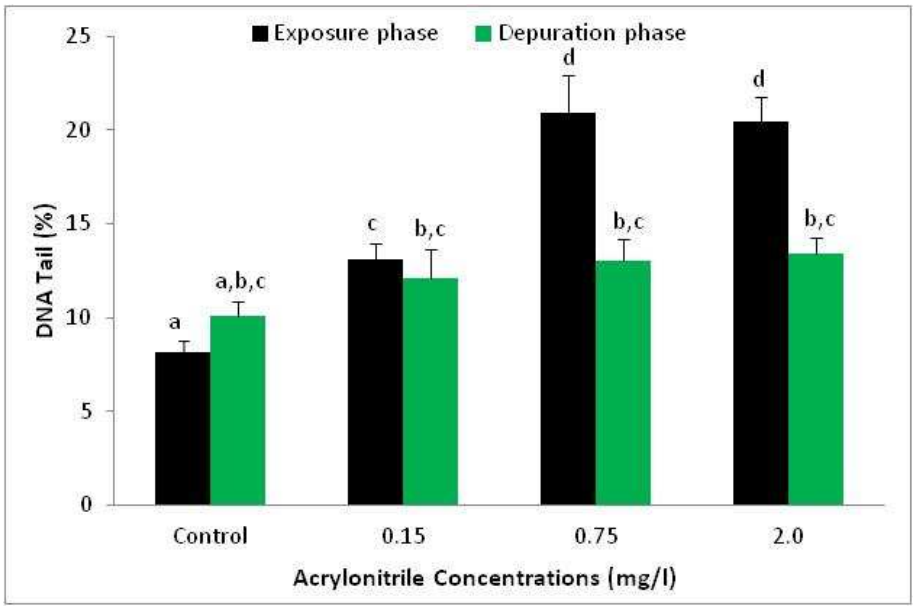


Figure 2 - Comet assay results (% of DNA in the tail) for the treatments: control, 0.15, 0.75 and 2 mg/l Acrylonitrile concentrations during the 15 days of exposure (■ Exposure phase) plus 8 days of depuration (■ Depuration phase). Values are presented as mean ± SE. Dissimilar letters denote statistical significant differences ($p < 0.05$).

3.5. Enzymatic activities

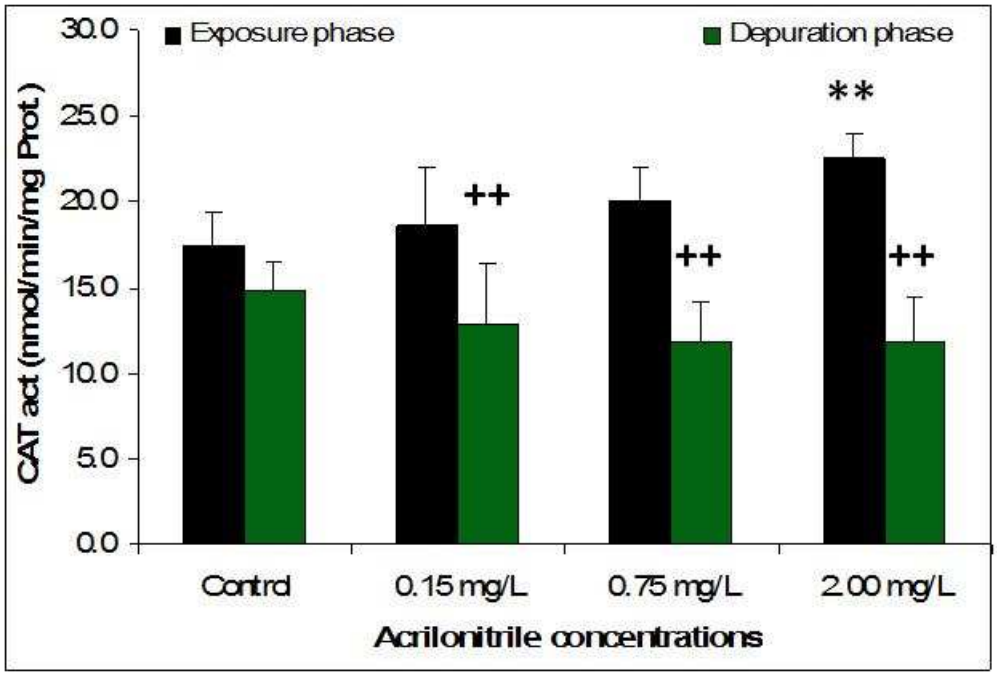
The determination of the antioxidant status of seabass exposed to acrylonitrile is important to study its mechanism of toxicity and to predict the potential damage in the organism. Also, it is very important to know the potential mechanisms of detoxification in the decontamination phase where fish were maintained for 7 days in clean seawater free of acrylonitrile.

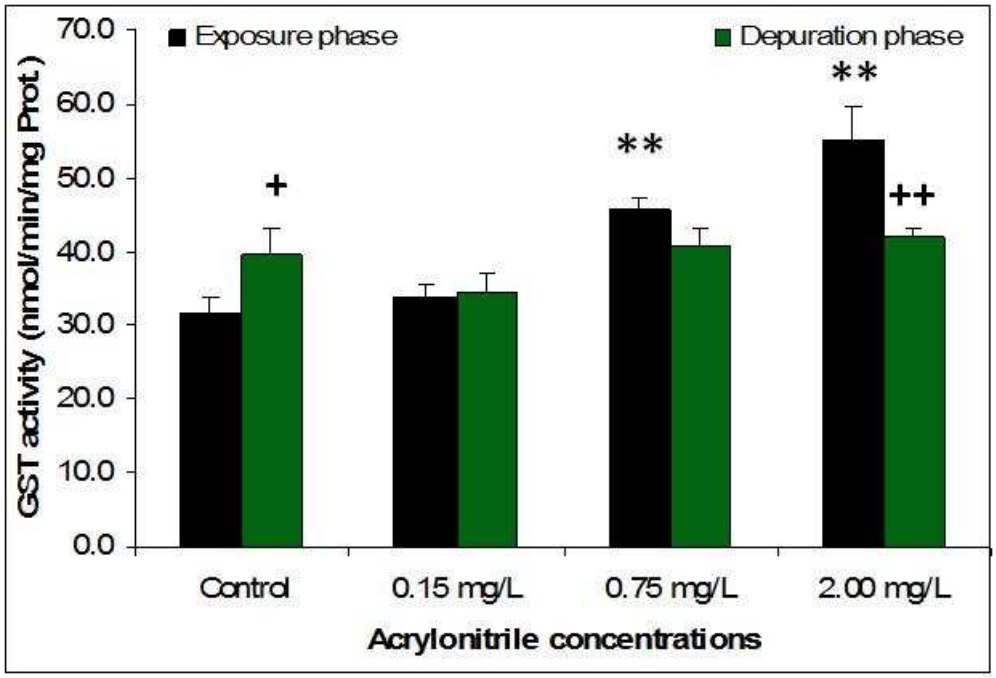
The activity of CAT, GST, SOD, LPO, and EROD in seabass liver, for the acrylonitrile exposure phase and subsequent depuration period, is displayed in Fig. 3.

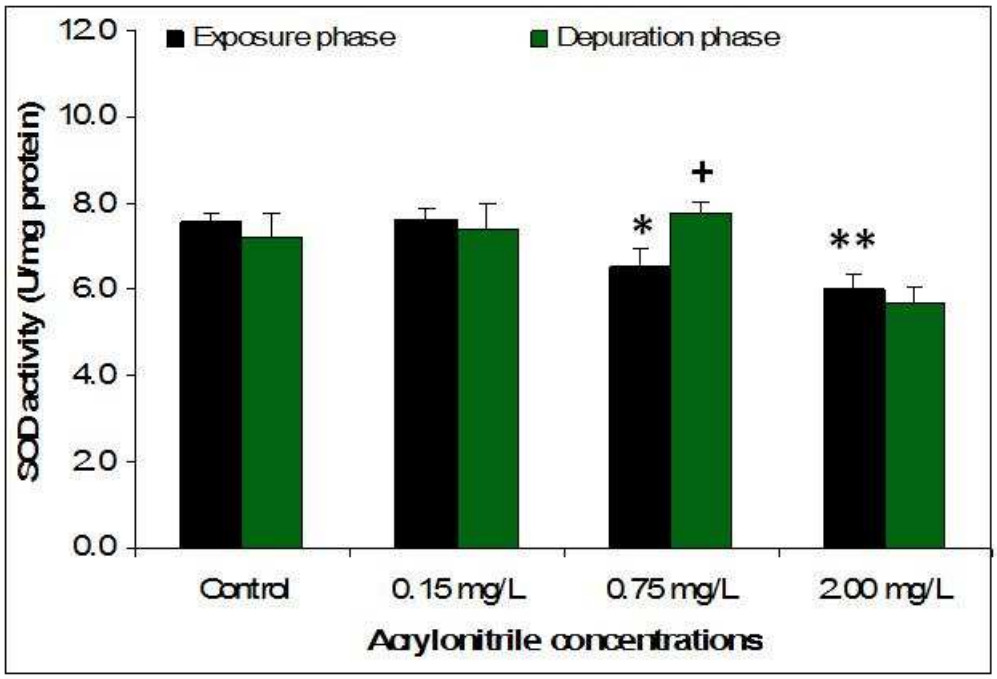
In the exposure phase, a significant induction of CAT and GST ($p<0.01$) was recorded on the highest acrylonitrile concentration (2mg/l for CAT and 0.75 and 2mg/l for GST). An inhibition of SOD activity was obtained for the two highest acrylonitrile concentrations ($p<0.05$ and $p<0.01$ respectively). for the LPO and EROD, no significant differences were observed between exposed and control groups, despite a decrease on the LPO levels was register for the highest acrylonitrile concentrations but not statistically significant.

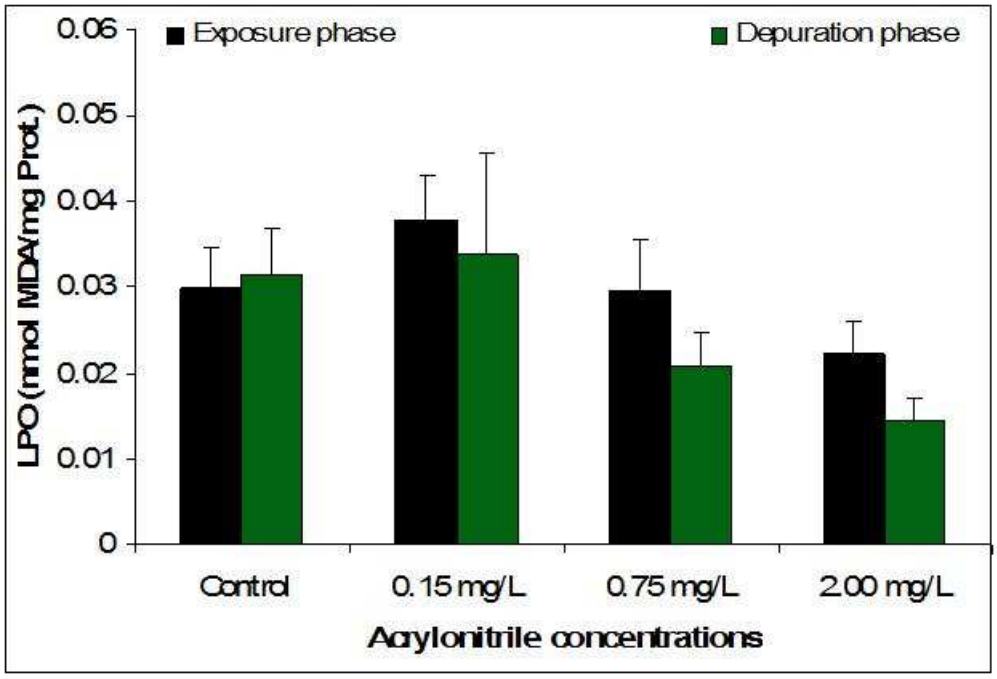
After 7 days of depuration, no significant changes were detected between the depurate acrylonitrile groups and control, and therefore all the activities of the tested enzymes in the depurated acrylonitrile groups were reduced to control levels. In the case

of CAT and GST activities, which were significantly induced in the exposure phase, a significant reduction was also recorded in some acrylonitrile groups of the depuration phase comparatively with the same acrytonitrile groups of the exposure phase (figure 3).






Exposure phase
Depuration phase
SOD activity (U/mg protein)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
*
+
**
Control
0.15 mg/L
0.75 mg/L
2.00 mg/L
Acrylonitrile concentrations


Exposure phase
Depuration phase
LPO (nmol MDA/mg Prot.)
0.06
0.05
0.04
0.03
0.02
0.01
0
Control
0.15 mg/L
0.75 mg/L
2.00 mg/L
Acrylonitrile concentrations

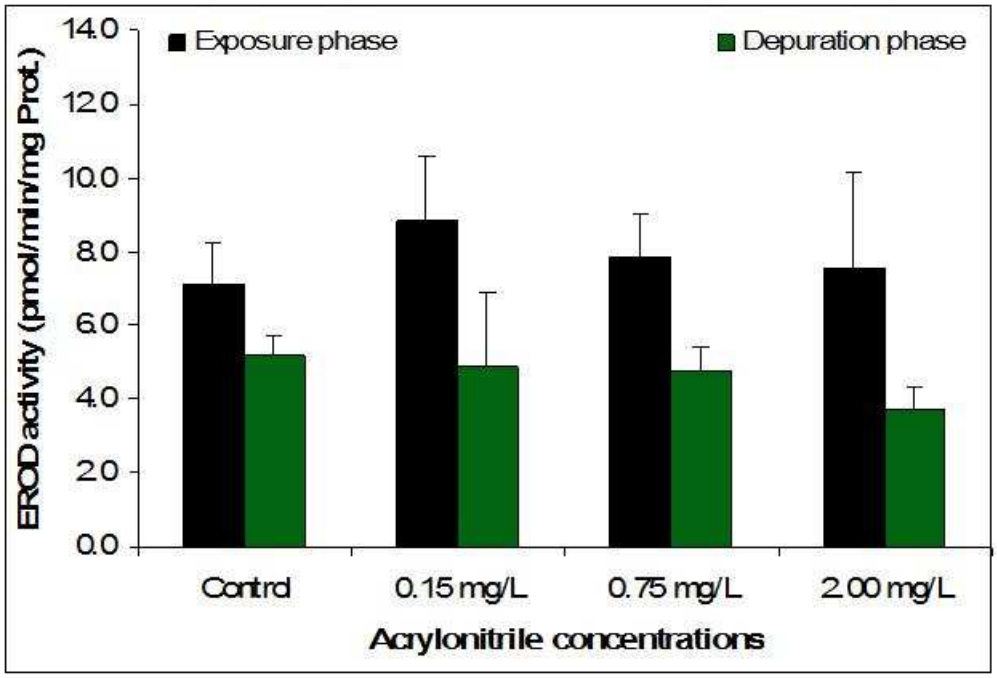


Figure 3 - Activity of the enzymes Catalase (CAT), Glutathione S-transferases (GST), Superoxide dismutase (SOD), lipid peroxidation (LPO) and Hepatic ethoxyresorufin O-deethylase (EROD), for the treatments control, 0.15, 0.75 and 2 mg/l acrylonitrile concentrations during the 15 days of exposure (■ Exposure phase) plus 7 days of depuration (■Depuration phase). Error bars indicate the standard errors; asterisks indicates differences from control of exposure phase: * - $p < 0.05$ and ** - $p < 0.01$, and plus indicate differences between exposure and depuration phase for each acrylonitile concentration: ++ $p < 0.01$.

4. Discussion

Acrylonitrile has been found to contaminate the environment due to accidental spills, or from industrial waste discharge (Watcharasit *et al*., 2009). Because of the large amounts of acrylonitrile transported by sea and its relatively high water solubility, this HNS is of high hazard to aquatic organisms. Acrylonitrile, is also a potent toxicant that can induce oxidative stress, immunotoxicity and neurotoxicity in mammalians (Gagnaire *et al*., 1998; Hamada *et al*., 1998; Watcharasit *et al*., 2009). Additionally, this HNS has been described to be mutagenic, teratogenic and carcinogenic in rodents after chronic exposure (Cole *et al*., 2008; Watcharasit *et al*., 2009) being classified by EPA as a environmental priority pollutant (Keith and Telliard 1979).

There are a considerately body of work that study the acrylonitrile toxicokinetic in mammalians, mainly in rodents and humans (Jiang *et al*., 1998; Whysner *et al*., 1998; IARC, 1999; Zhang *et al*., 2002; Chanas *et al*., 2003; Cole *et al*., 2008; Watcharasit *et al*., 2009). However, the current knowledge about the effects of acrylonitrile on marine

organisms is scarce, and also none is known about mechanism of acrylonitrile toxicity in fish. Therefore, the study here presented will be very important to predict the chronic effects of acrylonitrile in marine environment, its decontamination kinetics and to investigate if acrylonitrile produce DNA damage and oxidative stress to marine organisms, as induce in mammalians.

The results of the present study indicate that acrylonitrile showed several toxicity effects to seabass. Some mortality were recorded in the two higher acrylonitrile concentrations (0.75 and 2mg/l) and the fish from the 2mg/l acrylonitrile concentration presented the skin extremely dark and low swimming capacity comparatively with fish from the other acrylonitrile concentrations showing indications of acrylonitrile toxicity. Also, important alterations in several parameters at sub-individual level (biochemical markers) were observed, that were recovered after seven days of depuration.

In liver, acrylonitrile significantly induced the activity of Catalase (CAT) and Glutathione S-transferase (GST), and the Superoxide dismutase (SOD) activity was found to be significantly inhibited. Also, no significant effects were found on Lipid peroxidation (LPO) levels. 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. CAT and GST are very responsive enzymes to increasing levels of contaminant stimulated reactive oxygen species (ROS) production. CAT, is one of the first lines of antioxidant defenses, and its induction could indicate the presence of H_2O_2 and that the generation of H_2O_2 are still within the CAT elimination capacity. GST, as a multicomponent enzyme is involved in the detoxification of many xenobiotics and play an important role in protecting tissues from oxidative stress. Thus, the increased activity of these two antioxidant enzymes may reflect an adaptation to the chronic exposure to acrylonitrile, since this would confer increased protection from oxidative stress. These findings suggest that in seabass liver, the acrylonitrile was not implicated on an overall increase in 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, 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 LPO and ROS, 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. In the present study, no oxidative stress was produced by acrylonitrile in seabass liver, in agreement with the findings obtained in rats exposed to this HNS (Jiang *et al*. 1998).

In this study, the comet assay results revealed a significant dose dependent increase in DNA strand breaks in blood cells of seabass exposed to acrylonitrile compared with control. These results indicate potential genotoxic effects of acrylonitrile on seabass. These results also showed that the comet assay is a rapid and sensitive way to assess DNA damage induced by HNS chemicals. A number of studies have been shown that during the oxidative process, the overproduction of ROS can induce several kind of negative effects including the DNA damage. However, the potential mechanism of acrylonitrile genotoxicity in seabass requires a more specific investigation.

In the depuration 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 such reasons (1) some of acrylonitrile which may had been accumulated in fish tissues (liver and blood) was released to water; (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 depuration phase would be related with compensatory mechanisms that repaired the DNA strand breaks produced by the exposure to acrylonitrile.

5. Conclusions

The data generated by this study gathered information on the chronic toxicity and decontamination kinetics of the priority HNS acrylonitrile to seabass. These data will improve our knowledge about the effects of HNS on marine organisms.The importance of undertaking this research is essentially to assist relevant bodies to predict the adverse effects of acrylonitrile in marine ecosystems if an accidental spill occurs.

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