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LONG-TERM EFFECTS OF HAZARDOUS AND NOXIOUS SUBSTANCES IN CARCINUS MAENAS

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LIST OF ABBREVIATIONS

Ach - acetylcholine

AChE – acetylcholinesterase

AL - aniline

AN - acrylonitrile

BHT - butylatedhydroxytoluene

CEO - cyanoethylene oxide

CDNB - 1-chloro-2,4-dinitrobenzene

DTNB - 5,5'-dithiobis-(2-nitrobenzoic acid)

DTT – dithiothreitol

EDTA - ethylenediaminetetraacetic

ERA - Environmental Risk Assessment

GESAMP - Group of Experts on the Scientific Aspects of Marine Environmental Protection

GR – glutathione reductase

GSH - reduced glutathione

GST - glutathione *S*-transferases

GSSG - oxidized glutathione

GPx - glutathione peroxidase

H_2O_2 - hydrogen peroxide

HNS - Hazardous and Noxious Substances

IDH - $NADP^+$-dependent isocitrate dehydrogenase

IBR - Integrated Biomarker Response

IMO - International Maritime Organization

LDH - lactate dehydrogenase

LOD - limit of detection

LPO - lipid peroxidation

MARPOL 73/78 Convention - International Convention for the Prevention of Pollution from Ships

MDA – malondialdehyde

MDI - methylene diphenyl diisocyanate

NAD^+ - oxidized form of nicotinamide adenine dinucleotide

NADH - reduced form of nicotinamide adenine dinucleotide

$NADP^+$ - oxidized form of nicotinamide adenine dinucleotide phosphate

NADPH - reduced nicotinamide adenine dinucleotide phosphate

NOEC – No-Observed Effect Concentration

OPRC Convention - International Convention on Oil Pollution Preparedness, Response and Co-operation

OPRC-HNS Protocol - Protocol on Preparedness, Response and Co-operation to Pollution Incidents by Hazardous and Noxious Substances

PDMS-DVB - polydimethylsiloxane-divinylbenzene

PEC - Predicted Environmental Concentration

PMSF - phenylmethanesulfonylfluoride

PNEC - Predicted No Effect Concentration

PTFE - polytetrafluoroethylene

ROS - reactive oxygen species

SEBC - Standard European Behavior Classification

SPME - solid phase microextraction

SOLAS Convention - Safety Of Life At Sea Convention

TBA - thiobarbituric acid

TBARS - thiobarbituric acid reactive substances

TCA - trichloroacetic acid

UNCLOS Convention - United Nations Convention on the Law of the Sea Convention

WWTPs - Wastewaters Treatment Plants

RESUMO

O transporte marítimo é considerado o meio de transporte mais eficaz e comum de grandes volumes de compostos químicos. Tendo em conta a contínua procura da indústria por compostos químicos, espera-se, que até 2015, o seu transporte por mar atinja aproximadamente 215 milhões de toneladas. Na União Europeia têm sido transportadas por via marítima cerca de 50 000 Substâncias Perigosas e Nocivas (HNS, Hazardous and Noxious Substances), das quais 2 000 são movimentadas regularmente. Entre o ano de 1987 e 2007, cerca de 100 incidentes com HNS foram registados em águas Europeias. No entanto, os derrames de HNS são pouco compreendidos e reconhecidos no que se refere aos seus potenciais perigos ambientais. Existe pouca informação relativamente ao seu comportamento ambiental, bem como aos efeitos toxicológicos induzidos em animais marinhos devido a uma exposição aguda ou crónica. Deste modo, este trabalho teve como objetivo investigar as respostas biológicas induzidas no caranguejo, *Carcinus maenas*, pela exposição de longa duração a acrilonitrilo e anilina, sendo estudada a influência da concentração e do tempo de exposição.

Carcinus maenas é um invertebrado importante em estuários e sistemas costeiros europeus, sendo reconhecido como um bom indicador de contaminação. O acrilonitrilo é um importante composto industrial usado na produção de plásticos, borracha sintética, sendo também detetado no fumo de cigarro e em canos de escape. A anilina é usada na síntese de químicos de fotografia, borracha, tintas, pesticidas e produtos farmacêuticos.

Para a realização dos ensaios, espécimes de caranguejos foram recolhidos no estuário do Rio Minho. Os caranguejos foram expostos, durante 21 dias, a acrilonitrilo (0, 100 e 1000 µg/L) e anilina (0, 5 e 50 µg/L). Aos 7, 14 e 21 dias foram isolados tecidos de caranguejo para a determinação de biomarcadores de neurotransmissão, metabolismo energético, biotransformação, defesas antioxidativas e stress oxidativo, relacionados com o metabolismo e modo de ação destas HNS.

Na exposição a acrilonitrilo (1000 µg/L) verificou-se uma inibição significativa da atividade da acetilcolinesterase e da lactato desidrogenase no músculo, indução da atividade das glutationas *S*-transferase e inibição da glutationa peroxidase na glândula digestiva. A exposição a anilina (50 µg/L), por sua vez, levou a alterações na atividade da acetilcolinesterase no gânglio (inibição aos 14 dias e aumento da atividade aos 21 dias, relativamente aos controlos), diminuição da produção energética (na atividade da lactato desidrogenase e na da isocitrato desidrogenase dependente de $NADP^+$) e nos níveis de peroxidação lipídica. Os dados estão de acordo com uma atividade biológica global inferior, espectável devido à ação deste HNS como narcótico polar. No geral, foram observadas

alterações aos 7 dias após a exposição de *C. maenas* a acrilonitrilo ou anilina. Para ambos os compostos, os caranguejos pareceram conseguir adaptar-se à exposição de longa duração, recuperando até níveis semelhantes ao controlo, aos 21 dias. Este trabalho realça a importância dos estudos de toxicidade crónica com o objetivo de compreender os efeitos tóxicos induzidos pelas HNS, principalmente em animais marinhos, de forma a produzir um maior conhecimento científico, auxiliando na preparação de planos de contingência e cálculos para avaliação de risco ambiental.

ABSTRACT

Shipping is considered the most effective and common mean of transportation of high volumes of chemicals. Due to the continuous chemical demand, maritime transportation is expected to have reached around 215 million tonnes of chemicals transported worldwide by 2015. In European Union, 50 000 Hazardous and Noxious Substances (HNS) have been carried by sea up-to-date, of which 2 000 are carried on a regular basis. Between 1987 and 2007, about 100 incidents involving HNS were reported in European waters. However, HNS spills are poorly understood and recognized regarding their environmental hazards. There is a lack of information considering their fate and behavior, as well as their effects on marine biota due to acute or chronic exposure. This study aims therefore to investigate the biological responses induced by a long-term exposure to acrylonitrile and aniline in *Carcinus maenas*, the influence of concentration and duration of exposure being assessed.

Carcinus maenas is a key invertebrate of European estuaries and coastal systems, acknowledged as good indicator of contamination. Acrylonitrile is an important industrial chemical used in the production of plastics, synthetic rubber, also detected in cigarette smoke and auto-exhaust. Aniline is employed in the synthesis of photographic chemicals, rubber, dyes, pesticides or pharmaceuticals.

For both bioassays, specimens of male crabs were collected in Minho River estuary. The crabs were exposed to acrylonitrile (0, 100 and 1000 µg/L) and aniline (0, 5 and 50 µg/L) for a period of 21 days. At 7, 14 and 21 days crab tissues were isolated to determine biomarkers of neurotransmission, energy metabolism, biotransformation, antioxidant defenses and oxidative stress related to metabolism and modes of action of these HNS.

Acrylonitrile exposure (1000 µg/L) caused significant inhibition in the activity of acetylcholinesterase and lactate dehydrogenase in muscle, induction of glutathione *S*-transferases and inhibition of glutathione peroxidase in digestive gland. The responses observed suggest that low oxidative metabolism of AN may have occurred. Aniline (50 µg/L) was shown to significantly alter the activity of acetylcholinesterase in the ganglion (with inhibition at 14 days and increased activity at 21 days, relative to controls), decrease activity of energy production enzymes (lactate dehydrogenase and $NADP^+$-dependent isocitrate dehydrogenase) and lipid peroxidation levels. The data were in agreement with lower overall biological activity expected to be caused by this polar narcotic. In general, early alterations (7 days) were observed after *C. maenas* exposure to acrylonitrile or aniline. For both contaminants, crabs seem to be able to adapt to long-term exposure recovering to control levels at 21 days.

This study highlights the importance of long-term toxicological studies aiming at understanding the effects of HNS on marine animals, in order to produce more thorough knowledge supporting the development of contingency plans and calculations in environmental risk assessment.

COMMUNICATIONS

Within the scope of the work developed for this thesis the following communication has been *submitted* and accepted for presentation in one national scientific meeting.

Abreu, I.O., Monteiro, C., Rocha, A.C., Ferreira, M., Guimarães, L., Reis-Henriques, M.A. 2015. Long-term exposure of *Carcinus maenas* to acrylonitrile. Workshop ECORISK, under the theme “Monitorização e avaliação de risco ecológico de derrames químicos e contaminação ambiental por substâncias químicas: óleos, substâncias perigosas e nocivas, contaminantes clássicos e emergentes”. Porto (Portugal), 12th June. (Poster presentation)

I - GENERAL INTRODUCTION

I.1 - Hazardous and Noxious Substances

Over the years the increase in human activities has been causing a continuous contamination of marine environments (Cajaraville *et al.*, 2000). Agriculture, industries (Islam and Tanaka, 2004; Schwarzenbach *et al.*, 2006) and wastewaters from rural and urban areas (Islam and Tanaka, 2004; Schwarzenbach *et al.*, 2006; CEDRE, 2012) are among the major sources of marine contamination. Maritime transportation of chemical substances constitutes another relevant source of pollution (Rocha *et al.*, *submitted*).

Along with the development of chemical industries, a need for transportation of large quantities of chemical products has risen (HASREP, 2005). Shipping has been considered the most effective mean of transportation for high volumes of chemicals, therefore becoming the most common, especially for long distances (CEDRE, 2012). Nowadays, this industry covers more than 90% of the global trade (MKC, 2012). Europe, the United States, Japan and China are the main exporters and importers of chemical products (CEDRE, 2012).

Oil and chemical tankers represent a major risk to the aquatic environment and human health (Neuparth *et al.*, 2013). Increasing global demand for oils and other chemical substances (Radovic *et al.*, 2012) has led to increasing maritime traffic (Rocha *et al.*, *submitted*), especially in some areas such as China (Woolgar, 2008) and Europe, which enhance the risk of accidental spills (Kirby and Law, 2010).

Over the last decade, the number of oil spills has diminished especially due to the effectiveness of stringent regulations in marine transportation safety (Radovic *et al.*, 2012). Nevertheless, some high profile incidents (Neuparth *et al.*, 2012) and small spills continue to occur (Kirby and Law, 2010), including during the transfer to and from transport tankers in harbors. In order to tackle this serious problem, international and national authorities have instituted several measures to prevent and mitigate environmental and economic consequences which are presented in the International Convention on Oil Pollution Preparedness, Response and Co-operation (OPRC Convention) (Singhota, 1995). However, other chemical substances also transported by sea in large quantities can constitute a similar or greater risk and induce equal or more severe ecological effects as oils (Neuparth *et al.*, 2012). Thus, a new concept, “Hazardous and Noxious Substances” (HNS), was created (CEDRE, 2012). With the aim of improving the response to HNS spillage, the Protocol on Preparedness, Response and Co-operation to Pollution Incidents by Hazardous and Noxious Substances (OPRC-HNS Protocol) came into force in 2007 (Neuparth *et al.*, 2012). According to this protocol, 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 marine life, to damage amenities or to interfere with

other legitimate uses of the sea" (IMO, 2000). This definition covers from vegetable oils, used in food and pharmaceutical industries, to highly toxic compounds, such as corrosive gases, acids and alkalis and harmful volatile organic compounds (Harold *et al.*, 2014), highlighting the diversity of chemicals included in this group of substances.

Contrary to oil spills, HNS spills are less understood and recognized regarding their ecological hazards (Neuparth *et al.*, 2011; Cunha *et al.*, 2014). There is a lack of documentation considering their effects on marine biota, and the few ecotoxicological studies available come, in its majority, from experiments using freshwater animals(Neuparth *et al.*, 2012). Also, in most cases of HNS incidents, no monitoring programmes were performed following this disturbance (Purnell, 2009). This lack of information makes it difficult to extrapolate ecotoxicological data to marine animals, to develop appropriate contingency plans, and establish compensations for loss of biodiversity and natural capital when accidents occur (Purnell, 2009; Neuparth *et al.*, 2011).

Due to the inherent risk of HNS shipping, regulations were thus created to prevent and minimize the risks of contamination by these products. The OPRC-HNS Protocol was adopted by the International Maritime Organization (IMO). This organization was created in 1948, and is tasked with regulating marine safety on a global scale. The IMO has the following aims: 1) prevention of contamination of the marine environment, due to port facilities and shipping; 2) creation and adoption of safety standards related to shipping; and 3) to incentivize the collaboration between Member States, on maritime regulations (CEDRE, 2012).

Working with IMO, there is the Group of Experts on the Scientific Aspects of Marine Environmental Protection (GESAMP) which is responsible for creating chemical profiles which are based on the persistence of the pollutant in the environment, its aquatic ecotoxicity, mammalian toxicity, effects on human health and interference with uses of the sea (CEDRE, 2012).

Currently, 3 main international regulations are in effect:

1) the Safety Of Life At Sea Convention (SOLAS Convention), which started in 1980, with the aim of preventing accidents and guaranteeing the human life safety at sea (CEDRE, 2012);
2) the International Convention for the Prevention of Pollution from Ships (MARPOL 73/78 Convention), which started in 1983 and has a more specific aim: to prevent the pollution caused by ships (CEDRE, 2012) from operational or accidental causes (EMSA, 2007);

3) the United Nations Convention on the Law of the Sea Convention (UNCLOS Convention) which started in 1994 and establishes the guiding principles of international law on the boundaries defining maritime areas and the main rules applicable in the world's oceans (CEDRE, 2012).

Nevertheless, despite this effort in regulating and minimizing seaborne trade of chemicals, regulations based in accurate standard guideline values, inferred from effects that may be caused by concentrations that may occur in environmental matrices, are still in need to guarantee the protection of marine ecosystems. For that, more research must be pursued.

I.1.1 - HNS cargoes and its spillage into the marine environment

HNS cargoes can be transported in four different kinds of ships especially designed for those types of chemicals. These are: (1) bulk carriers, that carry solids in bulk as unpackaged dry cargoes (such as iron ore); (2) chemical tankers, that carry bulk liquid cargoes (such as benzene); (3) gas carriers, that carry cargoes of liquefied gas under pressure and/or reduced temperatures (such as methane); (4) container ships, that carry cargoes of packaged goods in intermodal containers which allow an efficient loading and unloading (CEDRE, 2012; ITOPF, 2011). Annually, a bulk trade of 165 million tonnes of chemicals is transported worldwide, and it is estimated that, by 2015, this value will rise to 215 million tonnes (Purnell, 2009). In European Union, 90% of the external trade is done by sea and up to date, around 50 000 HNS are carried by sea, within which 2 000 are carried on a regular basis (Purnell, 2009).

In 2007, it was estimated that a total of 155.6 million tonnes of liquid goods were transported. Petrochemical products (50%), such as benzene, and both animal and vegetal oils and fats (30%) were among those HNS followed by inorganic substances (10%), such as mineral salts and most ores, and a separated category of indiscriminate products (10%) (CEDRE, 2012). Vegetable oils, sulphuric acid, iron ore or fertilizers are among the substances most often spilt into the marine environment (CEDRE, 2012). Some reasons for these spills are presented in Figure 1.

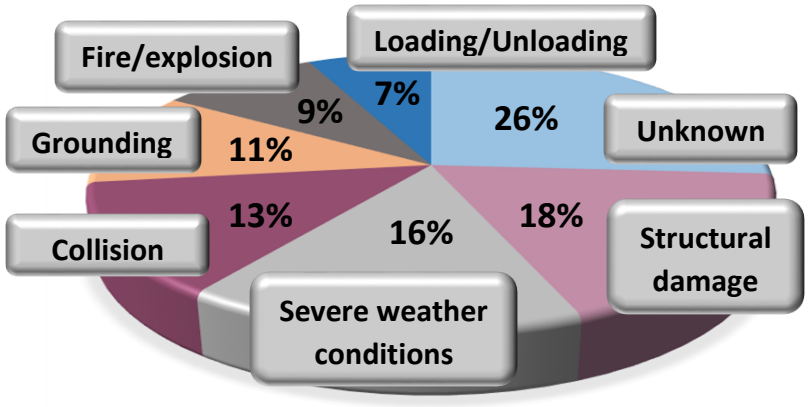

Figure 1: Causes of ships-source accidents involving HNS worldwide (between 1917 and 2010) (adapted from CEDRE, 2012).

Between 1987 and 2007, around 100 incidents involving HNS have been reported in European waters (CEDRE, 2009). Incidents such as Cason (1987, Spain) (Mamaca *et al.*, 2009), Anna Broere (1988, Netherlands), Alessandro Primo (1991, Italy), or more recently Ievoli Sun (2000, United Kingdom), Ece (2006, France), and MSC Napoli (2007, United Kingdom) (Neuparth *et al.*, 2011), are some examples. In addition, minor incidents or HNS discharges occur every year (Harold *et al.*, 2014). The knowledge collected upon the mentioned accidental spills allowed to understand some of the effects of the chemicals liberated to sea. This information is vital for the development of mitigation programs which could be applied upon the occurrence of another similar incident. However, the lack of monitoring programmes and established guidelines, together with the vast variety of substances with distinct physico-chemical properties belonging to HNS, are the main reasons for the weak preparedness and response after HNS spills (Kirby and Law, 2010; Neuparth *et al.*, 2011).

I.1.2 - HNS environmental behavior and impact

An important aspect regarding the spillage of chemicals in the marine environment is their behavior once released. Understanding their fate in the environment can be a useful tool for the development of a response strategy. The fate of a substance is based on its properties of volatility, solubility and density, which can be used to infer on its hazardousness (ITOPF, 2011). According to the Standard European Behavior Classification (SEBC) system, HNS are classified into 12 groups, considering their behavior in water (CEDRE, 2012; ITOPF, 2011, Figure 2). This variety makes it difficult to predict possible adverse effects as well as establish contingency plans (Rocha *et al.*, *submitted*). Also, other physico-chemical properties, besides the previously mentioned, can influence the chemical behavior, such as flammability, explosivity or oxidizing/corrosive hazard (ITOPF, 2011).

It has been previously shown that the presence of HNS in the environment represents a serious threat since these chemicals can induce toxic effects to animals. For instance, damage to internal organs, skeletal deformities (EMSA, 2007), alterations of biological responses or behavioral changes that could include reduction in reproductive success, feeding or social behaviors (Monserrat *et al.*, 2007; Neuparth *et al.*, 2012; 2014; Santos *et al.*, 2015), have been reported in literature. Depending on the persistence of HNS in marine environment, the adverse effects induced can be manifested not only at an individual level but also at population or community level (EMSA, 2007; Neuparth *et al.*, 2014; Santos *et al.*, 2015).

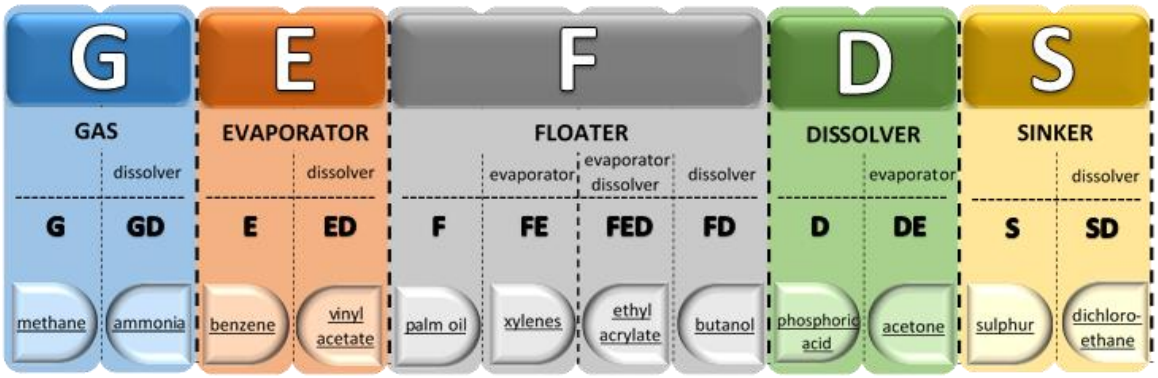

Figure 2: Classification of HNS according to Standard European Behavior Classification (SEBC) system with some examples for each group (adapted from CEDRE, 2012).

After a spill, HNS can disperse within a short period of time due to high dilution rates, especially for those which are categorized as dissolvers or evaporators. The concentration of a spilled substance tends to decrease gradually to a level at which lethal effects are not observed. Nevertheless, the presence of a contaminant even at low concentrations may still produce toxic sub-lethal effects in an extended area (EMSA, 2007). Therefore, there is a necessity to develop experiments in order to obtain information on HNS fate and behavior as well as the effects caused by acute and chronic exposure to these chemicals (Neuparth *et al.*, 2012).

Representative species of key marine and estuarine taxa of seawater and shoreline environments must be researched so that more reliable knowledge is produced regarding the toxicity of HNS towards these ecosystems (Neuparth *et al.*, 2012). The information obtained will support risk assessment of these substances to the coastal and estuarine environment, as well as the establishment of more effective preparedness and response measures to this sort of incidents.

Both short and long-term exposures are crucial to determine detrimental effects of a contaminant in an animal. However, studies conducted under this scope have been mainly focused on freshwater species, acute exposure and assessment of mortality as an endpoint

(Rocha *et al.*, *submitted*). Long-term exposure studies allow to determine sub-lethal effects potentially caused by exposure of animals to low concentrations of a toxic substance representing nowadays a more realistic and relevant scenario for hazard assessment. Two prioritized HNS for which the impact of long-term exposure to marine animals is still little understood are acrylonitrile (AN) and aniline (AL) (Neuparth *et al.*, 2011; 2012; Rocha *et al.*, *submitted*).

Acrylonitrile

Acrylonitrile (CAS No. 107-13-11) (Cole *et al.*, 2008) is a vinyl monomer (Campian *et al.*, 2002; Campian and Benz, 2008), and it is known by several names: acrylic acid nitrile, acrylon, carbacryl, cyanoethylene, fumigrain, propenenitrile, 2-propenenitrile, propenoic acid nitrile, propylene nitrile, ventox and vinyl cyanide (WHO, 2002, Figure 3).

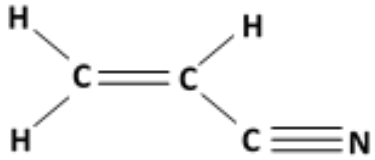

Figure 3: Chemical structure of acrylonitrile (adapted from WHO, 2002).

This man-made chemical is a volatile and flammable colorless liquid, at room temperature (WHO, 2002), with an onion or garlic-like odor (ATSDR, 1990; Smedt *et al.*, 2014). Its vapors are heavier than air, traveling long distances in the ground (Smedt *et al.*, 2014). It is a polar molecule due to the presence of a cyano group, being soluble in water and miscible with most organic solvents (WHO, 2002). Acrylonitrile is considered to be a dissolver/evaporater (Rocha *et al.*, *submitted*). Its physical and chemical properties are presented in table 1.

Table 1: Physical and chemical properties of acrylonitrile (adapted from WHO, 2002 and EURAR, 2004a).

Property	***Mean / Range***
Density (at 20°C; g/L)	806
Melting point (°C)	83.55 ± 0.5
Boiling point (at 1013 hPa; °C)	77.3
Vapour pressure (at 25°C; kPa)	11
Henry's Law Constant (at 25°C; Pa.m^3/mol)	11
Surface Tension (at 24°C; mN/m)	27.3
Water solubility (at 25°C; g/L)	75.1

Autoflammability (°C)	481
log Octanol/Water Partition Coefficient (log Kow)	0.25
log Organic Carbon/Water Partition Coefficient (log Koc)	1.06
log bioconcentration factor (log BCF) in fish	0.48 – 1.68
Half-life ($t_{1/2}$) in water (hours)	170

Acrylonitrile is an important industrial chemical used in the production of acrylic fibers, plastics, synthetic rubber, acrylamide (Campain and Benz, 2008; Cole *et al.*, 2008), resins (Cole *et al.*, 2008), dyes, pharmaceuticals and surface agents (Rocha *et al.*, *submitted*). It has also been detected in cigarrete smoke and auto exhaust (Byrd *et al.*, 1990). This chemical has been produced since 1940 (Cole *et al.*, 2008).

Due to the global economic recession, AN has experienced some decreases on its demand. However, since 2013 its average demand rate has been recovering, and it is expected a growth on its demand of about 2.6%, from 2013 to 2018 (IHS, 2014). In 2013, the global consumption of AN was led by China, followed by the Western Europe and the United States. The acrylic fibres are the main reason for its demand and responsible for 36% of world acrylonitrile consumption (IHS, 2014).

Acrylonitrile is also considered a highly toxic chemical (Gut *et al.*, 1981) that can be found easily in the air due to its rapid volatilization. However, industries and wastewater disposal can also be a considerable contribution to water and soil contamination. This chemical breaks down quickly in the air, contrary to what happens in water, where it can take 1 to 2 weeks varying with water conditions and on the quantity of chemical entering the system (ASTDR, 1990). It is also known that, via cytochrome P450, this chemical undergoes an oxidative biotransformation, generating cyanoethylene oxide (CEO) and, subsequently, cyanide (Abreu *et al.*, 1980; Gut *et al.*, 1984; Carrera *et al.*, 2007) that contribute to AN toxicity, since cyanide can easily diffuse through all body cells and interfere with its ability to use molecular oxygen (CEPA, 2000).

A wide range of concentrations of AN have been detected in the environment. According to the European Union Risk Assessment Report, concentration in surface water, groundwater and drinking water of 2.37×10^{-3} µg/L, 0.3×10^{-3} µg/L and 2.37×10^{-3} µg/L, respectively, were observed (EURAR, 2004a). Concentrations ranging from 0 to 4300 µg/L have also been detected in the vicinity of production or processing facilities. For instance, in the United Kingdom, a facility of acrylic fibres discharged into an estuary a concentration of acrylonitrile of 35 000 µg/L (EURAR, 2004a). Monitoring data indicates however that generally the concentration of AN in water is below the limit of detection (LOD) of 1 to 2 µg/L unless there is an accidental spill or inappropriate disposal (EURAR, 2004a). This

compound was included in a list of 23 priority HNS set by Neuparth *et al.* (2011) and it is considered a priority pollutant in the United States, Canada, Netherlands, Germany and China (Keith and Telliard, 1979; Tong *et al.*, 1999). This chemical is highly transported in European waters, occupying the 25th position in the traffic ranking of the 100 most transported chemicals in Atlantic European waters (HASREP, 2005).

Guideline values have been calculated and established in the European Union (EURAR, 2004a), e.g. the regional and continental Predicted Environmental Concentration (PEC), 2.81 and 0.41 µg/L, respectively, and the Predicted No Effect Concentration (PNEC) in water, 17 µg/L, which was based on the No-Observed Effect Concentration (NOEC) value (0.17 mg/L) found for *Pimephales promelas* exposed to AN for 30 days. This last value is however based on data regarding freshwater animals. The extrapolation of freshwater data to marine animals might not be accurate as the animals are adapted to different conditions and are likely to present different responses to contamination (Rocha *et al.*, *submitted*).

Aniline

Aniline (CAS No. 62-53-3) is a transparent to slightly yellow liquid (BASF, 2012), at room temperature, that darkens to brown to air or light exposure (EPA, 1994; Kahl *et al.*, 2005, Figure 4). It has a fishy odor (BASF, 2012) and burning taste (US EPA, 1985), and it occurs naturally in some foods, such as corn, grain, beans or tea (EPA, 1994). It is moderately soluble in water and miscible with most organic solvents (Kahl *et al.*, 2005).

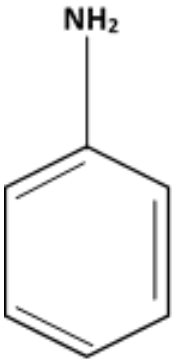

Figure 4: Chemical structure of aniline (adapted from EURAR, 2004b).

This compound is also known as aminobenzene, aminophen, benzenamine or phenylamine (Kahl *et al.*, 2005; Patnaik, 2007), and it is considered to be a dissolver (Santos *et al.*, 2015). According to Neuparth *et al.* (2011), this chemical takes part of a list of prioritized HNS established for European waters. AL physical and chemical properties are presented in table 2.

Table 2: Physical and chemical properties of aniline (adapted from EURAR, 2004b).

Property	***Mean***
Density (at 20°C, g/L)	1 022
Melting point (°C)	- 6.2
Boiling point (at 1013 hPa; °C)	184.4
Vapour pressure (at 20°C; hPa)	0.4
Henry's Law Constant (Pa.m^3/mol)	0.106
Surface tension (at 20°C; mN/m)	70.5
Water solubility (at 20°C; g/L)	35
Autoflammability	No flammable
log Octanol/Water Partition Coefficient (log Kow)	0.9
log Organic Carbon/Water Partition Coefficient (log Koc)	1.41
log bioconcentration factor (log BCF) in fish	2.6
Half-life ($t_{1/2}$) in water (hours)	360

Aniline is mainly used as a chemical intermediate, within which 76% is processed to 4,4'-methylenedialinine, the starting material of polyurethane plastics (CSTEE, 2003; EURAR, 2004b). Additionally, it is used in the synthesis of several products such as photographic chemicals, rubber (Patnaik, 2007), dyes, pesticides, coal liquefaction (Santos *et al.*, 2015; Patnaik, 2007), pharmaceuticals, resins, varnishes, perfumes, shoe blacks (EPA, 1994; Patnaik, 2007).

From 2009 to 2014, AL world consumption grew annually, at an average rate of nearly 7%. This increase in consumption was related to the growth in methylene diphenyl diisocyanate (MDI) production, which accounted for over 81% of world AL consumption in 2014 (IHS, 2015). Between 2014 and 2019, the estimates predict that the Middle East and China will experience the largest increases for AL demand (annually growth rates >10%), followed by the United States (4.6%), India (3.6%) and Western Europe (2.7%) (IHS, 2015).

Aniline is expected to be low volatile from aqueous solutions, bind to humic substances present in these solutions or in sediments/soils and suffer photolysis in surface waters (EURAR, 2004b). Tests in Wastewaters Treatment Plants (WWTPs) demonstrated that this chemical is readily biodegradable in water, under aerobic conditions, contrary to under anaerobic conditions, where it was found to be not biodegradable (EURAR, 2004b).

Aniline usually is cleared from the body through a combination of acetylation and hydroxylation reactions, originating acetanilide and 2- or 4-aminophenol, respectively. The N-acetylation of AL is catalyzed by hepatic N-acetyl-transferase, while the N-hydroxylation involves the cytochrome P450 enzyme system, producing N-phenylhydroxylamine

(EURAR, 2004b). The latter reaction is considered to be the principal route by which this chemical exert its toxicity (CSTEE, 2003; EURAR, 2004b). The conjugates resulting from the glucuronidation and sulfation of 4-hydroxyacetanilide represents the major urinary metabolites of AL (CSTEE, 2003).

Available information suggests three sources of occupational exposure: chemical industry, release of AL as a decomposition product during thermal degradation of plastics or use of products with residual AL, such as dyes or adhesives (EURAR, 2004b). AL is accidentally released into the environment through its principal sources of production, such as in plant protection agents or rubber industries, where this chemical is formed as a degradation product, or after thermal degradation of MDI-based polyurethane. In addition, this chemical has been detected in effluents of coal carbonization plants, ranging from 0.48 to 21 mg/L (EURAR, 2004b). In WWTPs, around 87% of AL is removed by biodegradation. However, this chemical can still to be found in the environment. For instance, the local PEC for water was established as ranging from < 0.14 to 590 µg/L, from sites that emit to rivers, and < 0.15 to 920 µg/L, from sites that emit into sea and estuaries (EURAR, 2004b).

I.2 - Estuarine systems

Estuaries, also known as transition zones or ecotones (Kennish, 2002), have been defined as a semi-enclosed and tidal coastal body of water, establishing a connection with the open sea, allowing the dilution of seawater in freshwater (Pritchard, 1967). This dilution has a strong influence on the physical and chemical dynamics, as well as ecology, of these ecosystems. Additionally, the water exchange controls the salinity gradients and stratification, and it is responsible for the transportation of silt, organic material and inorganic nutrients into estuaries (Flindt *et al.*, 1999).

These ecosystems are among the most important of the coastal zone and the most productive areas on Earth (Kennish, 2002). They are extremely important to life history and development of many aquatic animals, such as rearing, feeding, migration routes and nursery grounds (Chapman and Wang, 2001). Besides, estuaries are also characterized for sediment transfer between marine and fluvial systems (Ridgway and Shimmield, 2002).

Due to their geographical location and ecological richness, estuaries constitute desirable areas to establish industries, harbors and urban or recreational areas (Ridgway and Shimmield, 2002). However, this leads to alterations in natural habitats, changes in the structure and dynamics of biotic communities and contamination (Kennish, 2002) by HNS and other toxicants, diminishing water quality and resources (Kennish, 2001).

I.2.1 - *Carcinus maenas*: the study organism

Carcinus maenas, known as the European green crab (Klassen and Locke, 2007), is an ecologically important animal of European estuaries and coastal ecosystems (Mesquita *et al.*, 2011; Rodrigues *et al.*, 2015). This species can also be found in Africa, Australia and in the Pacific Coast of North America, where it is considered a non-indigenous invasive species. *C. maenas* is an epibenthic euryhaline animal (Crothers, 1968), which inhabits both estuaries and intertidal and subtidal zones (Mesquita, 2010), where it occupies a key position in food webs (Mesquita *et al.*, 2011).

This species belongs to the Class Malacostraca, Order Decapoda and Family Portunidae. It is characterized for having a medium size (broader than long), reaching a maximum of 9-10 cm of carapace width. It lives up for 4-7 years (Klassen and Locke, 2007). Males and females are dimorphic and can be easily distinguished through the abdomen shape: in males it is triangular, with five segments, contrary to females in which the abdomen is broad and round with seven segments (Figure 5). Also, both have pleopods under the abdomen, however the female presents a higher number of these appendixes in order to support the eggs during its transportation (Crothers, 1967).

Figure 5: Dorsal and ventral aspects of a *Carcinus maenas* male.

Crabs mate during summer after females molt (Broekhuysen, 1936; Klassen and Locke, 2007). Following fertilization, the females bury themselves in the sand in order to protect the eggs. Each female can release 185 000 eggs at a time (Klassen and Locke, 2007). The eggs take several months to hatch. *C. maenas* has 3 different stages in its larval period: protozoea, zoea and megalopa (Lebour, 1928), lasting about 50 days to develop from the first stage to the last one (Klassen and Locke, 2007). After hatching, the egg gives therefore origin to a protozoea, also called the first zoea. This animal has maxillipeds, which help the larvae to swim. Later in the development, the zoea gives origin to the megalopa

(Crothers, 1967).

From zoea stage until megalopae this species is temporary planktonic and migrate vertically, a process endogenously controlled (Queiroga *et al.*, 1997). Therefore, during these stages, crab larvae rise to the surface in the evening to feed and sink during the day (Crothers, 1967). This process of migration helps the larvae to move from areas with higher to lower salinity (in the stage of zoea and megalopae, respectively) (Klassen and Locke, 2007). At the stage of megalopa, it starts to look more like a crab (Crothers, 1967) and it migrates to estuaries (where the salinity is lower) to settle and metamorphose, originating a juvenile crab (Crothers, 1967; Queiroga, 1998).

The crabs are characterized by its rigid skeleton, which provides them support and protection. Nevertheless, at its full hardness, the skeleton cannot change its shape, so it becomes no longer fitting since the animal cannot grow. Consequently, the animal loses its skeleton in a process called molt which will allow it to grow before the formation of a new skeleton. More specifically, when close to molting, the animal begins to create a new cuticle under the oldest. Once out of the old skeleton, the increase in size is rapid through water absorption. Thereafter, the new cuticle starts to harden (Crothers, 1967). *C. maenas* molts approximately 18 times during its life cycle, after its first crab stage (Crothers, 1967; Mesquita *et al.*, 2011). This process could take from 3 to 16 days (Broekhuysen, 1936), depending on the size of the animal, temperature, pH and Ca^{2+} concentration in the water. During the process of hardening, the crab is unable to feed, defend itself and hardly moves, becoming an easy target (Crothers, 1967).

C. maenas has been extensively used in laboratory and field studies aiming to assess the adverse effects caused by contaminants (Mesquita *et al.*, 2011; Rodrigues *et al.*, 2015) mainly due to its sensitivity to a broad range of aquatic pollutants (Rodrigues *et al.*, 2014a; Rodrigues and Pardal, 2014). This species is acknowledged as a good biological indicator, reflecting the levels of environmental contamination (Bamber *et al.*, 1997; Rodrigues and Pardal, 2014). Due to its important ecological role and its widespread geographical distribution, *C. maenas* was recommended for use in Portuguese monitoring programmes, more specifically for biomarker measurements (Picado *et al.*, 2007).

I.3 - Multibiomarker approaches

Environmental Risk Assessment (ERA) is based on the comprehension of the potential risks associated with the presence of pollutants in the environment (van der Oost *et al.*, 2005). For each community or ecosystem, it is presumed that there is a range of conditions considered to be normal, and that the presence of pollutants will interfere with these conditions (Depledge and Fossi, 1994), representing a threat to ecosystems structure

and functions (Cairns and McCormick, 1992). Over the last decades, it has been recognized that assessing merely the levels of pollutants present in the environment was not enough to evaluate the risks associated to a certain contaminant (van der Oost *et al.*, 2005) because chemical analysis alone do not provide information regarding the effects that the chemicals and concentrations detected may have on the biota (Cajaraville *et al.*, 2000). Hence, there has been a change in the assessment of the environmental impact of a contaminant.

Deleterious effects can derive from an acute exposure to a toxic amount of pollutant or become only visible when animals have been exposed to a pollutant for a long period of time. In this last case, when the contamination is perceived, the problem can be beyond remediation (van der Oost *et al.*, 2003). Therefore, the early diagnose of effects of chemical contaminants is essential to maintain and protect ecosystems health. On this regard, biomarkers have been used as early-warning signals of exposure to and effect of environmental contaminants in aquatic species (Cajaraville *et al.*, 2000). A biomarker is a biological response, from molecular to behavioral levels, that provides information regarding the exposure and/or effects caused by a certain pollutant or a group of pollutants (Rodrigues and Pardal, 2014). These biological responses are considered sensitive indicators (McCarthy and Shugart, 1990) and are often divided in two types: those that confirm that the animal was exposed to a pollutant, the exposure biomarkers, and those related to the animal's response to a pollutant, the effect biomarkers (Cajaraville *et al.*, 2000). Due to their relatively rapid and easy handling and cost effectiveness (Rodrigues and Pardal, 2014), biomarkers have been commonly used to evaluate the effects of contaminants in estuarine animals and unravel their modes of action (Luís and Guilhermino, 2012; Neuparth *et al.*, 2013), including in field and laboratory experiments with *C. maenas* (Rodrigues *et al.* 2014a; Rodrigues and Pardal, 2014 and references herein). Effective evaluations and information are obtained when using multibiomarker approaches combining parameters related to the mode of action of toxicants and involved in vital physiological functions (Rodrigues *et al.*, 2015).

Among the biological responses that have been successfully used in the detection of adverse effects towards animals are biomarkers of neurotransmission, energy metabolism, biotransformation and oxidative stress (Garrigues *et al.*, 2001), specifically in green crab (Rodrigues and Pardal, 2014; Rodrigues *et al.*, 2015). To determine these responses, specific tissues may be selected according to specificity of the biomarkers to be used, *e.g.*, ganglion, muscle and digestive gland.

The thoracic ganglion is an important organ of the nervous system (Crothers, 1967), with a role in ventilation due to its composition in central pattern generating neurons (Simmers and Bush, 1983). Thoracic ganglion has a higher enzymatic content than cerebral ganglion (Walop and Boot, 1950). This organ has been used to determine the activity of

acetylcholinesterase (AChE), an enzyme involved in the cholinergic neurotransmission, being responsible for the degradation of the neurotransmitter acetylcholine (ACh) (Mesquita, 2010).

Muscle tissue is linked to locomotion in the green crab (Soreson, 1973) and it is known for its high levels of energy (Walsh and Henry, 1990). Additionally to AChE determinations, this organ has been used to determine the activity of lactate dehydrogenase (LDH) and $NADP^+$-dependent isocitrate dehydrogenase (IDH). These enzymes are related to energy production through an anaerobic and aerobic pathway, respectively. When the animal is under stress as, for instance, under toxicant exposure, these pathways may suffer alterations to provide additional energy (Rodrigues *et al.*, 2013a) for detoxification and maintenance of homeostasis.

The digestive gland comprises the functions of a vertebrate liver, pancreas and small intestine. Also, it is the main site of enzyme secretion (Crothers, 1967). Thus, this organ is important in the detoxification of xenobiotics, having high levels of biotransformation and antioxidant enzymes (Livingstone, 1998). This tissue has been used to measure the activity of glutathione *S*-transferases (GST) and glutathione peroxidase (GPx) and levels of lipid peroxidation (LPO). GST has an important role in the conjugation of electrophilic compounds with reduced glutathione (GSH), which becomes oxidized (GSSG), and is also involved in antioxidative defenses (Mesquita, 2010). GPx has an antioxidant role, being responsible for the conversion of the hydrogen peroxide (H_2O_2), formed during cellular metabolism, into water and oxygen (Mesquita, 2010). LPO (oxidation of polyunsaturated fatty acids) is a biomarker of oxidative damage caused by reactive oxygen species (ROS) (van der Oost *et al.*, 2003) generated during the detoxification processes. This oxidation starts with the reaction of a free radical with an unsatured fatty acid and is followed by its propagation. This leads to the destruction of the lipid structure of cellular membranes and later to cell death (Lima and Abdalla, 2001).

I.4 - Objectives

The main aim of this thesis was to investigate long-term effects of AN and AL in *Carcinus maenas* using a multibiomaker approach. The biomarkers were selected from those mentioned above for their potential involvement in the mode of action of the compounds and role in detoxification. For that, separate long-term bioassays (21 days) were performed. In each the test-organisms were exposed to three treatments (0, 100 and 1000 µg/L of AN or 0, 5 and 50 µg/L of AL) and analyzed at 7, 14 and 21 days of exposure.

The working hypotheses assumed herein were that: i) long-term exposure to AN or AL would cause alterations in the selected biomarkers, relative to controls, possibly

influencing the health status of *Carcinus maenas*; ii) low and high exposure concentrations would elicit different biomarker responses; and iii) different responses would be observed over time.

II – MATERIAL AND METHODS

II.1 - Crab sampling and acclimation

Intermolt male crabs were collected in Minho River estuary (NW Portugal), in January and April of 2015 (Figure 6). This estuary is classified as a NATURA 2000 site (Guimarães *et al.*, 2012). It is known for its low levels of human influence and environmental contamination (Ferreira *et al.*, 2003; Guimarães *et al.*, 2012; Rodrigues *et al.*, 2014b; Capela *et al.*, 2016).

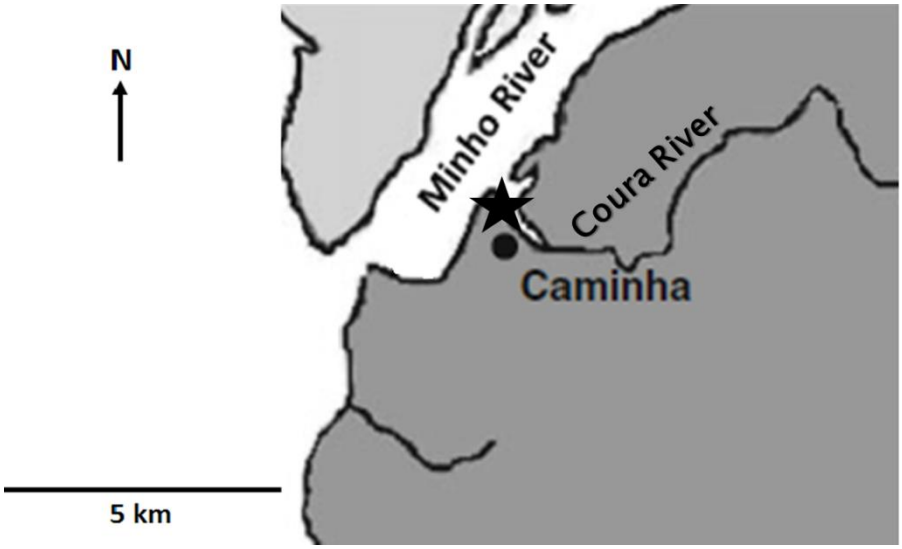

Figure 6: Location of Minho River in the northwestern Iberian coast. The sampling site is indicated by a star.

Molting is an essential process in *Carcinus maenas* life cycle that comprises hormonal changes which could interfere with experimental results (Mesquita, 2010). Furthermore, during the process of molting, the crabs become more vulnerable and, consequently, easy targets for cannibalism by other crabs. Therefore, only intermolt crabs were selected for the experiments. The capture was performed two hours after low tide, using baited hand nets.

Water abiotic factors (temperature, dissolved oxygen, conductivity and pH) were measured at the time of capture (Table 3), using a portable multiparametric sensor (WTW multi 340i) with appropriate probes (pH Sen Tix 41 and Tetracon 325).

Table 3: Values of abiotic factors measured at the study location, for each sampling.

Abiotic factors	January	April
Temperature (°C)	12.1	15.0
Dissolved oxygen (mg/L)	12.2	11.2
Salinity (psu)	17.5	13.2
pH	7.3	7.6

After capture, the animals were immediately transported to the laboratory in refrigerated ice boxes. Once in the laboratory, the crabs were kept for approximately 3 weeks in a tank of 300 L, maintained under low luminosity and with continuous aerification. Crabs were fed with cubes (1x1x1 cm) of frozen squid, twice a week, followed by water renewal. Both salinity and temperature were maintained at similar values to those measured at the time of collection (salinity 15±1 psu and temperature 14±1 °C). This adaptation period was used to minimize the stress caused by transportation.

Sampling and all animal experiments were conducted in compliance with the ethical guidelines of the European Union Council (Directive 2010/63/EU of 22nd September) for the protection of animals used for experimentation and other scientific purposes.

II.2 - Chemicals and equipment

Acrylonitrile and AL used for contaminating the experimental media, as well as, the reagents needed for enzymatic analyzis were purchased from Sigma-Aldrich Chemical (Steinheim, Germany). The Bio-Rad protein assay dye reagent was purchased from Bio-Rad Laboratories, Inc (Munich, Germany).

For tissue homogenization, a Precellys 24 homogenizer and both an Eppendorf 5810 R and VWR Micro Star 17 R centrifuges were used. Microplate analyzis was done using a BioTek Power Wave 340 spectrophotometer to determine the activity of several enzymes.

II.3 - Experimental design

Separate chronic bioassays (21 days) were performed for two widely transported HNS: AN and AL.

Following the adaptation period, 108 crabs were randomly selected and placed in 7 L aquaria. The tanks were filled with 4 L of filtered seawater (15±1 psu) and oxygen levels were maintained around 80%. Temperature was kept at 14±1 ºC. These parameters were measured regularly. The crabs were kept in the tanks for a period of 4 days before the

beginning of the experiment to allow their acclimation to the experimental conditions.

In each bioassay and replicates, animals were exposed to three treatments: control (filtered seawater at 15 psu) and two levels of nominal concentrations of either AN (100 and 1000 µg/L) or AL (5 and 50 µg/L) in a semi-static daily replacement regime. These concentrations were based on the environmental data provided above to investigate possible differences in effects caused by low environmental concentrations and high hazardous levels (EURAR, 2004a; 2004b; van Vlaardingen *et al.*, 2007; van Herwijnen, 2009). For AN, the highest concentration (C2) tested is equivalent to the serious risk concentration (SRC, 1.3 mg/L), while the lowest concentration (C1) is ten times lower. For AL, the lowest concentration (C1) is five times higher and C2 fifty times higher than the maximum admissible concentration (MAC, 1 µg/L). Nine replicate aquaria were prepared per treatment, each containing 4 crabs. Every 7 days, 3 replicas of each treatment were dismantled for tissue collection; at the end of 7, 14 and 21 days (7d, 14d and 21d, respectively) after the beginning of the experiment. The aquaria distribution was as shown in the Figure 7.

	C2 7 R3	C1 14 R3	C 14 R3	C2 21 R3	C1 7 R3	C 7 R3	C2 14 R3	C 21 R3	C2 7 R2	C1 7 R2	C 14 R2	C2 14 R2	C1 21 R3
C1 7 R1	C 7 R1	C2 14 R1	C1 21 R1	C 21 R1	C2 7 R1	C1 14 R1	C 14 R1	C2 21 R1	C1 21 R2	C 7 R2	C1 14 R2	C 21 R2	C2 21 R2

Figure 7: Experimental design related to aquaria placement. Three aquaria were assigned to control and to each of the test concentrations at each time point. Codes: C 7 – Control 7 days; C1 7 – Concentration 1 7 days; C2 7 – Concentration 2 7 days; C 14 – Control 14 days; C1 14 – Concentration 1 14 days; C2 14 – Concentration 2 14 days; C 21 – Control 21 days; C1 21 – Concentration 1 21 days; C2 21 – Concentration 2 21 days.

All animals were measured at the beginning of the experiments and at the dismantlement of each treatment (before tissue collection). Additionally, twelve crabs were maintained in the same conditions, as previously described and analyzed at the beginning of the experiment (T0).

II.4 - Tissue sampling

At days 7, 14 and 21d, the crabs were anesthetized on ice for about 30 minutes, weighed and measured (length and width in cm, and weight in g). Samples of digestive gland, ganglion, muscle and gills were then isolated from each crab (Figure 8). The samples were put in microtubes, immediately placed in a thermic bottle containing liquid nitrogen, and then stored at -80°C until further analyzis.

These tissues were used for biomarkers analyzis. The remaining tissues were collected and stored at -20°C, and used for quantification of AN and AL.

Figure 8: Collection of samples from internal organs and tissues of male shore crabs. 1: Digestive gland; 2: Thoracic ganglion; 3: Muscle.

II.5 - Chemical analyzis

Ten mL of experimental media were sampled, from each treatment, at day 2, 6, 13 and 20 into dark flasks and frozen at -20°C. These aliquots were gathered right after the addition of the contaminant to the tanks (0h) and in the following day, before the renewal of the medium (24h). This process aimed at quantifying the concentrations to which the animals were actually exposed, as well as, verifying stability of the experimental media. Aliquots of stock solution were also collected and frozen at -20°C. For determination of chemical concentrations of the test substances in tissues, composite samples of whole soft tissues (the remaining parts after collection for biomarkers analyzis) from several individuals were used to allow for five replicate measurements. The tissue was then homogenized with an Ultra-turrax blender (Ika).

For AL analyzis, test conditions were derived from previous experiments (Santos *et al.* 2015) while quantification in tissues is still under development. For analyzis of AN, 2 g of sodium chloride (NaCl) (p.a., Merck) was added to each vial and the vials were immediately closed with a plastic stopper with a polytetrafluoroethylene (PTFE) faced septum and vortexed for 30s to allow salt dissolution and homogenization. Headspace solid phase microextraction (SPME) was performed using an autosampler CombiPal model (CTC Analytics) fitted with a 65 µm thickness of polydimethylsiloxane-divinylbenzene (PDMS-

DVB, polar) fiber from Supelco. The instrument was set to 40ºC with 15 min extraction. AN quantification were performed using a Varian Saturn 2000 mass spectrometer (Walnut Creek, CA) coupled to a Varian 3900 gas chromatograph equipped with a split/splitless injector port, a SPME liner (0.75 mm ID), a microseal septum system (Merlin, Half Moon Bay, CA) and a VF-5ms column (60 m length x 0.25 mm diameter, 0.25 µm film thickness) from Agilent. The carrier gas was helium of high purity (99.9995% from Air Liquide) at a constant flow of 1.0 mL/min. SPME fiber was thermally desorbed directly in the injection port at 270ºC for 5 min. The oven temperature program was as follows: initial temperature 35ºC held for 2 min, then raised at 8ºC/min up to 100ºC, then raised at 20ºC/min up to 240ºC and held for 4 min. Transfer line temperature was set at 250ºC and the ion trap at 230ºC. The mass spectrometer was operated in SIM mode by selecting the most sensitive mass-to-charge ratio for each individual compound in order to reach both lowest detection limits and highest sensitivity. Calibration standards were prepared using a stock standard solution of CLP Volatiles Calibration Mix (Supelco). LOD for AN was 15 µg/L for water samples, and 20 ng/g wet weight for tissue samples.

II.6 - Biochemical determinations

A set of biomarkers involved in neurotransmission, energy metabolism, biotransformation and oxidative stress pathways was chosen to assess the sub-lethal effects induced by the contaminants in the animals. The determinations were performed according to established methods adapted to *Carcinus maenas*, as previously described (Rodrigues *et al.,* 2013b).

The protein content was determined in each tissue using the Bradford method (Bradford, 1976) adapted to microplate. This colorimetric method is based on the color intensity, meaning that color intensity is proportional to protein content. As a standard, bovine γ-globulin (1 mg/mL) was used.

The thoracic ganglion or a piece of muscle tissue (≈ 0.020 g) were homogenized in 500 µL of phosphate buffer 100 mM: potassium chloride (KCl, 150 mM): ethylenediaminetetraacetic acid disodium salt (Na_2EDTA, 1mM, pH 7.4), in a Precellys homogenizer (5600 rpm, 30 seconds) and centrifuged at 6.000 *g*, for 5 minutes, at 4°C. AChE activity was measured according to Ellman's method (Ellman *et al.*, 1961) using acetylthiocholine 0.075 M and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) 10 mM. The reaction solution consisted of phosphate buffer, acetylthiocholine and DTNB. The reaction between thiocholine and DTNB results in an increase of color (yellow) that is directly proportional to an increase in absorbance at 412 nm (Rodrigues *et al.*, 2012; 2013a). The enzymatic activity is expressed in nmol of substrate hydrolysed per min per mg of protein.

For determining LDH activity, pieces of muscle tissue were homogenized with 1000 µL of Tris/NaCl buffer, pH 7.2 (Precellys homogeneizer: 5600 rpm, 30 seconds) and centrifuged at 6.000 *g*, for 3 minutes, at 4°C. The activity was measured according to Vassault's method (Vassault, 1983). The Tris/NaCl buffer was used to obtain the solutions Tris/NaCl/reduced form of nicotinamide adenine dinucleotide (NADH) 0.256 mM and Tris/NaCl/Pyruvate 12.2 mM. This enzyme reduces the pyruvate, oxidizing the NADH during this process, to originate lactate and oxidized form of nicotinamide adenine dinucleotide (NAD^+), resulting in pyruvate consumption (Rodrigues *et al.*, 2012; 2013a). The enzyme activity was measured at 340 nm and expressed in nmol of consumed pyruvate per minute per mg of protein.

For determining IDH activity, portions of muscle (≈ 0.020 g), were homogenized with 1000 µL of Tris/NaCl buffer (pH 7.8) in a Precellys homogeneizer (5600 rpm, 30 seconds) and centrifuged at 15.000 *g*, for 15 minutes, at 4°C. The IDH activity was measured according to the method of Ellis and Goldberg (1971). The activity was determined in the presence of 2 mM manganese chloride ($MnCl_2$), 7 mM isocitric acid (DL) and 0.5 mM nicotinamide adenine dinucleotide phosphate oxidized ($NADP^+$). The method is based on the regeneration of reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), verified by an increase in absorbance at 340 nm (Rodrigues *et al.*, 2012; 2013a). The activity was expressed in nmol of regenerated NADPH per minute per mg of protein.

The digestive gland (≈ 0.035 g) was homogenized in 500 µL of phosphate buffer 100 mM: KCl 150 mM: Na_2EDTA 1 mM: dithiothreitol (DTT) 1 mM: phenylmethanesulfonylfluoride (PMSF) 0.1 mM (pH 7.4) in a Precellys homogenizer (5600 rpm, 30 seconds). The homogenate was centrifuged at 12000 rpm, for 20 minutes, at 4°C, and the supernatant was divided in aliquots, each one for a specific biomarker.

Glutathione *S*-transferases activity was measured according to Habig's method (Habig *et al.*, 1974), using 10 mM GSH and 60 mM 1-chloro-2,4-dinitrobenzene (CDNB). Absorbance was read at 340 nm. The activity was estimated by the conjugation between the GSH and CDNB (Rodrigues *et al.*, 2012; 2013a) and expressed in nmol of conjugated substrate per minute per mg of protein.

For determining GPx activity, the Mohandas's method was employed (Mohandas *et al.*, 1984), using 40mM GSH, 10mM sodium azide, 2.4mM NADPH and 2mM H_2O_2. The analyzis is based on the decrease of NADPH, using H_2O_2 as substrate (Rodrigues *et al.*, 2013a) and was measured by reading absorbance at 340 nm. The activity of GPx was expressed in nmol of reduced NADPH per minute per mg of protein.

Lipid peroxidation was measured according to Ohkawa' method (Ohkawa *et al.*, 1979) and Bird and Draper's method (Bird and Draper, 1984) as previously described (Capela *et al.*, 2016). The sample was centrifuged with trichloroacetic acid (TCA) 100%.

Afterwards, 0.1M ethylenediaminetetraacetic (EDTA), 1% thiobarbituric acid (TBA) and 0.025% butylatedhydroxytoluene (BHT) were added to the supernatant. This mixture was boiled for 30 min. After cooling down absorbance was read at 532 nm to measure the formed thiobarbituric acid reactive substances (TBARS) (Capela *et al.*, 2016). Lipid peroxidation levels were expressed in pmol of malondialdehyde (MDA) per mg of protein.

II.7 - Data analyzis

Data of each biomarker were first checked for normality (Kolmogorov-Smirnov test) and homogeneity of variances (Levene's test), and appropriate transformations were made when necessary. Values more than 2.5 standard deviations above their mean were considered as outliers and removed from the analyzes, since they were interfering with normality of data distribution and/or producing lack of variances homogeneity.

A possible effect of the test aquaria was first investigated using a one-way analyzis of variance (ANOVA). Given that no effect was observed the measurements obtained for each crab were treated as independent. Concentration (controls and AN or AL exposure) and Duration of exposure were taken as sources of variation and full-factorial two-way ANOVA was done in order to test for their influence on the biomarker responses measured. When significant differences were found either for the interaction term or the main factors, data were then analyzed with post-hoc tests (the Dunnett test when comparing treatment groups or duration of exposure; the Least Significant Difference Test when analyzing the interaction term).

Carcinus maenas exposed to AN and AL were collected in different seasons: winter and spring, respectively. A comparison was made to determine if baseline differences in biomarker levels could be detected. Therefore, a t-test for independent samples was conducted for each biomarker measured in crabs from T0 of both bioassays. All statistical analyzes were carried out in SPSS IBM v23.0 and significant differences were established at $p<0.05$.

In order to provide an overall interpretation of the multibiomarker responses, the Integrated Biomarker Response (IBR) index was used as a stress indicator. This index was calculated based on Beliaeff and Burgeot (2002) and the afterward modification described by Guerlet *et al.* (2010), for each duration of exposure as follows: individual areas A_i connecting the i^{th} and the $(i+1)^{th}$ radius coordinates of the star plot were obtained through the formula $A_i = ½\ sin(2\pi /n)S_iS_{i+1}$, where S_i and S_{i+1} represent the individual biomarker scored (calculated from standardized data) and their successive star plot radius coordinates, and n represents the number of radii corresponding to biomarkers used in the survey. The IBR index correspond to the summing-up of all the star plot triangular areas

(IBR=$\sum A_i$), corrected for the number of biomarkers used and their expected responses due to these HNS' mode of action. Higher IBR values indicate higher stress levels.

III - RESULTS

III.1 - Acrylonitrile

In exposures to AN the mortality rate was 1.7%, with only 2 dead crabs in a total of 120 animals. Those deaths occurred both in 21d exposure, one in the control and one in the highest test concentration. Salinity (mean ± standard deviation, 15.32 ± 0.33 psu), temperature (14.16 ± 0.42°C), pH (6.94 ± 0.56) and dissolved oxygen (89.42 ± 17.86%) were stable along the assay and within the expected values.

Table 4 summarises the actual concentrations of AN measured during the exposure experiments. No AN was detected in control groups; in all test aquaria the levels determined were below the LOD (15 µg/L). Levels measured in the contaminated experimental media after 24 hours (t_{24}) of AN addition, prior to the daily water renewal, were generally stable along the assays (Table 4). AN concentrations near the nominal selected ones (100 and 1000 µg/L) were fairly achieved under the test conditions employed. In days 13 and 20, the AN levels of the 100 µg/L treatment were 31 and 46% lower than the nominal concentrations selected, denoting some loss of AN. The concentration of AN measured in the soft tissues was below the LOD (20 ng/g), even at the end of the 21d of exposure, suggesting that no accumulation of AN occurred.

Table 4: Nominal and measured concentrations of acrylonitrile (AN, µg/L) in water samples collected at days 2, 6, 13 and 20, after 24h of AN addition and prior to the daily renewal of the experimental media. Data expressed as mean ± standard deviation.

		Day 2	Day 6	Day 13	Day 20
0 µg/L[a]	t_{24}	< 15[b]	< 15[b]	< 15[b]	< 15[b]
100 µg/L[a]	t_{24}	101 ± 13	92 ± 27	69 ± 12	54 ± 1
1000 µg/L[a]	t_{24}	917 ± 97	701 ± 33	814 ± 83	738 ± 103

[a] Nominal acrylonitrile concentration.

[b] Limit of detection.

III.1.1 - Biochemical analyzis in tissues

Thoracic ganglion

Acetylcholinesterase activity ranged from 154.83 to 1059.39 nmol/min/mg protein, with a mean value of 545.02 ± 210.87 nmol/min/mg protein. Comparing AChE activity determined in T0 crabs (dashed line, Figure 9) to that obtained for crabs exposed to non-contaminated medium (control group), a significant increase of 47% in the enzyme activity was observed after 14d of exposure, denoting some variation in enzyme activity over the duration of the assay. Controls from 7 and 21d showed no differences when compared to

data from T0. No significant differences were found either for the Concentration, the Duration of exposure or their Interaction (Table 5), suggesting that exposure to the selected AN concentrations had no effect on ganglion neurotransmission.

Table 5: Results of the full factorial two-way ANOVA performed to investigate the effects of Concentration (Conc) and Duration of exposure to acrylonitrile on the biomarkers analyzed in the tissue of organisms exposed for 21 days to AN (0, 100, 1000 µg/L). Activity of acetylcholinesterase in ganglion (AChEg) and muscle (AChEm), lactate dehydrogenase (LDH) and $NAPD^+$-dependent isocitrate dehydrogenase (IDH) in muscle, glutathione *S*-transferases (GST), glutathione peroxidase (GPx) and levels of lipid peroxidation (LPO) in digestive gland.

Parameter	**Source of variation**	***df***	***F***	***P***
Neurotransmission				
AChEg	Conc	2, 99	0.998	0.372
	Duration	2, 99	0.231	0.794
	Conc x Duration	4, 99	1.323	0.267
AChEm	Conc	2, 99	1.194	0.307
	Duration	2, 99	1.070	0.347
	Conc x Duration	4, 99	3.233	0.015
Energy metabolism				
LDH	Conc	2, 99	4.613	0.012
	Duration	2, 99	4.029	0.021
	Conc x Duration	4, 99	0.963	0.432
IDH	Conc	2, 99	2.082	0.130
	Duration	2, 99	2.806	0.065
	Conc x Duration	4, 99	1.430	0.230
Biotransformation and antioxidant defenses				
GST	Conc	2, 99	4.031	0.021
	Duration	2, 99	1.623	0.202
	Conc x Duration	4, 99	4.278	0.003
GPx	Conc	2, 99	1.873	0.159
	Duration	2, 99	2.080	0.130
	Conc x Duration	4, 99	2.619	0.039
Oxidative damage				

LPO	Conc	2, 99	0.081	0.922
	Duration	2, 99	5.160	0.007
	Conc x Duration	4, 99	0.802	0.527

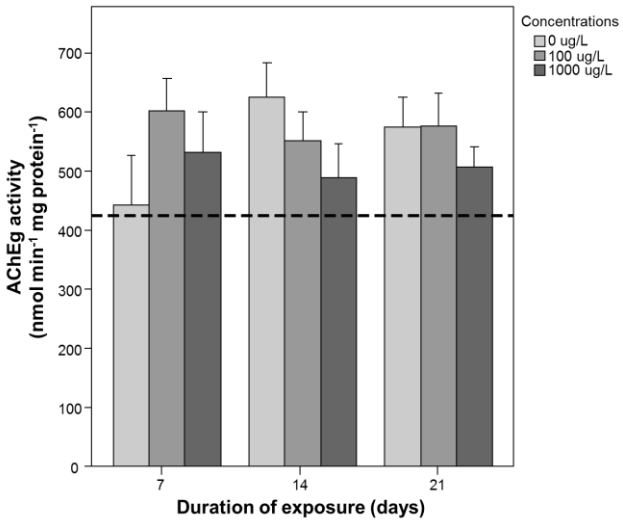

Figure 9. Activity of acetylcholinesterase in ganglion (AChEg, mean ± standard error), in *Carcinus maenas* exposed to acrylonitrile (0, 100 and 1000 µg/L) for 21 days. The dashed line represents data regarding T0 organisms.

Leg muscle

Concerning AChE activity in muscle, values varied between 7.29 and 41.64 nmol/min/mg protein, with an average value of 21.64 ± 7.47 nmol/min/mg protein. Statistically significant differences in AChE activity were found between the control groups at 7 and 14d and T0 (dashed line), with an increase of 35 and 38%, respectively, being registered. Significant differences were detected for the Interaction between Concentration and Duration of exposure (Table 5), indicating the low and the high AN concentrations caused different effects over time. Crabs exposed to 100 µg/L showed activity levels near control values, however, at 21d, a significantly decrease (-28%) in the enzyme's activity was observed for the 100 µg/L AN treatment when compared to data from 7d ($p<0.05$, Figure 10). In contrast, crabs exposed to the highest AN concentration showed an increase in activity with the duration of the exposure, despite the significant decrease in AChE activity (-31%), in relation to control group ($p<0.01$, Figure 10), was found after 7d of exposure. No significant differences relative to controls were observed after 14 and 21d of exposure. This suggests a decrease in neuromuscular transmission, caused not only by short-term (7d) exposure to a high AN concentration, but also that AN was able to influence AChE activity, following a prolonged exposure to a low concentration.

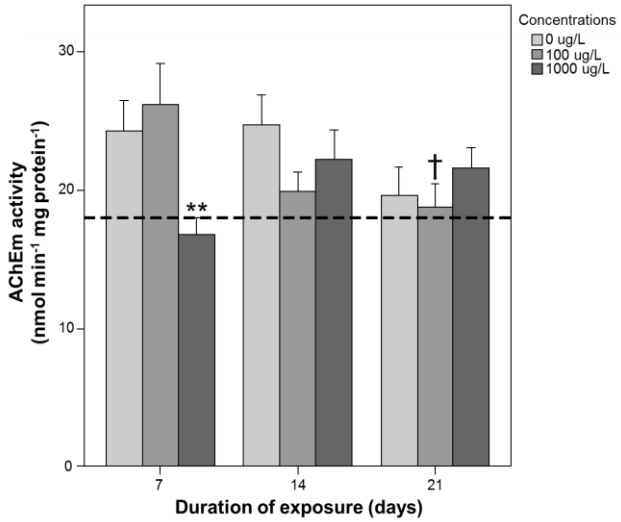

Figure 10. Activity of acetylcholinesterase in muscle (AChEm, mean ± standard error), in *Carcinus maenas* exposed to acrylonitrile (0, 100 and 1000 µg/L) for 21 days. The dashed line represents data regarding T0 organisms; † symbols indicate differences within each treatment compared to its equivalent at 7 days of exposure; asterisks indicate differences within each day of exposure, compared to the respective control group (Two-way ANOVA; ** p< 0.01).

Lactate dehydrogenase activity ranged from 39.56 to 170.52 nmol/min/mg protein, with a mean value of 110.28 ± 28.14 nmol/min/mg protein. No significant differences in LDH activity were found between T0 (dashed line) and control groups analyzed at 7, 14 or 21d. Under exposure to AN, LDH activity was significantly influenced by the two tested factors: Concentration and Duration of exposure (Table 5). In AN groups, LDH activity showed an increased over time. Lower values were detected at 7d exposure to AN compared to 14d and 21d (increase significantly for 21d, 17%). At 7d of exposure, a significant decrease (-25%) in activity was verified for the 1000 µg/L AN treatment in relation to control group ($p<0.05$, Figure 11). No significant differences among treatments were found after 14 and 21d of exposure.These data suggest that AN was able to inhibit LDH activity in the short term. However, in the long-term the crabs was able to cope with the exposure recovering the anaerobic pathway of energy production to control levels.

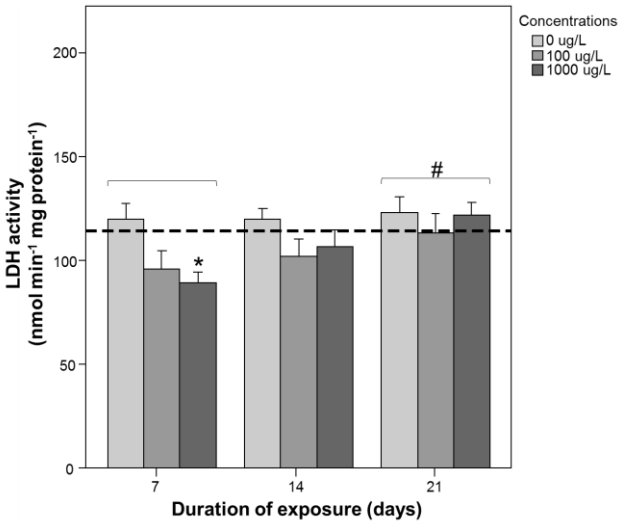

Figure 11. Activity of lactate dehydrogenase in muscle (LDH, mean ± standard error), in *Carcinus maenas* exposed to acrylonitrile (0, 100 and 1000 µg/L) for 21 days. The dashed line represents data regarding T0 organisms; asterisks indicate differences within each day of exposure, compared to the respective control group; hashes represent differences between time points, with no differentiation for the treatment (Two-way ANOVA; * p< 0.05).

$NADP^+$-dependent isocitrate dehydrogenase activity varied between 4.40 and 33.03 nmol/min/mg protein, with an average value of 15.97 ± 6.25 nmol/min/mg protein. IDH activity registered in crabs from T0 (dashed line, Figure 12) and control groups showed no statistically significant differences. In spite of the variation observed, neither AN concentrations or the Duration of exposure had a significant effect on the enzyme activity (Table 5 and Figure 12).

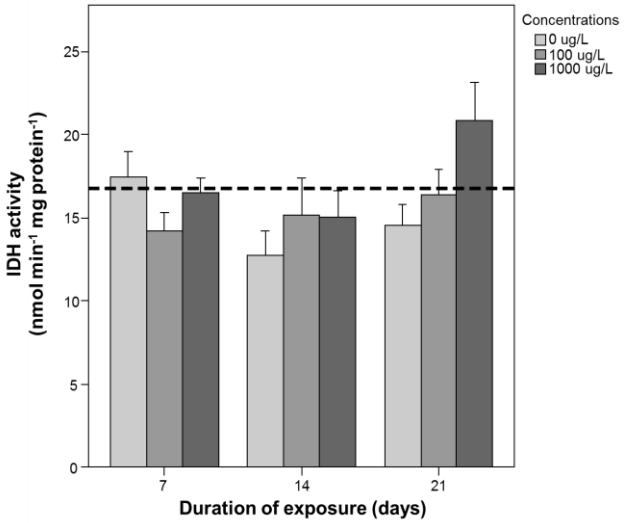

Figure 12. Activity of $NADP^+$-dependent isocitrate dehydrogenase (IDH, mean ± standard error), in *Carcinus maenas* exposed to acrylonitrile (0, 100 and 1000 µg/L) for 21 days. The dashed line represents data regarding T0 organisms.

Digestive gland

The activity of phase II biotransformation enzymes, GST, varied between 9.23 and 105.03 nmol/min/mg protein, with a mean value of 55.16 ± 22.22 nmol/min/mg protein. No significant differences were found between control groups at 7, 14 or 21d and T0 (dashed line, Figure 13). Significant differences were registered for the Concentration of AN and the Interaction between Concentration and Duration of exposure (Table 5): the low and high AN concentrations elicited different short and long-term effects. Low AN concentration caused induction (+63%) of GST after 7d of exposure, relative to the respective control group, which decreased thereafter ($p<0.01$). A significant decrease (-37%) in activity was observed after 21d of exposure, comparing to 7d ($p<0.01$). GST activity of crabs exposed to the high AN concentration was near control levels after 7d of exposure. However, after 21d of exposure, significant induction (+68%) was found compared to the respective control group ($p<0.01$, Figure 13).

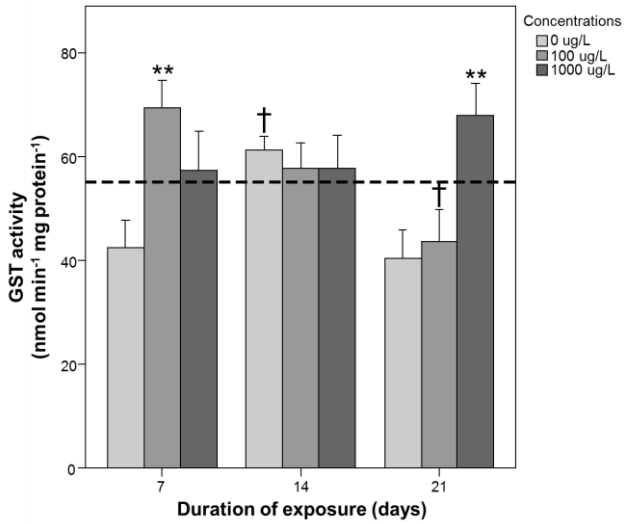

Figure 13. Activity of glutathione *S*-transferases in digestive gland (GST, mean ± standard error), in *Carcinus maenas* exposed to acrylonitrile (0, 100 and 1000 µg/L) for 21 days. The dashed line represents data regarding T0 organisms; † symbols indicate differences within each treatment compared to its equivalent at 7 days of exposure; asterisks indicate differences within each day of exposure, compared to the respective control group; (Two-way ANOVA; ** $p< 0.01$).

The activity of GPx ranged between 4.65 and 15.54 nmol/min/mg protein, with an average value of 10.11 ± 2.26 nmol/min/mg protein. No significant differences were found in the enzyme's activity between control groups at 7, 14 or 21d and T0 (dashed line, Figure 14). A statistically significant effect of the Interaction term on GPx activity was also detected (Table 5), showing that antioxidant defenses were affected by AN exposure. Within 7d of exposure, a significant decrease in GPx activity was observed, comparing to the control group, when the crabs were exposed to AN (a 22% decrease for both AN treatments, $p<0.01$). In what concerns the duration of the exposure, GPx slightly decreased, in control

group (-20%), at 21d, when globally compared to 7d ($p<0.05$). Also, the low AN concentration caused an increase (+22%) in GPx activity from 7 to 14d of exposure ($p<0.05$), decreasing thereafter to control levels.

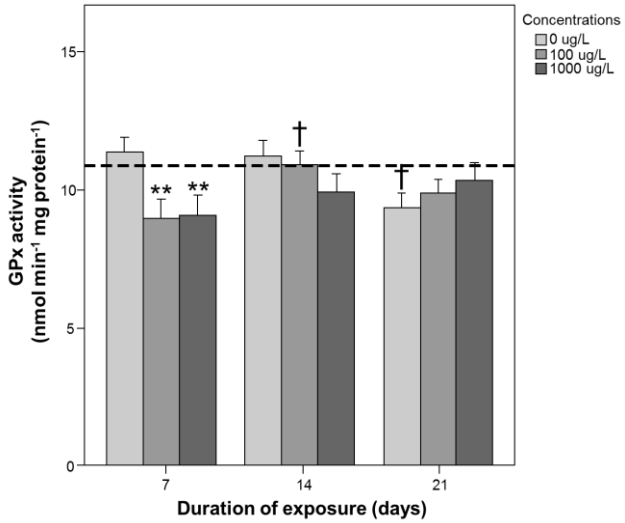

Figure 14. Activity of glutathione peroxidase in digestive gland (GPx, mean ± standard error), in *Carcinus maenas* exposed to acrylonitrile (0, 100 and 1000 µg/L) for 21 days. The dashed line represents data regarding T0 organisms; † symbols indicate differences within each treatment compared to its equivalent at 7 days of exposure; asterisks indicate differences within each day of exposure, compared to the respective control group; (Two-way ANOVA; ** $p< 0.01$).

Lipid peroxidation levels, ranged from 20.72 to 556.36 pmol/min/mg protein, with a mean value of 181.65 ± 104.83 pmol/min/mg protein. Between T0 and control groups at 7, 14 or 21d (dashed line, Figure 15), no significant differences were found. Only the Duration of exposure had a significant influence in LPO levels (Table 5 and Figure 15). Globally, at the end of the 21d experiments LPO was 50% higher than at 7d ($p<0.01$).

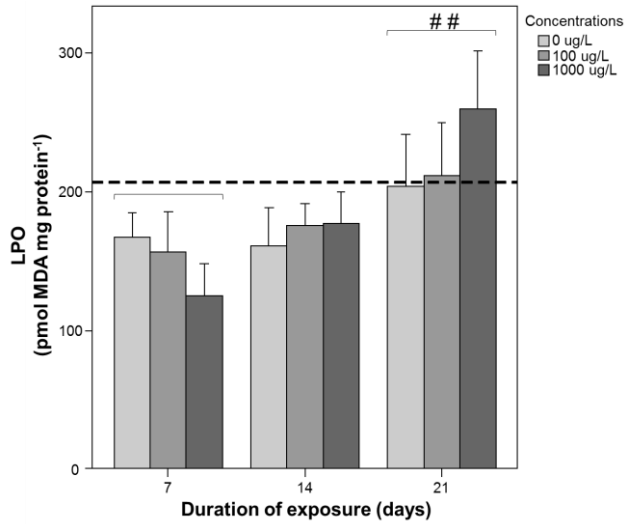

Figure 15. Levels of lipid peroxidation in digestive gland (LPO, mean ± standard error), in *Carcinus maenas* exposed to acrylonitrile (0, 100 and 1000 µg/L) for 21 days. The dashed line represents data regarding T0 organisms; hashes represent differences between time points, with no differentiation for the treatment (Two-way ANOVA; ## $p< 0.01$).

III.1.2 – Integrated Biomarker Response

The Integrated Biomarker Response index showed distinct stress levels among treatments, within each duration of exposure, providing a qualitative measure of the chemical stress to which the crabs were exposed (Figure 16 and 17). At the end of the first 7d, IBR was higher in crabs exposed to AN than in the control group, particularly in the 100 µg/L treatment. After 14d of exposure, stress level had decreased by about half. After 21d, IBR was higher in the 1000 µg/L group, compared to both the control and the lower AN concentration which showed fairly similar stress levels.

At 7d, the discrimination between control and AN concentrations was given mainly by GST, GPx and LDH. Within 21d, GST and LPO distinguished the high AN concentrations from the remaining treatments (Figure 17).

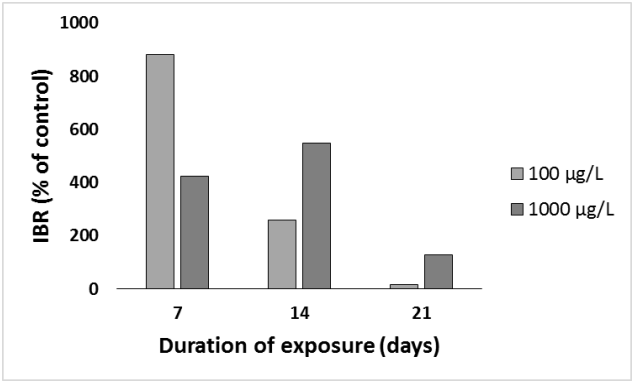

Figure 16: Integrated Biomarker Response (IBR) index (in % of control) calculated with the biomarkers evaluated in *Carcinus maenas* exposed to acrylonitrile (0, 100 and 1000 µg/L), for 21 days.

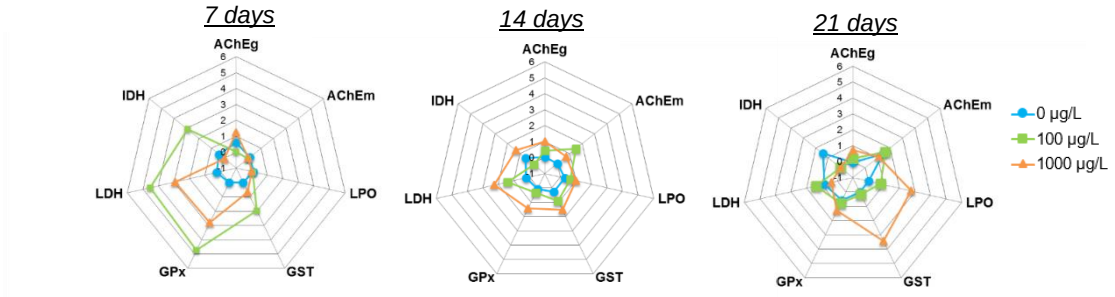

Figure 17: Integrated Biomarker Response (IBR) index (in % of control) calculated with the biomarkers evaluated in *Carcinus maenas* exposed to acrylonitrile (0, 100 and 1000 µg/L), for 21 days. The star plots represent the contribution of each biomarker to the IBR value, at 7, 14 and 21 days of exposure. Activity of acetylcholinesterase in ganglion (AChEg) and muscle (AChEm), lactate dehydrogenase (LDH) and $NADP^+$-dependent isocitrate dehydrogenase (IDH) in muscle, glutathione *S*-transferases (GST), glutathione peroxidase (GPx) and levels of lipid peroxidation (LPO) in digestive gland.

III.2 - Aniline

In this bioassay, a mortality rate of 9.2% was recorded, with 11 dead crabs in a total of 120 animals. Those deaths occurred at 14 and 21d of exposure, in all concentrations, with 5 dead animals in control, 5 in the lowest concentration and 1 in the highest concentration. Salinity (15.29 ± 0.33 psu), temperature (14.47 ± 0.40), pH (7.04 ± 0.56) and dissolved oxygen (87.49 ± 12.54 °C) were stable along the assay and within the expected values.

III.2.1 - Biochemical analyzis in tissues

Thoracic ganglion

Acetylcholinesterase activity in the ganglion, ranged from 83.47 to 830.79 nmol/min/mg protein, with a mean value of 369.43 ± 177.21 nmol/min/mg protein. No statistically significant differences were found between control groups at 7, 14 or 21d and T0 (dashed line, Figure 18). AChE activity was significantly influenced by exposure to AL, as indicated by significance of main factor Concentration and the Interaction between Concentration and Duration of exposure (Table 6). Significant changes in AChE activity were detected after 21d of exposure to 5 µg/L AL (+52%, compared to the respective control group, $p<0.05$), and after 14d (-44%, relative to control) and 21d (+66%, relative to control), for the highest concentration ($p<0.01$ (Figure 18). Additionally, a significant increase of 52% was detected between 7 and 21d of exposure for 50 µg/L AL ($p<0.05$).

Table 6: Results of the full factorial two-way ANOVA performed to investigate the effects of Concentration (Conc) and Duration of exposure to aniline on the biomarkers analyzed in the tissue of organisms exposed for 21 days to aniline (0, 5, 50 µg/L). Activity of acetylcholinesterase in ganglion (AChEg) and muscle (AChEm), lactate dehydrogenase (LDH) and $NADP^+$-dependent isocitrate dehydrogenase (IDH) in muscle, glutathione *S*-transferases (GST), glutathione peroxidase (GPx) and levels of lipid peroxidation (LPO) in digestive gland.

Parameter	**Source of variation**	***df***	***F***	***P***
Neurotransmission				
AChEg	Conc	2, 99	3.776	0.026
	Duration	2, 99	1.421	0.246
	Conc x Duration	4, 99	4.693	0.002
AChEm	Conc	2, 99	0.332	0.718
	Duration	2, 99	1.296	0.278
	Conc x Duration	4, 99	0.885	0.476

Energy metabolism				
LDH	Conc	2, 99	2.161	0.121
	Duration	2, 99	4.208	0.018
	Conc x Duration	4, 99	5.523	0.000
IDH	Conc	2, 99	3.499	0.034
	Duration	2, 99	25.750	0.000
	Conc x Duration	4, 99	0.577	0.680
Biotransformation and antioxidant defenses				
GST	Conc	2, 99	0.927	0.399
	Duration	2, 99	0.195	0.823
	Conc x Duration	4, 99	0.389	0.816
GPx	Conc	2, 99	4.687	0.011
	Duration	2, 99	7.013	0.001
	Conc x Duration	4, 99	1.663	0.165
Oxidative damage				
LPO	Conc	2, 99	3.246	0.043
	Duration	2, 99	0.987	0.376
	Conc x Duration	4, 99	1.528	0.200

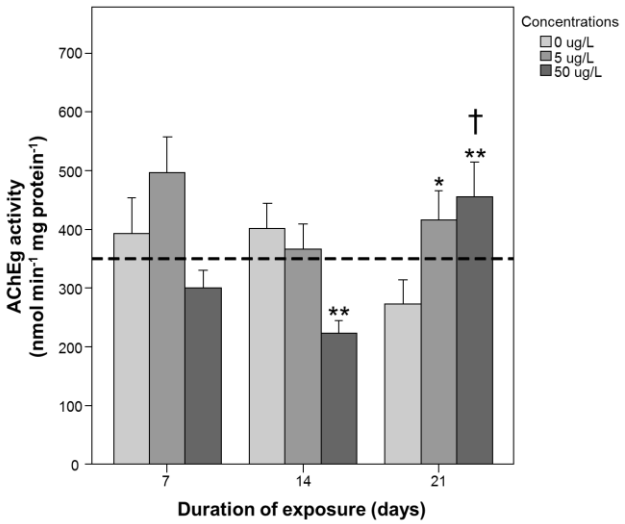

Figure 18. Activity of acetylcholinesterase in ganglion (AChEg, mean ± standard error), in *Carcinus maenas* exposed to aniline (0, 5 and 50 µg/L) for 21 days. The dashed line represents data regarding T0 organisms; † symbols indicate differences within each treatment compared to its equivalent at 7 days of exposure; asterisks indicate differences within each day of exposure compared to the respective control group (Two-way ANOVA, * p< 0.05, ** p< 0.01).

Leg muscle

In relation to AChE activity in muscle, the values varied between 10.03 and 38.62 nmol/min/mg protein, with an average value of 21.89 ± 6.30 nmol/min/mg protein. No significant differences were detected in AChE activity between T0 (dashed line, Figure 19) and control groups at 7, 14 and 21d exposure. No significant differences were found among the tested Concentrations, different Durations of exposure or the Interaction Concentration vs Duration of exposure (Table 6 and Figure 19), suggesting that AL had no effect on neuromuscular transmission.

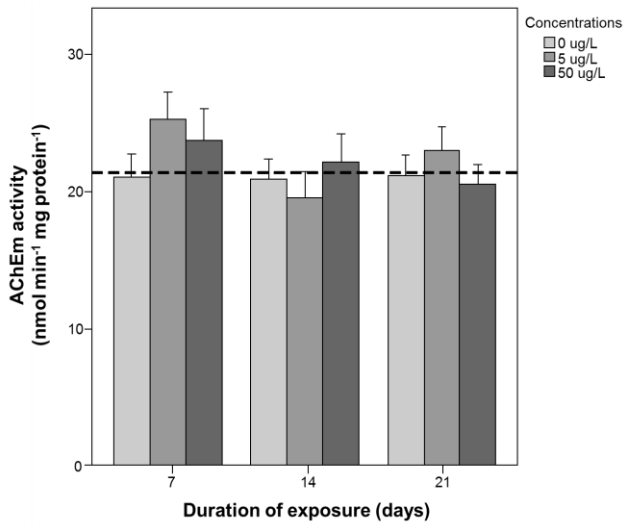

Figure 19: Activity of acetylcholinesterase in muscle (AChEm, mean ± standard error), in *Carcinus maenas* exposed to aniline (0, 5 and 50 µg/L) for 21 days. The dashed line represents data regarding T0 organisms.

The activity of LDH ranged between 68.24 to 220.94 nmol/min/mg protein, with a mean value of 139.91 ± 33.47 nmol/min/mg protein. Activity of the control group measured after 14d of exposure was slightly lower than T0 (-19%, dashed line, Figure 20). Activity of LDH was influenced by the Duration of exposure and the Interaction term (Table 6, Figure 20). In crabs exposed to the low AL concentration, LDH activity was significantly lower (-23%) at 21d of exposure than in the respective control ($p<0.01$). In crabs exposed to 50 µg/L AL, there was a significant decrease (-17%) in activity after 7d of exposure ($p<0.05$), followed by a significant increase (+35%) after 14d of exposure ($p<0.01$) and again a decrease in activity (for both AL concentrations) relative to the control, at 21d ($p<0.05$, Figure 20). Comparing data for Duration of exposure, significant differences were observed in relation to the exposure concentration. LDH activity in controls decreased by 30% at 14d when compared to values obtained at 7d ($p<0.001$). For 5 µg/L AL, lower activity (-19%) was measured at 21d compared to values at 7d ($p<0.05$). A slight significant decrease (-13%) in activity was found globally at 14d relative to measurements at 7d ($p<0.05$, Figure 20).

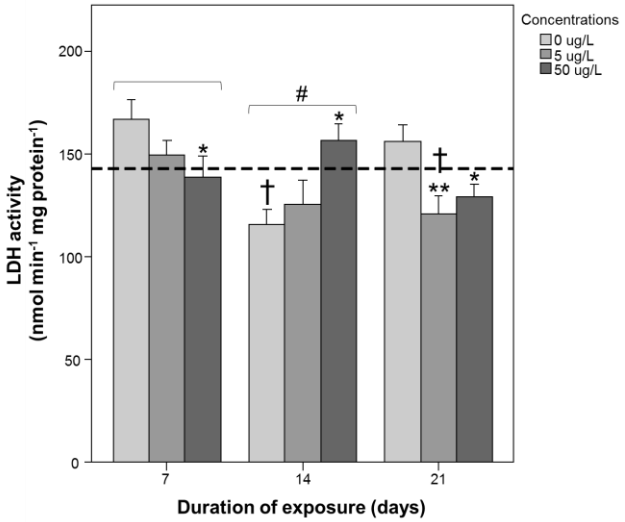

Figure 20. Activity of lactate dehydrogenase in muscle (LDH, mean ± standard error), in *Carcinus maenas* exposed to aniline (0, 5 and 50 µg/L) for 21 days. The dashed line represents data regarding T0 organisms; † symbols indicate differences within each treatment compared to its equivalent at 7 days of exposure; asterisks indicate differences within each day of exposure compared to the respective control group; hashes represent differences between time points with no differentiation for the treatment (Two-way ANOVA, * and # p< 0.05, ** p< 0.01).

Activity of IDH varied between 2.42 and 28.72 nmol/min/mg protein, with an average value of 10.82 ± 5.00 nmol/min/mg protein. Data from control group at 7d and T0 (dashed line, Figure 21) were significantly different, a 130% increase in IDH activity being found. IDH activity was significantly influenced by both Concentration and Duration of exposure to AL (Table 6). The enzyme activity was decreased (by about 30%) in crabs exposed to 50 µg/L ($p<0.05$), after 7d of exposure. Over time, at 14 and 21d of exposure significantly lower IDH activity was measured, compared to that of animals exposed only for 7d to AL (-39 and -43%, respectively, $p<0.001$, Figure 21).

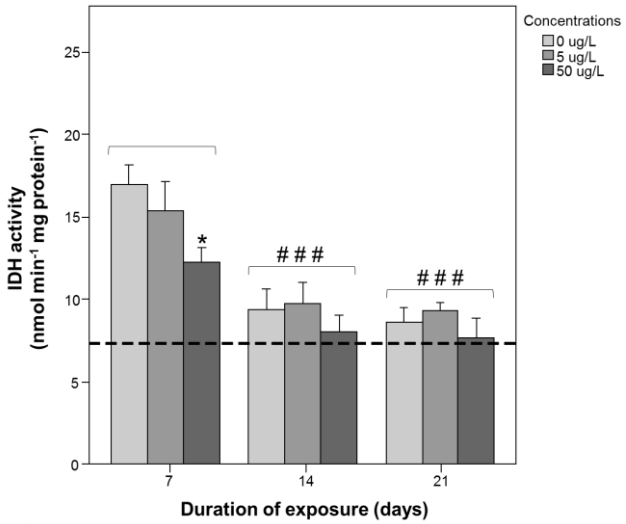

Figure 21. Activity of $NADP^+$-dependent isocitrate dehydrogenase in muscle (IDH, mean ± standard error), in *Carcinus maenas* exposed to aniline (0, 5 and 50 µg/L) for 21 days. The dashed line represents data regarding T0 organisms; asterisks indicate differences within each day of exposure compared to the respective control group; hashes represent differences between time points with no differentiation for the treatment (Two-way ANOVA, * p< 0.05, ### p< 0.001).

Digestive gland

Activity of glutathione *S*-transferases ranged from 3.51 to 78.93 nmol/min/mg protein, with an average value of 30.48 ± 19.40 nmol/min/mg protein. No significant differences were found between GST activity measured in the different control groups and T0 (dashed line, Figure 22). Data exhibited in Table 6 and Figure 22 show no significant effects of Concentration, Duration of exposure or the Interaction between both factors on the activity of GST (Table 6). This suggests that the exposure to AL caused no notable variation in this biotransformation pathway.

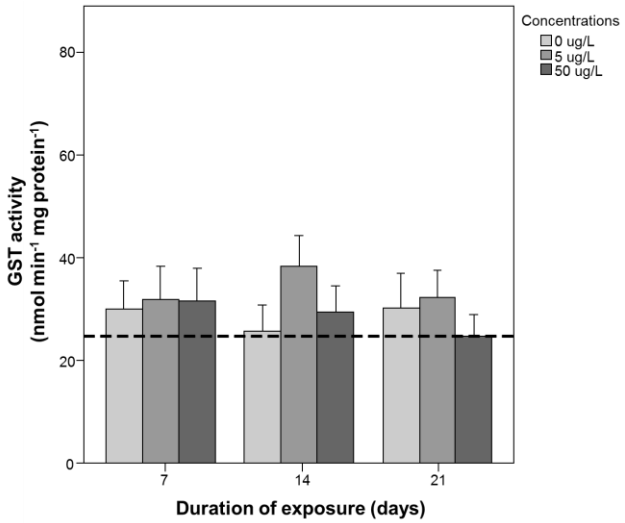

Figure 22. Activity of glutathione *S*-transferases in digestive gland (GST, mean ± standard error), in *Carcinus maenas* exposed to aniline (0, 5 and 50 µg/L) for 21 days. The dashed line represents data regarding T0 organisms.

Concerning GPx, its activity ranged on average from 4.37 to 19.85 nmolmin/mg protein, with a mean value of 11.21 ± 3.24 nmol/min/mg protein. T0 and control groups showed no significant differences in GPx activity, except for control group at 21d, which showed higher activity (+23%) than T0 crabs. Concentration and Duration of exposure showed significant influence on the activity of this antioxidant enzyme (Table 6). A significant decrease (-29%) in activity, compared to controls, was observed after 7d of exposure to 5 µg/L AL ($p<0.05$). Additionally, overall GPx activity was significantly higher (+18%) at 21d than at 7d ($p<0.01$, Figure 23).

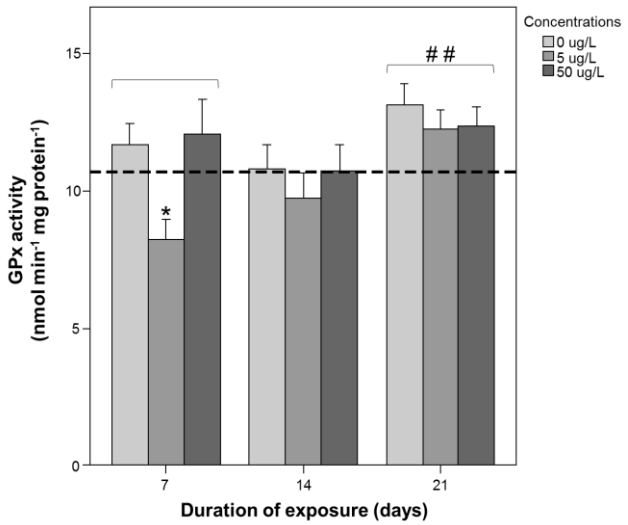

Figure 23. Activity of glutathione peroxidase in digestive gland (GPx, mean ± standard error), in *Carcinus maenas* exposed to aniline (0, 5 and 50 µg/L) for 21 days. The dashed line represents data regarding T0 organisms; asterisks indicate differences within each day of exposure compared to the respective control group; hashes represent differences between time points with no differentiation for the treatment (Two-way ANOVA, * p< 0.05, ## p< 0.01).

Lipid peroxidation levels varied between 19.16 and 437.83 pmol of MDA/mg protein, with an average value of 173.46 ± 95.40 pmol of MDA/mg protein. Only the Concentration of AL was found to have a significant effect on oxidative damage to lipids (Table 6). Exposure to 50 µg/L, after 7d of exposure caused a decrease in LPO levels in relation to the control group (-40% decay, p<0.05, Figure 24).

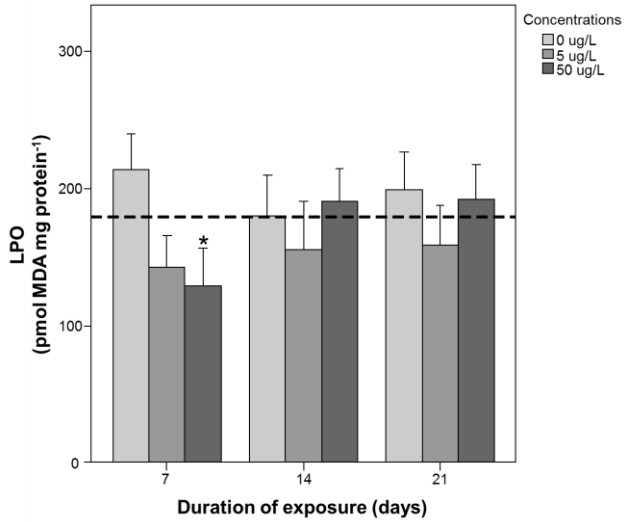

Figure 24. Levels of lipid peroxidation in digestive gland (LPO, mean ± standard error), in *Carcinus maenas* exposed to aniline (0, 5 and 50 µg/L) for 21 days. The dashed line represents data regarding T0 organisms; asterisks indicate differences within each day of exposure compared to the respective control group (Two-way ANOVA, * p< 0.05).

III.2.2 - Integrated Biomarker Response

Distinct stress levels among the tested concentrations were suggested by IBR values, within each duration of exposure (Figure 25). Highest levels of IBR were detected for 50 µg/L treated crabs at 7d, compared to control. At 21d of exposure IBR was fairly similar among treatments (Figure 26).

After 7d of exposure, biomarkers LDH, GPx, GST and LPO distinguished the high AN group from the remaining ones.

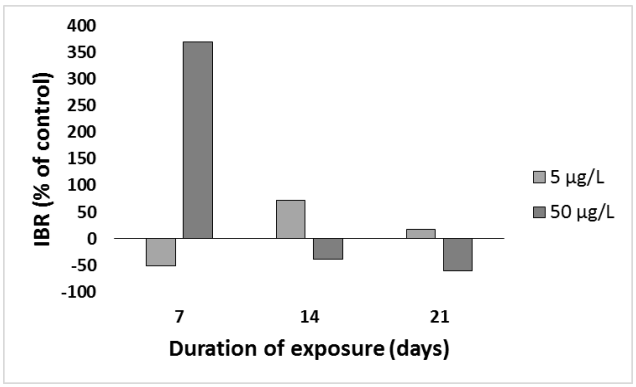

Figure 25: Integrated Biomarker Response (IBR) index (in % of control) calculated with the biomarkers evaluated in *Carcinus maenas* exposed to aniline (0, 5 and 50 µg/L), for 21 days.

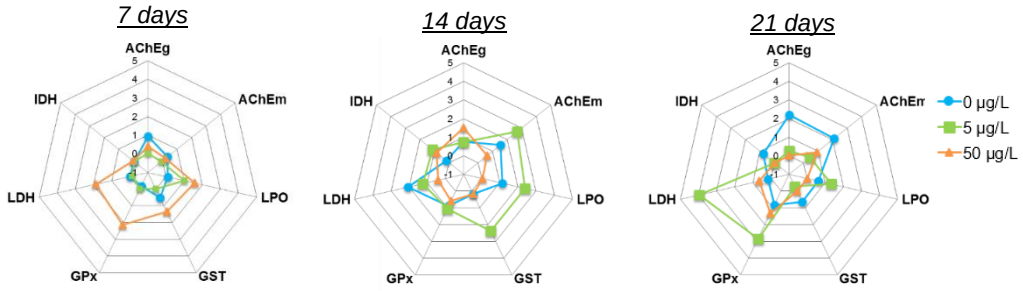

Figure 26: Integrated Biomarker Response (IBR) index (in % of control) calculated with the biomarkers evaluated in *Carcinus maenas* exposed to aniline (0, 5 and 50 µg/L), for 21 days. The star plots represent the contribution of each biomarker to the IBR value, at 7, 14 and 21 days of exposure. Activity of acetylcholinesterase in ganglion (AChEg) and muscle (AChEm), lactate dehydrogenase (LDH) and $NADP^+$-dependent isocitrate dehydrogenase (IDH) in muscle, glutathione *S*-transferases (GST), glutathione peroxidase (GPx) and levels of lipid peroxidation (LPO) in digestive gland.

III.3 - Acrylonitrile and Aniline: T0

The statistical analyzis performed in T0 crabs, from AN and AL bioassays showed significant differences for LDH, GST ($p<0.05$), IDH and LPO, suggesting some level of seasonal variation ($p<0.01$, Table 7). LDH activity was lower in the winter than spring. IDH and GST activity, as well as LPO levels, were higher in the winter.

Table 7: Comparison of the biomarkers values measured in T0 organisms of both acrylonitrile and aniline bioassays. Data expressed as mean ± standard error. Activity of acetylcholinesterase in ganglion (AChEg) and muscle (AChEm), lactate dehydrogenase (LDH) and $NADP^+$-dependent isocitrate dehydrogenase (IDH) in muscle, glutathione *S*-transferases (GST), glutathione peroxidase (GPx) and levels of lipid peroxidation (LPO) in digestive gland.

Parameters	**AChEg**	**AChEm**	**LDH**	**IDH**	**GST**	**GPx**	**LPO**
Acrylonitrile	424.39 ± 53.20	17.97 ± 1.22	114.33 ± 7.98	16.77 ± 2.88	55.19 ± 8.97	10.87 ± 0.71	206.26 ± 20.26
Aniline	350.50 ± 59.99	21.31 ± 4.12	142.89 ± 9.95	7.36 ± 0.93	24.69 ± 7.28	10.68 ± 0.84	120.65 ± 15.55
P	0.367	0.445	0.036	0.005	0.015	0.860	0.003

IV – DISCUSSION

Acrylonitrile and AL are HNS frequently and widely transported by sea, raising environmental concern over the last years. These pollutants have been considered priority HNS, as they are prone to induce hazardous effects on aquatic ecosystems once liberated to the marine environment (Neuparth *et al.*, 2011).

Mammals, such as rodents and humans, have been the main focus of toxicological studies concerning AN (Cole *et al.*, 2008; Neuparth *et al.*, 2013) and AL (CEPA, 1999; EURAR, 2004b). On the other hand, knowledge is still limited for aquatic animals, with only a few studies yet available for either AL (Bhunia *et al.*, 2003; Dom *et al.*, 2010; Luís and Guilhermino, 2012) or AN (Hawkins, 1991; Tong *et al.*, 1996; Neuparth *et al.*, 2013). Additionally, most ecotoxicological studies addressed acute toxicity and mortality has been the most assessed endpoint (reviewed in Rocha *et al.*, *submitted*). Research is therefore needed to better understand the sub-lethal effects induced by those HNS in marine animals and improve their environmental risk assessment. Sub-lethal hazards are produced at lower concentrations of contaminants than those needed to cause mortality (EMSA, 2007). They involve manifestation of important physiological, behavioral and biochemical alterations, among others (Monserrat *et al.*, 2007; Neuparth *et al.*, 2012), which can produce severe consequences in the long-term not only at individual but also at population levels (EMSA, 2007; Neuparth *et al.*, 2014). This information is relevant to establish accurate and ecosystem-protective environmental standard guidelines. The work presented herein, therefore, intended to evaluate potential long-term effects of AN and AL on *Carcinus maenas*. For that, several biomarkers, mainly biochemical parameters with important role in cell metabolism, were selected and three time points (7, 14 and 21d) were chosen to assess the animals' responses to prolonged exposure. All abiotic factors (temperature, dissolved oxygen, salinity and pH) were stable during the experiment and near those found in the environment, therefore minimizing the effect of these potential confounding variables.

Exposure assays were carried out in different seasons (winter for AN and spring for AL). Differences in LDH, IDH, GST and LPO were recorded between T0 crabs, denoting some seasonal variability in these biomarkers. Several abiotic parameters, such as temperature, pH, dissolved oxygen, as well as, internal factors that control the life cycle and physiology of animals, such as age, maturity, nutritional state or reproduction, are known to vary along seasons (Mesquita, 2010), which may explain the reported differences. Similar responses have also been seen in other studies involving either vertebrates (Guimarães *et al.*, 2009) or invertebrates (Mesquita, 2010), including *C. maenas* (Rodrigues *et al.*, 2014a). Compared to data from monitoring campaigns carried out in the same sampling site and in the same season, LDH, IDH and GPx activity levels were similar to those reported values,

while AChEm and GST were higher (Rodrigues *et al.*, 2014a).

Low mortality rates were recorded in all the experiments. Those rates being lower than the maximum established (20%) for the validation of a long-term experiment (Commons *et al.*, 1996). Some of the deaths occurred due to cannibalism following molting of the old exoskeleton. In fact, the concentrations selected were based on environmental guideline values previously established, not susceptible of causing death, and in preliminary experiments carried out to determine the lethal range for *C. maenas*.

Biomarkers can serve as diagnostic and prognostic early warning signals for exposure to pollutants before adverse effects on individual animals or populations are observed (Abrahamson, 2007). Herein, biomarkers of utmost relevance for neurotransmission, energetic metabolism, biotransformation of xenobiotics, antioxidant defenses and oxidative stress were selected to better understand the modes of action of AN and AL in estuarine crustacean.

IV.1 – Biochemical analyzis in tissues

Acrylonitrile

Acetylcholinesterase is a serine hydrolase responsible for the cleavage of acetylcholine, in both synapses and neuromuscular junctions, being able to end the transmission of nervous impulses to postsynaptic cells. The blockage of its activity could lead to paralysis and respiratory arrest (Rodrigues *et al.*, 2015).

No significant effect of AN on AChEg was found, though a non-monotonic tendency of variation appeared to occur in the short-term (7d). The activity of AChE was less pronounced in muscle than in ganglion as expected and observed in other studies with *C. maenas* (Rodrigues *et al.*, 2015). AN was shown to influence the activity of AChE in muscle after 7d of exposure. When exposed to 1000 µg/L AN, the activity of AChEm was significantly inhibited. However, recovery of activity to control levels was found at 14 and 21d of exposure, indicating the crabs were able to adapt and cope with exposure to the toxicant in the long-term.

It has been previously shown that exposure to low levels of toxicants can induce a compensatory response, which could lead to a biphasic dose-response relationship, known as hormesis (Yaunqing *et al.*, 2013). This process is characterized by a dose-response event in which opposite effects are observed at low doses, when compared to high doses, for the same parameter (Calabrese and Blain, 2011). Hormesis was even proposed to explain the effects of AN on AChE activity in rats' blood and brain (Yuanqing *et al.*, 2013). Previous studies with mammals also suggested that AN is responsible for causing

cholinergic toxicity through acetylcholine (ACh) mimetics and AChE inhibition. Exposure to AN, in mice, was reported to compromise cell's capacity to deal with the stress, leading to a decrease in AChE activity (Yuanqing *et al.*, 2013). Nonetheless, this subject is still controverse since, despite studies corroborating the inhibition in AChE activity induced by AN, this HNS may also stimulate AChE activity, or induce no effect, on other animals, e.g., in rats' brain (Klimwa, 1974) and in beetles such as *Tribolium castaneum* larvae (exposed to 0.53 and 1.05 mg/L) and adults (0.40 and 0.79 mg/L) or *Trogoderma granarium* larvae exposed to 0.47 and 0.93 mg/L (Rajendran and Muthu, 1980).

Neurotoxicity of AN is usually associated to acute exposure, with formation of cyanide via oxidative biotransformation through the cytochrome P450 pathway (Abreu *et al.*, 1980; Gut *et al.*, 1984; Carrera *et al.*, 2007). However, in invertebrates contribution of this pathway to biotransformation is considered residual, compared to that of vertebrate animals. This could help explaining the contradictory results apparently found.

Lactate dehydrogenase is involved in the anaerobic pathway of energy production and it is related with metabolic processes such as glycolysis and gluconeogenesis (Walsh and Henry, 1990). This alternate pathway is often active when rapid additional energy may be needed to cope with detoxification or locomotion requirements, with reported augment in LDH activity under natural (e.g., to cope with salinity change) or chemical stress (Diamantino *et al.*, 2001; Rodrigues *et al.*, 2012). In the present study, AN (1000 µg/L) inhibited activity of LDH in crabs exposed for 7d to the contaminant. However, activity was stimulated as the duration of exposure increased, reaching control values at 14 and 21d of exposure. The first 7d of exposure to AN may have inhibited the anaerobic pathway of energy production perhaps in detriment to a defense mechanisms against AN toxicity. An increase in LDH activity has been previously reported for mice exposed to AN (Tandon *et al.*, 1988) and is usually expected considering that cyanide can easily diffuse through all body cells and interfere with their ability to use molecular oxygen (CEPA, 2000). The present results are therefore in support of low bioactivation of AN into its toxic metabolite in crabs, possibly due to limited CYP450 involvement and GSH conjugation of AN and its major oxidation metabolite. This is also consistent with the fact that no effect of AN on IDH activity was detected in exposed crabs.

$NADP^+$-dependent isocitrate dehydrogenase (IDH) is another enzyme of energetic metabolism, which is involved in the aerobic pathway of energy production. This pathway is well more efficient in ATP production than LDH (Moreira *et al.*, 2006; Rodrigues *et al.*, 2013b). These two enzymes often present opposite activities: in other words, decay in IDH activity is expected to be followed by an increase in LDH activity, aiming to compensate the decline in energy production (Rodrigues *et al.*, 2013b). This enzyme is also associated with antioxidant defenses, providing NADPH to glutathione reductase (GR) regeneration of

GSSG into GSH (Jo *et al.*, 2001; Lee *et al.*, 2002; Rodrigues *et al.*, 2012). Many contaminants are known to interact with GSH. IDH activity is expected to increase in exposed animals to deal with the stress inflicted by metabolism of contaminants and the cell redox balance. Enhancing the regeneration of NADPH will indirectly supply the animal with the necessary GSH (Selvakumar *et al.*, 2006; Sunil and Neelam, 2011) to allow a better performance of GST and GPx (Mesquita, 2010) and avoid oxidative damage.

Glutathione *S*-transferases are involved in phase II biotransformation, being responsible for the detoxification of xenobiotics by conjugation with GSH (Rodrigues *et al.*, 2015). GST also has a defense role against oxidative damage, eliminating some of the secondary ROS (Veal *et al.*, 2002; Neuparth *et al.*, 2013). In mammals, AN suffers both non-oxidative and oxidative metabolism: i) nucleophilic reaction with GSH and proteins, and ii) activation through CYP450 that leads to production of epoxide cyanoethylene oxide, which is then converted into cyanide, and other molecules, in extensive secondary metabolism. Reaction with GSH appears to be the major detoxification pathway (Mirkin, 2015). Both AN and the epoxide metabolite may react with GSH to form conjugates subsequently metabolized to mercapturic acids and excreted in urine. In particular, an important mode of action of this HNS seems to be linked to GSH depletion. This decrease in the antioxidant molecule will cause a decline in cell's antioxidant capacity, increase in intracellular ROS and consequent oxidative damage (Kamendulis *et al.*, 1999). AN can also bind covalently to sulfhydryl groups (-SH) of proteins (Friedman *et al.*, 1965; Nerland *et al.*, 2001) and it is supposedly responsible for the inhibition of various –SH dependent enzymes (EURAR, 2004a).

Previous studies in aquatic animals exposed to AN detected alterations in GST activity. More specifically, GST activity was stimulated in seabass' liver exposed to 2 mg L^{-1} for 15 days (Neuparth *et al.*, 2013). As mentioned, AN metabolism would be expected to result in the formation of cyanide. This molecule is known for its toxicity, is a potent generator of ROS (by inhibition of the mitochondrial respiratory chain) (Mirkin, 2015), and has been shown to induce oxidative stress, such as lipid peroxidation, in rats and cell lines (Johnson *et al.*, 1987; Kamendulis *et al.*, 1999). In the study presented herein, GST was induced by 100 µg/L AN at 7d and by 1000 µg/L AN at 21d of exposure, indicating that low and high exposure concentrations may be handled through different metabolisation pathways. GST appears to provide a detoxification response when crabs are exposed to low AN levels, or to high (1000 µg/L) AN concentration in the long-term. This data also suggest that GST was activated probably to act as an antioxidant defense, especially after a long exposure to the contaminant.

Acrylonitrile is able to inhibit the activity of several antioxidant enzymes, including GPx (Mirkin, 2015). In adult Sprague-Dowley rats, for example, inhibition of GPx activity

was recorded in several brain regions after exposure to AN (Rongzhu *et al.*, 2009). In agreement with this, AN exposure decreased GPx activity in crabs at 7d. The observed inhibition could also be due to the metabolic depletion of GSH, which is an important molecule for GPx activity. Glutathione peroxidase is an important component of cell defense system (Rocha *et al.*, *submitted*), being responsible for the conversion of hydrogen peroxide into oxygen and water, using GSH as a cofactor (Livingstone, 2001; Rodrigues *et al.*, 2012). At 14 and 21d, GPx activity in AN-treated crabs was similar to that of controls. Moreover, LPO alterations relative to controls were never detected. Lipid peroxidation is a meaningful detrimental consequence of oxidative stress being highly investigated (Stegeman *et al.*, 1992; van der Oost *et al.*, 2003) to measure oxidative damage to macromolecules (Rodrigues *et al.*, 2014a). This suggests that crabs were able to cope with the stress imposed by exposure to AN, and comes also in support of low extent of oxidative metabolism in these animals. Nevertheless, this HNS is known for covalently binding to proteins and other macromolecules such as lipids (ASTDR, 1990), therefore showing propensity to cause lipid peroxidation (Johnson *et al.*, 1987; Jiang *et al.*, 1998). In a long-term study, in which rats were exposed to AN for 14d, MDA was found to increase in the brain cortex. Hence, compensatory increases of other antioxidant defenses or repair mechanisms (Jiang *et al.*, 1998) may also have occurred in exposed crabs.

IBR index provided a rough qualitative overall measure of the health status of the organisms under study. Distribution of biomarkers in the star plot indicated that AN exposure caused high stress initially (7d). But over time crabs were able to cope with the exposure, showing low stress values at 21d. This may be due to limited bioactivation of AN through CYP450 oxidative pathway. It is of note that although no accumulation could be detected in tissues, AN was able to induce effects in exposed crabs. Considering that this HNS has evaporation properties, and crabs are able to displace freely, if the animals are to overcome the initial chemical stress imposed by the exposure, AN spills may have limited impact on this species.

Aniline

Scarce information is available about AL modes of action in aquatic animals. AL is a polar narcotic, which toxicity is thought to be due to disturbance of the structure and functioning of biomembranes through non-covalent bioreactivity (Bradbury *et al.*, 2008). In mammals, this HNS undergoes p-hydroxylation, catalyzed by multiple CYP450 isozymes, in liver microsomes (La Bella and Queen, 1995). However, though bioactivation reactions are known to occur in mammals, earlier studies suggested metabolic activation may not be involved in its acute toxicity towards rainbow trout (*Oncorhynchus mykiss*) (Bradbury *et al.*,

1990). Studies with rainbow trout have also shown alterations in respiratory-cardiovascular responses of spinally transected fish exposed to AL (Bradbury *et al.*, 1989). Treated animals exhibited strong slowing of all respiratory-cardiovascular functions, as well as decreased ventilation volume and oxygen consumption.

In this study, AL influenced the activity of AChEg, with different temporal responses caused by low and high concentrations, but not that of AChEm. The thoracic ganglion of *C. maenas* has a role in ventilatory function (Simmers and Bush, 1983) while muscle tissue is involved in locomotion. This ganglion is composed of interneurons and motoneurons that are a crucial part of its ventilatory central pattern generator. The motoneurons innervate the levator and depressor muscles controlling the ventilatory appendages (Simmers and Bush, 1983). Inhibition of AChEg elicited by 50 µg/L AL at 14d suggests that ventilatory function might be negatively affected by high concentration of AL, possibly increasing crabs' risk of predation and difficulty to nourish. However, on continued exposure AChEg activity increased above control levels, in a U-shape response over time. Detrimental consequences may also arise from such responses. Augmented AChE activity, or expression, beyond control levels has been recognized has a marker of apoptosis (Zhang and Greenberg, 2012). Effects of AL on biomarkers of estuarine or marine species have seldom been addressed. A recent study investigating short-term (96h) effects of AL (0.5 - 16 mg/l) in the common goby (*Pomatoschistus microps*) and the common prawn (*Palaemon serratus*) indicated that induction of neurotoxicity and energetic alterations took place in both species (Luís and Guilhermino, 2012).

Low (5 µg/L) and high (50 µg/L) AL concentrations triggered different temporal changes in LDH activity. In both cases, however, significant inhibition was noted at 21d of exposure, suggesting that AL may be able to interfere with the enzyme's behavior. IDH activity was inhibited by both AL concentrations (being significant for 50 µg/L), only after 7 days of exposure. In the long-term exposed crabs were able to adapt to the exposure, recovering IDH activity to control levels. Decreased energy production through both anaerobic and aerobic pathways is in agreement with generalized depression of biological activity that could be expected from the narcotic substance.

Phase II biotransformation remained unaltered throughout exposure to AL, but the antioxidant enzyme GPx suffered a significant inhibition triggered by exposure 5 µg/L for 7d. In the short-term (7d) oxidative damage was decreased, probably due to depression of biological activity caused by narcosis. Crabs apparently dealt well with the exposure concentrations because GPx alterations were no longer observed at 14d and 21d of exposure. Previously, AL was found to cause lipid oxidative damage in estuarine common goby fish but not in common prawn (Luís and Guilhermino, 2012). Differences in biotransformation enzymes were also found between the two species, leading the authors

to highlight the need to investigate effects of chemicals in different animals occupying the same ecological niche to improve the risk assessment of HNS spills in the marine environment. The present work supports those observations. Stress levels caused by exposure to AL were clearly detectable at 7d of exposure. At 21d, differences relative to control animals almost vanished, suggesting a possible adaptation to prolonged exposure.

Results for IBR index, at 7d, suggested that exposure to 50 µg/L would cause high stress in the animals, compared to control and 5µg/L treatment. However, as the exposure continues the crabs appear to adapt to this concentration. In crabs exposed to low AL concentration (5 µg/L) stress levels peaked at 14d of exposure. As a polar narcotic, AL is expected to interfere with enzyme's behavior, causing a depression in biological activities. As AN, the metabolite responsible for AL's principal route of toxicity (N-phenylhydroxylamine) is a product of CYP450 activity. Due to residual action of this oxidative pathway in invertebrates, the AL's exposure does not seem to prevent crabs adaptation over time.

V - CONCLUSIONS

Early alterations (7d) were observed after *C. maenas* exposure to AN or AL. Biomarkers of neurotoxicity and anaerobic energy metabolism in muscle, and biotransformation and antioxidant defenses in the digestive gland, were affected by exposure to both low and high concentrations of AN. The responses observed suggest that low oxidative metabolism of AN may have occurred. Biomarkers of neurotoxicity, related to ventilatory function, anaerobic and aerobic energy production and oxidative damage were altered by exposure to AL. Their levels were in agreement with lower overall biological activity expected to be caused by this polar narcotic. This may cause difficulty of crabs to escape their predators, finding adequate food, or displace to less contaminated areas. For both contaminants, crabs seem to be able to adapt to long-term exposure recovering to control levels (21d).

Further research, would involve the analyzis of these selected biomarkers in the gills of experimental crabs to bring further insight into the toxicant metabolism and adaptation. This organ is of great importance due to its direct interaction with the external environment, being therefore a main target of contaminants. In the green crab, it is considered an important route for uptake, bioconcentration and excretion of pollutants (Jebali *et al.*, 2011). Analyzis of other antioxidant defenses such as catalase, superoxide dismutase and GSH levels, damage caused to DNA (e.g. through comet assay), and the development of behavioral experiments (e.g. evaluation of respiratory and cardiac output and locomotion) can also bring important understanding on the modes of action of these HNS and ecological consequences.

The results indicate that long-term exposure is determinant to comprehend the extent of stress caused by these HNS. For risk assessment purposes, they highlight the importance of conducting long-term exposures for accurate risk characterization. Due to scarcity of empirical data, hazard quotients are often derived from short-term assays using either assessment factors or extrapolation corrections to account for long-term worst-case scenarios and empirical data obtained for freshwater species. The research presented herein indicates that more long-term empirical data is needed if reliable calculations, avoiding overestimation but sufficiently protective, are to be obtained and efficient predictive models developed. These long-term evaluations should account for species differences in response to exposure, as well as effects of mixtures of aquatic toxicants on marine species.

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