DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

A short-term sublethal *in situ* toxicity assay with snails to assess and monitor river water quality: a tool for a functional approach

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia, realizada sob a orientação científica do Professor Doutor Rui Ribeiro (Universidade de Coimbra) e da Doutora Matilde Moreira dos Santos (Universidade de Coimbra)

Vânia Isabel Neves Correia

2012

AGRADECIMENTOS

Gostaria de agradecer à Fundação para a Ciência e Tecnologia (FCT, Portugal) por parcialmente financiar este estudo através dos projetos "FRAMEFFECTIVE – Can bioassays be cost-effectively integrated in a predictive model approach for rivers in compliance with the Water Framework Directive?" (referência PTDC/AAC AMB/105411/2008) e "ECOTOXTOOLS - Ecotoxicological tools for assessing agriculture associated environment risks in Southern Europe big man-mad freshwater reservoirs" (referência PTDC/AAC-AMB/103547/2008).

Gostaria de agradecer ao meu orientador Dr. Rui Ribeiro e co-orientadora Dr.ª Matilde Moreira-Santos por me terem aceitado como aluna, pelo acompanhamento, ajuda e motivação que em deram na realização deste trabalho por tudo o que aprendi com eles. Também gostaria de agradecer por toda a ajuda na revisão e melhoramento do manuscrito.

Um agradecimento a todos os membros do IMAR, por o tornarem um lugar agradável para trabalhar, especialmente aos elementos do grupo de ecotoxicologia aquática. Um agradecimento especial à Rita e à Tânia pela ajuda prestada na realização deste trabalho.

A todos os meus amigos que me têm acompanhado neste percurso, pelo apoio, motivação e também pelos momentos de descontração que sempre ajudaram a aliviar a pressão.

Um agradecimento muito especial à minha família, ao meu irmão e especialmente aos meus pais. Não há palavras suficientes para agradecer tudo o que fizeram por mim. Muito obrigado por todo o apoio, motivação e por sempre acreditarem que eu era capaz.

Finalmente, gostaria de agradecer ao Rui por estar sempre a meu lado. Obrigado por toda a motivação, ajuda.

TABLE OF CONTENTS

AGRADECIMENTOS iii

TABLE OF CONTENTS v

ABSTRACT vii

RESUMO ix

Chapter 1 Introduction 1

1.1 Objectives 7

Chapter 2 Materials and Methods 9

2.1 Assay organisms 10

2.2 *In situ* assay chambers and procedures 11

2.3 Study sites 14

2.4 Feeding quantification 17

2.5 Endpoint sensitivity 19

2.6 Influence of environmental exposure conditions on postexposure feeding 20

2.7 *In situ* assay effectiveness 21

2.8 Data analysis 22

Chapter 3 Results 25

3.1 Feeding quantification 26

3.2 Endpoint sensitivity 26

3.3 Influence of environmental exposure conditions on postexposure feeding 27

3.4 *In situ* assay effectiveness 30

Chapter 4 Discussion 35

4.1 Feeding quantification 36

4.2 Endpoint sensitivity 39

4.3 Influence of environmental exposure conditions on postexposure feeding 42

4.4 *In situ* assay effectiveness 43

Chapter 5 Conclusion 45

References 47

ABSTRACT

In the last decades, pollution has greatly increased in aquatic systems due anthropogenic activities, leading to the need to develop tools to assess and monitor river water quality. Faced with the need to enlarge the existent battery of toxicity tools to assess and monitor water quality, particularly in rivers, the aim of the present study was to develop a short-term sublethal *in situ* assay based on the postexposure feeding of the freshwater snail *T. fluviatilis*. To achieve this purpose, a method to precisely quantify the feeding rate of *T. fluviatlis* was first developed under laboratory conditions, consisting of a 3-hours feeding period on 150 defrosted *nauplii* of *A. franciscana* in darkness. The sensitivity of the postexposure feeding response was then tested by comparing it with lethality after exposing the organisms to a range of cadmium concentrations. The 48-hours effective median cadmium concentration for postexposure feeding (EC50) was 179 µg/L and for lethality (LC50) was 208 µg/L, whereas the 96-hours LC50 was 141 µg/L. Therefore, the lethal and sublethal responses showed a similar sensitivity to cadmium. Faced with this finding, the feeding inhibition and mortality were integrated into a recently proposed single population parameter – immediate inhibition of population consumption (popEC50) – since the feeding depression at the population level is caused by both the dead organisms and the feeding inhibition alive ones. The 48-hours popEC50 after exposure to cadmium was calculated through two methods which originated similar values, 157 and 150 µg/L, and revealed that at the 48-hours LC50 concentration (179 µg/L), the immediate inhibition of population consumption would be 79%, which corresponds to a high disruption of the snails ecosystem function. The influence of environmental conditions on the postexposure feeding response was evaluated by performing an *in situ* assay at five reference sites covering different exposure conditions regarding the variables

temperature, current velocity, hardness, conductivity, and sediment organic matter content and particle size distribution (percentages of gravel, sand and silt). Yet, temperature was not included in the model because the variation among sites was minimal and neither conductivity nor percentages of silt and gravel due to strong collinearity with other variables. The regression model showed no significant influence of either environmental condition on the postexposure feeding response, demonstrating the assay can be performed at sites with differences in all investigated environment conditions within the tested ranges. Finally, the effectiveness of the developed assay was evaluated by exposing organisms at three reference and five contaminated sites within the Mondego and Sado river basins. After a 48-hours *in situ* exposure, organisms at the single impacted site were mortality was not 100% (survival of 63%) presented a feeding inhibition relatively to the correspondent reference site (by 46%), showing that the postexposure feeding of *T. fluviatilis* is a potential valid response to detect contaminant effects. Therefore, the developed assay constitutes an important addition to the already existing battery of *in situ* assays used to assess and monitor rivers water quality, even though further studies should be performed mainly on its sensitivity to wider ranges of environmental variables and different types of contaminants.

Keywords: Lotic systems, Cadmium, Postexposure feeding, *In situ* assay, *Theodoxus fluviatilis*

RESUMO

Nas últimas décadas os níveis de poluição aumentaram consideravelmente nos sistemas aquáticos devido a atividades antropogénicas, levando à necessidade do desenvolvimento de ferramentas para avaliar e monitorizar o estado ecológico da água. Face à necessidade de alargar o conjunto de ferramentas ecotoxicológicas já existentes para avaliar e monitorizar a qualidade da água, particularmente em rios, o objetivo deste trabalho foi desenvolver um ensaio *in situ*, subletal e de curta duração, baseado na alimentação após a exposição de um caracol de água doce, o *T. fluviatilis*. Para alcançar este objetivo foi desenvolvido, em condições laboratoriais, um método para quantificar com precisão a taxa de alimentação de *T. fluviatilis*, que consiste em fornecer 150 *nauplii* descongelados de *A. franciscana* durante um período de 3 horas no escuro. A sensibilidade da resposta alimentação após exposição foi depois testada através da sua comparação com a letalidade, após expor os organismos a uma gama de concentrações de cádmio. A concentração efetiva mediana de cádmio às 48 horas para a alimentação após exposição (EC50) foi de 179 µg/L e para a mortalidade (LC50) de 208 µg/L, e o valor de LC50 às 96 horas foi de 141 µg/L. Assim sendo, a resposta letal e subletal mostraram ter uma sensibilidade semelhante ao cádmio. Face a estes resultados, a inibição da alimentação e a mortalidade foram integradas num único parâmetro, recentemente proposto – inibição imediata do consumo da população (popEC50) – já que uma depressão na alimentação ao nível da população é causada tanto pelos organismos que morreram como pela inibição na alimentação dos que permaneceram vivos. O popEC50 para as 48 horas de exposição ao cádmio foi calculado através de dois métodos, que originaram valores semelhantes, 157 e 150 µg/L, e revelaram que para o LC50 às 48 horas (179 µg/L) a inibição imediata no consumo da população seria de 79%, o que corresponde a uma forte disrupção da função dos caracóis no

ecossistema. A influência das condições ambientais na resposta da alimentação após exposição foi avaliada realizando um ensaio *in situ* em cinco locais de referência abrangendo diferentes condições de exposição relativamente às variáveis temperatura, velocidade da corrente, dureza, condutividade, e conteúdo de matéria orgânica e distribuição do tamanho de partículas do sedimento (percentagens de cascalho, areia e finos). Não foram incluídas no modelo a temperatura, porque a variação entre os locais foi mínima, nem a condutividade e a percentagem de cascalho e finos devido a uma forte colinearidade com outras variáveis. O modelo da regressão não mostrou uma influência significativa das condições ambientais na resposta da alimentação após exposição, demonstrando que o ensaio pode ser realizado em locais com diferenças em todas estas variáveis ambientais investigadas dentro dos intervalos testados. Finalmente, a eficácia do ensaio *in situ* desenvolvido foi avaliada expondo os organismos em três locais de referência e cinco locais contaminados nas bacia dos rios Mondego e Sado. Após 48 horas de exposição *in situ*, os organismos do único local contaminado onde a mortalidade não foi 100% (sobrevivência de 6%) apresentaram uma inibição na alimentação após exposição relativamente ao local de referência da mesma bacia (de 46%), mostrando que a alimentação após exposição do *T. fluviatilis* é uma resposta potencialmente válida para detetar efeitos de contaminantes. Assim sendo, o ensaio desenvolvido constitui uma importante adição para o conjunto de ensaios *in situ* já existentes para avaliar e monitorizar a qualidade da água de rios, embora mais estudos devam ser realizados principalmente para investigar a sensibilidade do ensaio proposto a maior gama de valores das variáveis ambientais e diferentes tipos de contaminantes.

Palavras-chave: Sistemas lóticos, Cádmio, Alimentação após exposição, Ensaios *in situ*, *Theodoxus fluviatilis*

Chapter 1 Introduction

Nowadays, aquatic systems are frequently exposed to toxicants, mainly in the form of wastes, leading to a considerably increase in pollution and habitat degradation due to anthropogenic activity. Toxicants may enter into aquatic systems through diffusion, like agricultural runoff from land, urban runoff, dredged sediment disposal, contaminated ground water and bottom sediments, and atmosphere fallout, or through point sources, such as industrial effluents, hazardous waste disposal sites and wastewater treatment plants (Rand, 1995; Islam & Tanaka, 2004; Rodriguez-Mozaz *et al.*, 2004). After entering water bodies, toxicants can act as agents that produce adverse effects in the system, causing harm to living resources including human health, hindering aquatic activities, including fishing, impairing water quality (e.g. for drinking), reducing amenities, eventually damaging the structure and function of the ecosystems (Rand, 1995). Therefore, it is of major importance to evaluate the effects of contaminants in the environment, for which it is necessary to develop assessment and monitoring tools (Allan *et al.*, 2006; Roman *et al.*, 2007; Wharfe *et al.*, 2007).

At present, the need to assess water quality is a component transversal to major pieces of environmental legislation across Europe, like the Water Framework Directive (WFD) (European Community, 2000). The WFD aims to improve, protect and prevent the further deterioration of water quality and to obtain and ensure the good ecological status of all water bodies until 2015 (Marín-Guirao *et al.*, 2005; Allan *et al.*, 2006; Wharfe *et al.*, 2007). To achieve this propose, it is necessary to determine the environmental risks associated with water bodies and implement management plans for ecosystem rehabilitation and monitoring, which require the establishment and use of low-cost quality assessment tools to complement and provide a more holistic estimation of environmental quality evaluations (Allan *et al.*, 2006; Roman *et al.*, 2007).

Laboratory ecotoxicity tests emerged into current quality evaluation strategies in many countries because they offer rapid measurements of environmental effects or of the potential environmental harm posed by contaminants (Rand, 1995; Chapman *et al*., 2002; Wharfe *et al*., 2007). The integration of toxicity tests into environmental quality assessments allows to evaluate the effects of chemicals on living organisms under laboratory controlled and reproducible conditions (Rand, 1995), which provides a causal linkage between contaminants and their biological effects, complementing chemical characterization and the monitoring of indigenous biota (Chapman, 1996; Burton *et al*., 2002). However, toxicity tests performed under laboratory controlled conditions have some limitations: (1) the collection and manipulation of samples may alter toxicity, (2) dynamic systems like estuaries and rivers are very difficult to simulate, (3) interactions among species are not taken into account, (4) fluctuating environmental conditions are not integrated, and (5) standard exposure media do not simulate contaminants adsorption kinetics (Adams, 2003; Chapman *et al*., 2003; Burton *et al*., 2005; Crane *et al*., 2007). It is also important to consider that in natural systems organisms are generally exposed not to a single chemical but rather to a myriad of substances which mixture is difficult to simulate in the laboratory (Rand, 1995). Therefore, most frequently, the observed effects in laboratory cannot be reliably extrapolated to real contamination scenarios (Connell *et al.*, 1999; Burton *et al.*, 2005; Liber *et al.*, 2007).

In situ toxicity testing has been recognized as an useful approach in ecotoxicity assessments as it can be used, either alongside or instead, to transcend the disadvantages of laboratory tests (Crane *et al*., 2007). By exposing organisms in cages at the field sites, *in situ* assays incorporate field fluctuations, are less subjected to artifacts

associated with sample collection and manipulation and the exposure is under more realistic conditions, which result in a more ecologically relevant exposure scenario in a cost-effective way (DeWitt *et al*., 1999; Castro *et al*., 2004; Crane *et al*., 2007; Liber *et al*., 2007; Wharfe *et al*., 2007). However, it is important to be aware that *in situ* assays may also pose some limitations because there are many variables difficult to control, like the stress induced to the test organisms due to their transport to the field and caging (DeWitt *et al*., 1999), the exposure to the fluctuating environmental conditions that may complicate the establishment of a causal link between exposure and effects (Maltby *et al*., 2000; Adams, 2003), deployments in deep or fast-moving systems, the selection of appropriate reference sites, and even the vandalism of chambers during exposure (Burton *et al*., 2005).

The most commonly used test endpoint in aquatic toxicity testing is mortality (Rand, 1995). However, most often mortality does not provide enough information on contamination effects since chemicals are usually discharged at concentrations that induce ecologically relevant effects before killing the organisms (Gerhardt, 1996; Maltby *et al*., 2002; Mills *et al*., 2006; Pestana *et al*., 2007). Regarding sublethal endpoints, the most commonly used in toxicity assays are reproduction and growth (Pereira *et al*., 2000; Ringwood & Keppler 2002; Castro *et al*., 2003). However, measurements of the latter sublethal responses imply long-term exposures and consequently huge efforts in time and money, especially when *in situ* assays are conducted. To circumvent these problems, the feeding response has recently been proposed as a sublethal response in toxicity tests, because an inhibition in feeding is known as a fast and sensitive response to a vast array of contaminants (Maltby, 1999; Maltby *et al.*, 2002; McWilliam & Baird, 2002; Castro *et al*., 2004).

Feeding is a physiological function to organisms linked with growth, reproduction and survival and a depression in feeding rate, namely due to contaminants, may have direct effects on parameters at the individual level. These effects are successively transferred to higher levels of biological organization, which can compromise the success of populations and communities (Taylor *et al*., 1998). Moreover, a depression in feeding can also have direct and immediate effects on ecosystems, by hampering key functions (e.g. grazing, organic matter decomposition, secondary production), long before the effects at the individual level (e.g. reproduction, growth) have consequences at successively higher levels of biological organization (Forrow & Maltby, 2000; Krell *et al*., 2011; Agostinho *et al*., 2012). Given that feeding rates during exposure, particularly in the field during *in situ* testing, are difficult to measure and it is known that the feeding response to contaminants usually persists in the period immediately after the exposure, the feeding rate can be quantified just after the exposure (before physiological recovery) – being the test endpoint designed as postexposure feeding – which allows tests to be methodologically simple (Taylor *et al*., 1998; McWilliam & Baird, 2002; Moreira *et al*., 2005; Moreira *et al*., 2006a).

When feeding depression occurs at partially lethal concentrations, the immediate inhibitory effect on the population consumption is due not only to a reduced population size, but also to a decrease on the feeding rate of the organisms that continue alive. Therefore, it is important to integrate survival and feeding into a single toxicity parameter – the immediate inhibition of population consumption – to improve ecological realism (Agostinho *et al.*, 2012).

In ecotoxicological assays, the choice of the test species is an important aspect as it may highly influence the ecological relevance of the test results, both in field and in laboratory approaches (Baird *et al*., 2007). Yet, because it is impossible to acquire all the necessary understanding on the great majority of the species, it is necessary to focus on relevant species, i.e., if possible, on species that have a key role in the structure and function of the ecosystems, like keystone, sentinel or dominant species, and also the chosen species should be abundant (Baird *et al*., 2007; Amiard-Triquet, 2009).

Snails are an integral component of freshwater lotic and lentic communities both as primary consumers (Ruehl & Trexler, 2011) and as prey for invertebrate (leeches, flies and beetles) and vertebrate taxa (fish) (Brown & DeVries, 1985; Lodge, 1986; Reed & Janzen, 1999). Their grazing activity influences the dynamics of the macrophyte epiphyte system by reducing shading and competition for nutrients and through significant shifts in algal communities (Brönmark, 1989). Degradation of water quality may be an important factor affecting the declining populations of freshwater snails (Crichton *et al*., 2004; Besser *et al*., 2007). The reduction or elimination of a population of snails from the freshwater community may have significant consequences in the structure and function of communities (Lefcort *et al*., 2000; Ruehl & Trexler, 2011). Freshwater snails in lakes polluted with metals failed to exhibit antipredator behaviour (Lefcort *et al*., 2000). In the case of grazers, reduced feeding rate in organisms exposed to contaminants may have indirect effect on the algal community structure (Evans-White & Lamberti, 2009).

Theodoxus fluviatilis (L.) is a Prosobranchia Neritidae freshwater snail with a large distribution throughout Europe and Anatolia in lakes, streams, rivers, canals, and

even estuaries and brackish waters, up to 60 m in depth (Zettler *et al.*, 2004; Bunje, 2005; Kirkegaard, 2006; Bunje & Lindberg, 2007). Its distribution is favoured by rapid currents, high content of calcium, carbon dioxide, dissolved oxygen, and high conductivity (Carlsson, 2000; Bunje, 2005; Kirkegaard, 2006; Graça *et al*., 2012), being absent in lakes with high contents of humic substances (Carlsson, 2000). *Theodoxus fluviatilis* is a scraper that feeds on biofilms, ingesting mainly diatoms, but also feeds on cyanobacteria and detritus (Zettler *et al.*, 2004; Kirkegaard, 2006). In rivers, *T. fluviatilis* is mainly fixed to the substrate, preferably stones where they scrap food, in shallow areas and is less present in vegetation (Zettler *et al*., 2004; Graça *et al*., 2012). The densities of the populations of the *T. fluviatilis* are variable. According to Graça *et al.* (2012), the density of *T. fluviatilis* in the spring source of Anços river (Portugal) can vary from approximately 10 to 9 000 individuals per square meter, but decreases to zero 3 800 m downstream. In the littoral zone of Lake Esrom (Denmark), a local population showed a variable density, between 556 and 2 100 individuals per square meter, with a mean annual abundance of 1 084 individuals per square meter (Kirkegaard, 2006).

1.1 - Objectives

The aim of the present study was to develop and evaluate a short-term sublethal toxicity *in situ* assay based on the postexposure feeding of the freshwater snail *T. fluviatilis* to complement river quality assessments and monitoring. To achieve this purpose, four specific objectives were delineated: (1) to develop a method to easily, precisely and rapidly quantify the feeding rate of *T. fluviatilis* in the laboratory, while preventing organisms' physiological recovery, (2) to investigate the sensitivity of the postexposure feeding response relatively to survival, after exposure to the model

toxicant cadmium, (3) to determine the influence of environmental factors on the postexposure feeding response, to infer upon the need to discriminate potential environmental from contaminant effects, and (4) to evaluate the effectiveness of the proposed *in situ* assay by deploying it at several reference and allegedly impacted sites within the Mondego and Sado River basins.

Chapter 2 Materials and Methods

2.1 - Assay organisms

Organisms of *T. fluviatilis* were collected at the spring source of the Anços River (Central Portugal: 39º58'43.40"N, 8º34'23.30"W), with a mean (± standard error) annual water temperature of 16.1ºC (±0.1) (Graça *et al*., 2012). This site was previously classified as "minimally disturbed" based on (i) classes of water quality for multiple uses by the National Water Agency (INAG, Portugal), (ii) species composition and abundance in the riparian corridor, (iii) channel morphology, and (iv) urbanization and industrial activities in the catchment area (Feio *et al*., 2010), and presently considered as being in a good ecological status for support of aquatic life according to the physico-chemical parameters listed under the national implementation of the Water Framework Directive (M.J. Feio, personal communication).

Organisms were handpicked from stream-bed stones and transported in thermally insulated boxes filled with local water to the laboratory or directly to the *in situ* assays sites. For the laboratory experiments (feeding quantification and endpoint sensitivity), organisms were conditioned for transportation (30 minutes trip) in plastic containers (length × width × height of 21 × 15.4 × 9.4 cm). In the laboratory, cultures were maintained in a temperature controlled room at 19 to 21ºC under a 14:10-hours light:dark cycle, in plastic containers with aerated culture medium (approximately 5-cm height) and local stones, as *T. fluviatilis* is a scraper feeding on biofilm (Zettler *et al.*, 2004; Kirkegaard, 2006). Culture medium consisted initially of 100% local water and within the first week was gradually changed to 100% reconstituted hard water (ASTM, 2002), hereafter referred to as ASTM medium; medium was renewed three times a week and plastic containers were renewed once a week. Each collected batch of organisms was maintained under these controlled laboratory conditions during a maximum period

of two weeks. For the *in situ* assays, organisms were collected at the same day as deployment and conditioned to be transported to the study sites in 50-ml polypropylene vials (10 organisms/vial; see section 2.2) filled with local water.

The size of each experimental batch of organisms was estimated at the end of each experiment/assay through measurements of the shell height and length in a sample of 20 organisms, according to the scheme presented in Figure 1 (Zettler *et al.*, 2004).

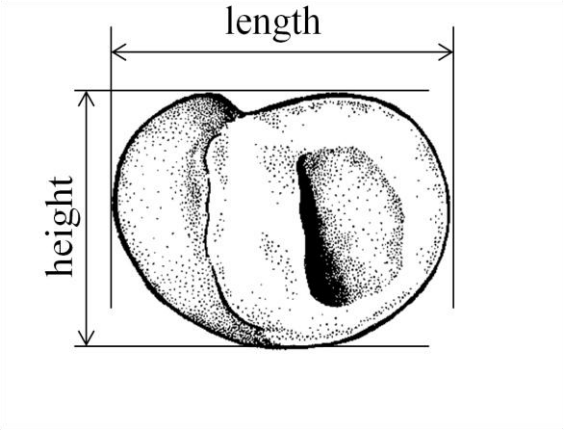

Figure 1 – Scheme of the shell dimensions used to measure the length and height of the shell of *Theodoxus fluviatilis* (adapted from Zettler *et al.*, 2004).

2.2 – *In situ* assay chambers and procedures

The assay chambers used were those previously developed by Moreira *et al.* (2005). Each assay chamber (Figure 2) consisted of a 20-cm long and 5 cm inner diameter acrylic tube with open ends with 0.5 cm wall thickness, being the bottom edge slimmed for 1 cm to facilitate penetration into the sediment. The exchange of overlying water (at the water-column level) and porewater (at the sediment level) between the interior and exterior of the chamber was guaranteed by two opposite rectangular windows (4 x 10 cm) covered with a 200-µm nylon mesh. To cover the top end of the

assay chamber during exposure, a nylon mesh (15 x 15 cm), held by an elastic band, was used.

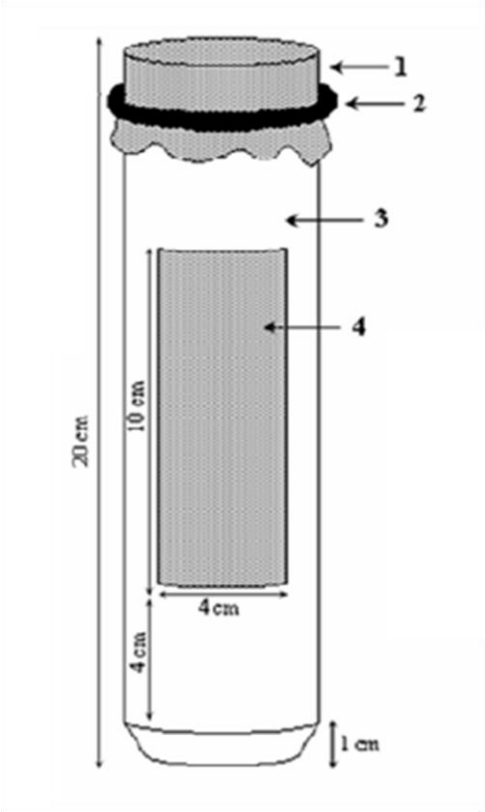

Figure 2 – Schematic view of assay chamber for the 48-hours *in situ* assay with *Theodoxus fluviatilis*; (1) 200-µm nylon mesh cover (15 x 15 cm), (2) 1-cm wide elastic band, (3) transparent plastic tube (2-m long and 4-mm inner diameter), (4) 200-µm mesh lateral widow (4 x 10 cm) (adapted from Moreira *et al*., 2005).

To deploy chambers, they were carefully pushed into the sediment to a depth of about 10 cm, so that the lateral windows allowed both the flow of the overlying water at the water-column level and of porewater at the sediment level. When needed, a holding structure made from a 10 x 21 x 25-cm basket of plastic coated metal wire was used to protect assay chambers from strong water flows (Soares *et al.*, 2005). Three replicate chambers were deployed at each site and 10 organisms were deployed per chamber. Although only 8 organisms were required to quantify postexposure feeding rates, the

deployment of 10 organisms per chamber, while respecting field densities (Kirkegaard, 2006; Graça *et al.*, 2012) facilitated organism retrieval at the end of the assay. After organisms' deployment chambers were covered with the 200-µm nylon mesh.

After the 48 hours exposure period, chambers were retrieved from the sediment and individually placed into trays to retrieve the organisms from the sediment. Organisms from each replicate were placed into the same 50-ml polypropylene used for transportation to the field sites filled with local water to prevent organisms' physiological recovery, and transported to the laboratory inside thermally insulated boxes at approximately 20º C. A 5-hours period since the retrieval of the chambers until the beginning of the postexposure feeding quantification was established to provide similar conditions among sites. After this period, individuals were allowed to feed according to the procedures described below (section 2.4).

At each site, conductivity (Wissenschaftlich Technische Werkstatten Cond315i/SET conductivity meter, WTW, Weilheim, Germany), dissolved oxygen (WTW OXI 92 oxygen meter) and current velocity (Valeport 108 MkIII current meter; Devin, United Kingdom) were measured at deployment (sections 2.6 and 2.7) and the former two parameters also at retrieval at the allegedly contaminated sites (section 2.7). Values of pH (WTW 537 pH meter) and hardness (only for *in situ assay* at reference sites; section 2.6) were measured in samples collected at the study sites immediately upon arrival to the laboratory and kept frozen until analysis, respectively, Temperature (Cond315i/SET) was measured at the beginning and end of all deployments.

At the end of all deployments, composite sediment samples were collected into black airtight plastic bags, transported to the laboratory and stored at 4ºC until processed for sediment characterization, i.e., humidity, organic matter content and particle size distribution. Water content was determined by measuring the loss of

sediment weight after oven drying at 60ºC for three days and was expressed as the mean percentage of the initial wet weight of three sediment subreplicates. Organic matter content (percent volatile solids) was determined by quantifying the loss of weight after ignition in a muffle furnace (Nabatherm L3, Lilienthal, Germany) at 600ºC for 6 hours and was expressed as the mean percentage of the oven-dry weight of the sediment (Buchanan & Kain, 1971). The sediment particle size distribution was analyzed by sieving the sediment (pooling of three subreplicates) into eleven fractions, ranging from 63 µm to 64 mm. The sediment remaining on each sieve was weighted, expressed as a percentage of the final weight and classified according to the Wentworth scale (Buchanan & Kain, 1971).

2.3 – Study sites

To evaluate the effect of the environmental conditions on the 48-hours postexposure feeding of *T. fluviatilis* (section 2.6) and the effectiveness of the proposed *in situ* assay (section 2.7), five reference sites (R1 to R5) were selected within the Mondego River basin (Central Portugal) and one reference (R6) and five sites allegedly impacted by a mine effluent, characterized by very low pH and high metal concentrations (I1 to I5), were selected within the Sado River basin (South Portugal).

The Mondego hydrological basin is located entirely in Portuguese territory, having about 6 670 km^2 (Marques *et al*., 2002). All reference sites within this river basin were selected based on their classification as "reference sites", identified through predictive models for water quality assessments in compliance with the WFD using macroinvertebrate communities as biological indicators and various environmental explanatory variables (Feio *et al*., 2007). The five reference sites were the following:

spring of the Anços River (R1: 39º58'43.40"N, 8º34'23.30"W), Anços River (R2: 39º59'53.45"N, 8º35'02.15"W), Arunca River (R3: 39º52'53.00"N, 8º37'12.65"W), Crespos Stream (R4: 39º54'28.45"N, 8º42'18.00"W) and São João Stream (R5: 40º05'58.50"N, 8º14'02.60"W) (Figure 3).

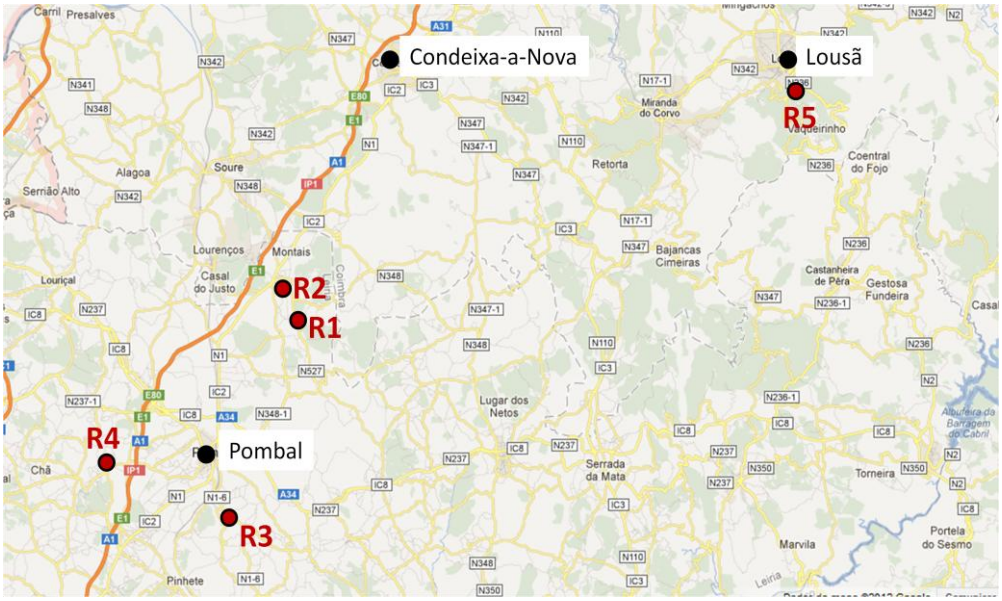

Figure 3 – Study sites selected as reference sites (R1 to R5) within the Mondego River basin (Central Portugal) for the *in situ* assay to evaluate the influence of environmental conditions on the 48-hours postexposure feeding of *Theodoxus fluviatilis* (from Google maps. (Consult. 27 jul. 2012) URL: http://maps.google.com/?ll=40.00912,-8.43613&z=10&t=h).

The Sado River basin is located in Alentejo (South Portugal) with an area of about 7 640 km^2 (Rocha, 1998; Burke *et al.*, 2011). The Aljustrel cupriferous pyrite mine is located within the area of this basin (Lopes *et al.*, 2000). It is one of the greatest mines within the Iberian Pyrite Belt, a world class volcanic-hosted massive sulphids metalogenetic province (Luís *et al.*, 2009; Alvarenga *et al.*, 2008). The mine effluent, with its origin on the mine tailings, runs through the small Água Forte Stream which drains into the Roxo River, an effluent of the Sado River. Low pH values and high

metal concentrations are present in the stream, mainly (concentrations, in mg/L, previously detected in the Água Forte Stream; Lopes *et al*., 2000) Fe (1095), Al (695), Zn (695), Cu (160), Mn (123), Co (3.6), Cd (1.51), and Ni (1.47); yellow precipitates of $FeOH_3$ (commonly referred to as "yellow-boy") contribute to a high water acidity (Harris *et al.*, 2003). The reference site was selected in the Roxo River upstream the Aljustrel mine contaminated area (R6; 37º56'29.60"N, 8º08'04.30"W), whereas the five impacted sites were located along the dilution gradient of the mine effluent in the Água Forte Stream (I1: 37º56'31.10"N, 8º08'53.35"W; I2: 37º56'39.05"N, 8º09'01.05"W; I3: 37º56'41.00"N, 8º09'01.90"W); I4: 37º56'42.70"N, 8º09'01.85"W; I5: 37º56'43.20"N, 8º09'02.06"W) (Figure 4).

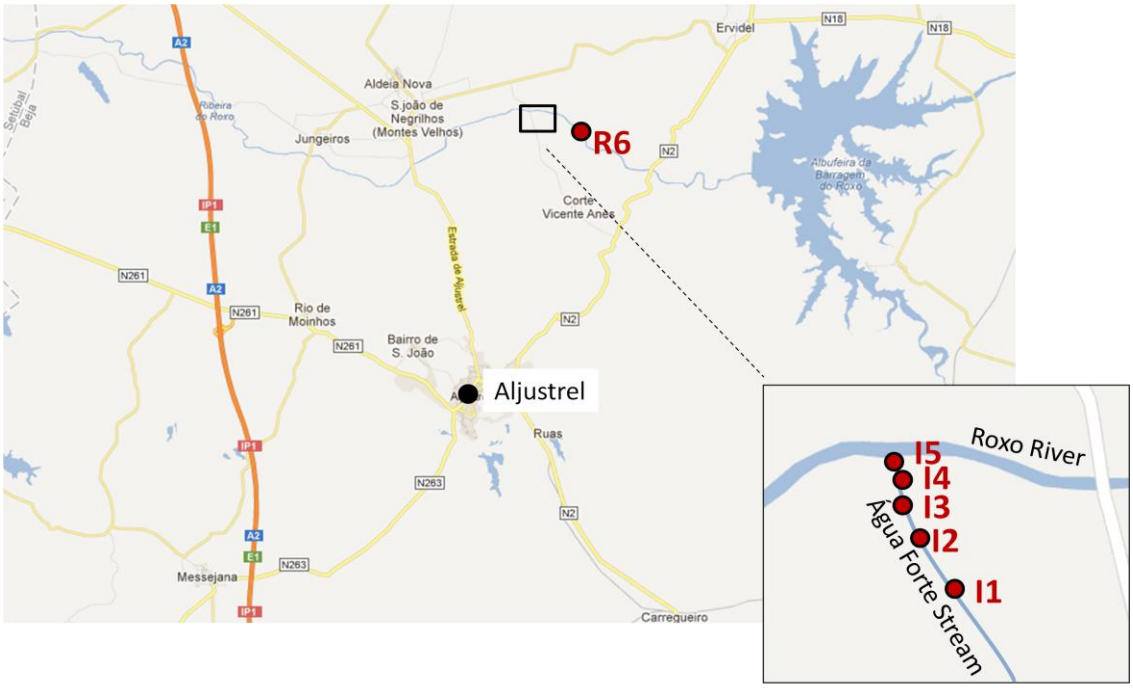

Figure 4 – Study sites selected as reference (R6) and allegedly impacted (I1 to I5) within the Sado River basin (South Portugal) for the 48-hours postexposure feeding *in situ* assay with *Theodoxus fluviatilis* to evaluate the effectiveness of proposed assay chambers and procedures (from Google maps. (Consult. 27 jul. 2012) URL: http://maps.google.com/?ll=37.91872,-8.14477&z=12&t=h).

2.4 - Feeding quantification

To develop a methodology to precisely quantify the postexposure feeding rates of *T. fluviatilis* in the shortest possible period, aiming at minimizing organisms' physiological recovery, preliminary and definitive experiments were conducted with organisms deprived of food during the previous 24 hours. Two food types were preliminary tested: dried spinach mats and defrosted *nauplii* (less than 24-hours old) of *Artemia franciscana*. The dried spinach mats were prepared based on the procedures described by Crichton *et al.* (2004). In brief, frozen spinach (Iglo, Porto Salvo, Portugal) was macerated with a hand-blender using ASTM medium (1g/1 ml) for 2 minutes, 5 ml of this mixture were used to fill the bottom of 8-cm diameter plastic Petri dishes, which were left to dry at 60ºC overnight. To quantify feeding rates on dried spinach mats, egestion was used as a surrogate of feeding following the methodology proposed by Krell *et al*., (2011), i.e., a three-stage procedure (at 20ºC in darkness): (1) a first stage for organisms to clean their guts from the food ingested during exposure (5 organisms/5-cm diameter plastic Petri dish filled with 12 ml ASTM medium for 60, 90, 120, and 180 minutes), (2) a second stage to allow organisms to feed on spinach (5 organisms/8-cm diameter plastic Petri dish filled with 25 ml ASTM medium for 1 hour), and (3) a final third stage to quantify egestion rates (5 organisms/5-cm diameter plastic Petri dish filled with 12 ml ASTM medium for 60, 90 and 120 minutes). The observed results showed a minimal consumption on spinach, a high variability in faecal pellets produced and difficulty in quantifying individual faecal pellets.

Nauplii of *A. franciscana* were obtained by incubating cysts (Creasel, Deinze, Belgium) in standard reconstituted seawater (salinity and pH of 35 and around 7.6, respectively) at 24 to 26ºC under continuous light (75 $\mu E/m^2/s$) during 24 hours (Soares

et al., 2005). They were then counted under a stereomicroscope into 1.5 ml eppendorfs with ASTM medium and stored at -20ºC up to a maximum period of two months. Preliminary experiments were conducted with different combinations of the following variables: vial shape and volume of ASTM medium (30-ml glass vials with a 2.2 cm diameter filled with 5 ml medium and 24-well microplates with 1 ml medium per well), organism density (1, 3 and 5), food quantity (100 and 150 *nauplii*), and feeding periods (1 up to 3 hours). Results showed *nauplii* to be actively consumed by *T. fluviatilis* relatively to spinach and thus a suitable food to quantify feeding rates.

Based on these preliminary results and to optimize the feeding rate of *T. fluviatilis* on *A. franciscana nauplii*, an experiment was conducted to investigate the influence of food quantity (100 *versus* 150 *naupii*). For each treatment, 20 replicates were set up in 24-well microplates with each replicate consisting of 1 organism/well filled with 1 ml ASTM medium. Organisms were allowed to feed for 3 hours in darkness. Organisms used in this experiment had a mean (± standard deviation [SD]; n = 20) shell height and length of 4.39 (±0.24) and 5.56 (±0.25) mm, respectively. Results obtained (see Results section) determined the following methodology to determine the feeding rates of *T. fluviatilis*: snails placed individually in 1 well of 24-well microplates containing 1 ml of ASTM medium and 150 defrosted *nauplii* of *A. franciscana* and allowed to feed in darkness for a period of 3 hours, to estimate feeding rates as number of consumed *nauplii*/organism/3 hours.

A second experiment was conducted to evaluate whether the time lag between organism collection and feeding quantification, i.e., laboratory culture age (1 up to 7 days) influences *T. fluviatilis* feeding rate. In parallel, the influence of organism size was also investigated. During seven days in a row, starting the day after organism collection, 20 replicate organisms with an overall mean (± SD) shell height and length

of 4.02 (±0.22) and 5.05 (±0.22) mm were collected from cultures and allowed to feed under the conditions above stated. At days 3 and 4, 20 organism of a different size class, i.e., with an overall mean (± SD) shell height and length of 4.86 (±0.22) and 6.12 (±0.33) mm were also used to quantify feeding rates as consumed *nauplii*/organism/3 hours.

2.5 - Endpoint sensitivity

The sensitivity of the postexposure feeding response of *T. fluviatilis* was evaluated by exposing organisms to lethal and sublethal concentrations of cadmium to compare the 48-hours median effect concentration (EC50) for postexposure feeding with the 48- and 96-hours median lethal concentration (LC50). An exposure period of 48 hours for the postexposure feeding test was chosen because feeding is expected to be a fast response to contaminants that persists in the period immediately after exposure (McWilliam & Baird, 2002; Moreira *et al.*, 2005; Pestana *et al.*, 2007; Krell *et al.*, 2011; Agostinho *et al*., 2012).

A stock solution of cadmium (5 g/L) prepared in nanopure water with cadmium chloride monohydrate (Merck, Darmstadt, Germany) was used to prepare the test solutions by adding the appropriate volumes to ASTM medium. For the 96-hours lethality test, a control (ASTM medium) and seven nominal cadmium concentrations were used using a dilution factor of 1.5 (750, 500, 333, 222, 148, 98.8, and 65.8 µg/L). To estimate the 48-hours LC50 and EC50 for postexposure feeding (using the same test and checking for dead organisms after quantifying postexposure feeding, see below), a control and the same seven nominal cadmium concentrations of the 96-hours lethal test were used. The mean (±SD; n = 20) shell height and length of the organisms used in the

96-hours lethal test was 4.25 (±0.21) and 5.37 (±0.21) mm, whereas the correspondent means of those used in the 48-hours test was 4.32 (±0.23) and 5.38 (±0.24) mm.

For both tests, five replicates were set up per test concentration. Each replicate consisted of 5 organisms in a 60-ml glass vial filled with test solution, covered with a 200-μm nylon mesh, held in place with an elastic band, and placed inside polyethylene terephthalate containers (5-cm diameter) filled with the test solution, so that the vials were submerged (to guarantee continuous organism exposure). No food was added during the tests. Both tests were conducted at 19 to 21°C under a 14:10-hour light:dark photoperiod. In the 96-hours test, solutions were renewed once after 48 hours to ensure that cadmium concentrations were maintained. After the 96-hours exposure, mortality was assessed by observing organisms under a stereomicroscope. For the organisms not showing movements, mortality was verified by touching the organisms with a needle and snails that did not react to the touch were counted as dead. In the 48-hours test, postexposure feeding was quantified immediately at the end of exposure following the procedures described above, and only after, not to induce stress to organisms, mortality was assessed. Conductivity, dissolved oxygen and pH were measured in two replicates of all fresh and old test solutions.

2.6 – Influence of environmental exposure conditions on postexposure feeding

The influence of environmental conditions on the background variability of the 48-hours postexposure feeding of *T. fluviatilis* was investigated *in situ*, following the procedures described in section 2.2. For this, an *in situ* assay was performed at five reference sites (R1 to R5) within the Mondego River basin (central Portugal; please see

section 2.3). These five sites were selected to cover different environmental exposure conditions regarding the variables temperature, current velocity, hardness, conductivity, and sediment organic matter content and particle size distribution, even though pH and dissolved oxygen were also measured (for further details see section 2.2). Besides, to maximise the number of different combinations involving sediment particle size and current velocity, four and two treatments were deployed at sites R1 and R5, respectively. The mean (±SD; n = 20) shell height and length of the organisms used in this assay was 4.23 (±0.13) and 5.28 (±0.14) mm.

2.7 – *In situ* assay effectiveness

The effectiveness of the proposed assay chambers and methodologies for toxicity evaluation based on the 48-hours postexposure feeding of *T. fluviatilis* was evaluated by performing an *in situ* assay at three reference (R1, R2 and R6) and five sites allegedly impacted by an acid mine drainage (for details on selected sites see section 2.3), following the procedures described in section 2.2. Whereas site R6 was selected as a reference within the same river basin as the impacted sites, sites R1 and R2 were selected as references within the same river basin where organisms were collected and the influence of environmental conditions on the background variability in postexposure feeding was evaluated. The mean (±SD; n = 20) shell height and length of the organisms used in this *in situ* assay was 4.40 (±0.15) and 5.44 (±0.14) mm.

2.8 - Data analysis

For the first experiment on the influence of food quantity (100 *versus* 150 *naupii*) on the feeding rate of *T. fluviatilis*, means were compared through the Student's *t*-test and differences between the coefficients of variation (CV) of the two means were tested through the Miller equation (Zar, 1996). In the second experiment, the effect of laboratory culture age (up to 7 days) on the feeding rate of the smallest organism size class was assessed by one-way analysis of variance (ANOVA). As for the effect of organism size on feeding rate, it was evaluated by a Student's *t*-test comparing feeding rates of the pooled organisms (n = 40) of the two size classes. This effect was further evaluated with Pearson's correlation. Prior to these analyses (second experiment), a one-way ANOVA confirmed that all sets of 20 organisms used during the 7-days experiment had a similar shell height and length ($F_{6,133} > 0.92$; $P > 0.30$), whereas Student's *t*-tests confirmed the feeding rates of the two groups of twenty organisms within each size class (two days feeding trial) to be similar ($t_{37\text{-}38} < 0.26$; $P > 0.18$) and the shell height and length between (the pooled 40) organisms of the two size classes to be significantly different ($t_{78} < 15$; $P < 0.001$). Prior to all analysis of variance, the assumptions of normality (Shapiro–Wilk's test) and homoscedasticity (Bartlett's test) were checked.

The 48- and 96-hours lethal concentrations (LC20 and LC50) for cadmium and the corresponding 95% confidence limits (CL) were calculated using the software PriProbit 1.63 (Sakuma, 1998) (http://bru.gmprc.ksu.edu/proj/priprobit/download.asp), which applies the probit transformation on the proportion of mortality and the logarithmic transformation on the concentration values. The 48-hours cadmium effective concentrations for postexposure feeding (EC20 and EC50) and the

correspondent 95% CL were estimated by fitting the feeding response to a logistic model (EC, 2004).

To integrate the effect of cadmium due to lethality and feeding inhibition into a single parameter (immediate inhibition of population consumption) two methods were used (Krell *et al.*, 2011; Agostinho *et al.*, 2012). The first method consisted of recalculating the 48-hours EC50 and EC20 by multiplying individual feeding rates by the respective proportional survival at each cadmium concentration. In the second method the concentration-response relationships relatively to feeding and to survival were integrated as x% of the population consumption inhibition induced by the cadmium concentration that simultaneously caused (i) a y% mortality (i.e., the LCy) and (ii) a w% depression on individual feeding (i.e., the ECw) (therefore LCy = ECw); x (in %) being quantified as:

$$x = [1 - (1 - y/100) \times (1 - w/100)] \times 100$$

To quantify the influence of abiotic parameters on the feeding rate, a multiple linear regression analysis was performed, using the software R 2.6.0 (http://cran.r-project.org/bin/windows/base/) and Brodgar 2.5.7 (Highland Statistics, Newburgh, UK). Explanatory variables included in the model were current velocity, hardness, organic matter content, and percentage of sand (particle size between 0.063 and 2.0 mm). Temperature and pH were not included in the model because their variation among sites was minimal. Conductivity and percentages of silt and gravel were excluded due to strong collinearity, being correlated with hardness, organic matter content and percentage of sand, respectively ($r = 0.89$, $r = 0.99$ and $r = 1.00$).

To evaluate the effectiveness of the proposed *in situ* assay, the postexposure feeding rate between the impacted site I5 (since survival was 0% at the remaining four impacted sites) and the reference site R6 within the same river basin (Sado) was

compared by one-way nested ANOVA. Prior to this analysis, postexposure feeding rates of the organisms from the three reference sites were tested by a one-way nested ANOVA to confirm that there were no differences between all reference sites.

Except otherwise stated, all analyses were performed using the software Statistica 8.0 (StatSoft, Aurora, CO, USA) and the level of significance was set at 0.05.

Chapter 3 Results

3.1 - Feeding quantification

When testing the influence of food quantity on the feeding rate of *T. fluviatilis*, significant more defrosted *nauplii* of *A. franciscana* were eaten when 150 rather than 100 items were given to each organism, with feeding rates of 13 and 22 *nauplii*/organism/3 hours, respectively (Student´s *t*-test: $t_{35} = 2.96$; $P < 0.01$). However, no significant differences were found between the CV values of the two means (49 and 46%, respectively; Z = 0.216; $P > 0.05$). Thus, to maximize the power of discrimination among treatments, 150 *nauplii* were used in all subsequent experiments/assays.

The laboratory culture age (up to 7 days) had no significant effect on the feeding rate of *T. fluviatilis* (one-way ANOVA: $F_{6,127} = 1.64$; $P = 0.14$); overall means ranged from 20.8 to 29.4 *nauplii*/organism/3 hours with CV values between 30 and 49%. Moreover, feeding rates were not significantly influenced by organism size within the two size classes considered (mean shell height and length of 4.02 and 5.05 mm and of 4.86 and 6.12 mm, respectively), as shown by Student´s *t*-test comparing feeding rates ($t_{78} = 0.40$; $P > 0.88$) and Pearson's correlation between feeding rate and shell size (height: r = 0.092; $P = 0.22$; length: r = 0.11; $P = 0.14$).

3.2 - Endpoint sensitivity

Overall, dissolved oxygen was always above 40% and pH and conductivity ranged within short optimal intervals, 7.23 to 7.87 and 547 to 595 µS/cm, respectively. In both the 48-hours postexposure feeding and 96-hours lethal tests for cadmium no control mortality was observed. In the 48-hours test, mortality was observed as of the 222 µg/L concentration and 100% mortality was observed in the two highest

concentrations (500 and 750 µg/L). The 48- and 96-h LC50 values (95% CL) of cadmium were 208 (186 – 230) and 141 (122 – 162) µg/L, respectively, whereas the correspondent LC20 values (95% CL) were 162 (136 – 180) and 91.6 (72.5 – 108) µg/L. The 48-hours EC50 and EC20 values (95% CL) for postexposure feeding were 179 (137 – 222) and 96.1 (54.7 – 137) µg/L, respectively.

Using the first and the second integration techniques, the 48-hours median immediate inhibition population consumption (popEC50) estimates for cadmium were 157 (95% CL: 134 – 181) and 150 µg/L, respectively; the latter resulting from a 15% mortality and a 41% individual feeding depression. At the median individual effective concentration (48-hours EC50 = 179 µg /L), the mortality would be 31% and the population-level feeding depression would be 66%. At the median lethal concentration (48-hours LC50 = 206 µg /L), the individual feeding depression would be 58% and the population feeding depression would be 79%.

3.3 - Influence of environmental exposure conditions on postexposure feeding

Results of the physico-chemical parameters measured in the water column during the *in situ* assay and of the sediment characteristics at each site are presented in Tables I and II, respectively. The required numbers of 8 organisms for postexposure feeding quantifications were rapidly retrieved from the chambers and no mortalities were recorded. The multiple linear regression analysis revealed that neither current velocity, nor hardness, nor sediment organic matter content, nor percentage of sand significantly influenced the 48-hours postexposure feeding rate of *T. fluviatilis* ($P > 0.15$).

Table I – Physico-chemical parameters measured in the water column at each of the five reference sites (R1 to R5) within the Mondego River basin (Central Portugal) to evaluate the influence of environmental exposure conditions on the 48-hours postexposure feeding of *Theodoxus fluviatilis*. Temperature (T), conductivity (Cond) and dissolved oxygen (DO) were measured at the field sites and pH and hardness at the laboratory in samples collected at the study sites. Each line corresponds to each combination of variable performed in order to cover different environmental exposure conditions.

Site	T (ºC)[b]	Sediment[c]	Current velocity (m/s)[d]	Cond (µS/cm)[d]	DO (mg/L)[d]	Hardness (mgCaCO3/L)[d]	pH[d]
R1[a]	16.3 - 16.6	medium-very coarse gravel	0.60	550	9.60	129.1	7.09
	16.3 - 16.6	medium-coarse sand	0.60	550	9.60	129.1	7.09
	16.3 - 16.6	medium-very coarse gravel	0.00	550	9.60	129.1	7.09
	16.3 - 16.6	medium-coarse sand	0.00	550	9.60	129.1	7.09
R2	16.4 – 17.0	coarse-very coarse sand	0.00	528	10.3	129.1	7.84
R3	15.5 - 17.3	very fine-medium sand	0.02	668	10.3	82.3	7.85
R4	15.9 - 17.2	fine-medium sand	0.25	175	9.60	62.7	7.05
R5[a]	14.4 - 16.1	medium-coarse gravel	0.20	45.7	13.2	20.9	7.13
	14.4 - 16.1	medium-coarse gravel	0.00	45.7	13.2	20.9	7.13

[a] more than one treatment was deployed to maximise the number of environmental variables combinations
[b] measured at assay deployment and retrieval
[c] sediment type based on particle size(s) with more than 50% (see Table II for more details on sediments particle size distribution)
[d] measured at assay deployment

Table II – Water and organic matter content (in %) and particle size distribution (in %) of the six sediments collected at the reference sites (R1 to R5; at R1 two different types of sediment were evaluated to maximise the number of environmental variables combinations) within the Mondego River basin (Central Portugal) to evaluate the influence of environmental exposure conditions on the 48-hours postexposure feeding of *Theodoxus fluviatilis.*

	R1 (gravel)[b]	**R1 (sand)**[c]	**R2**	**R3**	**R4**	**R5**
Water[a]	6.55 (±0.95)	18.0 (±0.46)	23.1 (±0.56)	60.8 (±1.3)	17.8 (±0.18)	7.59 (±0.65)
Organic matter[a]	0.81 (±0.72)	0.26 (±0.10)	1.4 (±0.28)	9.0 (±1.2)	0.57 (±0.034)	1.1 (±0.047)
Particle size						
32 - 64 mm	22.9	0.00	0.00	0.00	0.00	2.00
16 - 32 mm	3.50	0.00	0.372	0.00	0.664	26.8
8 - 16 mm	33.3	0.00	0.954	0.00	0.928	42.9
4 - 8 mm	18.1	0.166	3.24	0.00	0.685	20.4
2 - 4 mm	11.5	1.61	11.1	6.59	1.74	5.25
1 - 2 mm	4.93	17.5	31.5	12.4	5.99	2.36
500 µm - 1 mm	3.26	35.8	22.2	12.2	19.9	0.142
250 - 500 µm	2.01	36.0	16.4	13.6	26.7	0.0364
125 - 250 µm	0.285	8.37	10.1	27.3	26.7	0.0245
63 - 125 µm	0.0915	0.336	2.62	14.2	16.3	0.0206
<63 µm	0.138	0.145	1.44	13.7	0.434	0.0705

[a] Content is presented with associated standard deviations of the mean of three subreplicates
[b] R1 treatment with medium-very coarse gravel
[c] R1 treatment with medium-coarse sand

3.4 - *In situ* assay effectiveness

Results of the physico-chemical parameters measured during the 48-hours *in situ* assay and the characteristics of the sediment at each study site are present in the Tables III and IV, respectively. Overall, temperature ranged between 17 and 25ºC with small variations within each site (< 3ºC) and a marked gradient in conductivity (1255 – 4260 μS/cm) and pH (2.12 – 7.24) was observed along the allegedly impacted sites (from I1 to I5) (Table III).

The required numbers of 8 organisms for postexposure feeding quantifications were rapidly retrieved from the chambers and mortality was observed at all impacted sites, 100% at I1, I2, I3, and I4, and 37% at I5. The 48-hours postexposure feeding rates of *T. fluviatilis* were similar across all three reference sites (one-way nested ANOVA: $F_{2,56} = 2.02$; $P > 0.21$), confirming the use of R6 as a reference site (Figure 5). The postexposure feeding rate of organisms at the impacted site I5 was significantly inhibited (by 46%) relatively to the reference site R6 (one-way nested ANOVA: $F_{1,24} = 22.7$; $P < 0.001$), with mean (±SD) postexposure feeding rates of 20.7 (±12) and 44.9 (±14) *nauplii*/organism/3 hours, respectively (Figure 6). Integrating mortality and postexposure feeding inhibition (using the first integration technique) the percentage inhibition at the population level was 71%.

Table III – Physico-chemical parameters measured in the water column at each the two reference sites (R1, R2) in the Mondego River basin (Central Portugal) and at the reference site (R6) and five allegedly impacted sites (I1 to I5) in the Sado River basin (South Portugal) to evaluate the effectiveness of chambers and procedures of the proposed 48-hours *in situ* assay based on the postexposure feeding of *Theodoxus fluviatilis*. Temperature (T), conductivity (Cond) and dissolved oxygen (DO) were measured at the field sites and pH at the laboratory in samples collected at the study sites.

Site	T (ºC)[a]	Sediment[b]	Current velocity (m/s)[c]	Cond (µS/cm)[a]	DO (mg/L)[c]	pH[c]
R1	16.3 - 16.6	medium-coarse sand	0.02	550.0 – 546.0	9.60	7.09
R2	16.3 - 16.6	coarse-very coarse sand	0.00	528.0 – 558.0	10.3	7.84
R6	16.3 - 16.6	coarse sand-very fine gravel	0.00	1280 – 1206	3.60	7.50
I1	16.3 - 16.6	coarse-very coarse sand	0.00	4220 – 4260	9.50	2.13
I2	16.4 – 17.0	medium-very coarse sand	0.00	3050 – 2480	17.7	2.52
I3	15.5 - 17.3	very coarse sand-fine gravel	0.00	2060 – 1735	12.0	5.45
I4	15.9 - 17.2	fine-coarse sand	0.00	1650 – 1345	10.0	7.01
I5	14.4 - 16.1	medium-very coarse sand	0.00	1540 – 1255	10.2	7.24

[a] measured at assay deployment and retrieval
[b] sediment type based on particle size(s) with more than 50% (see Table II for more details on sediments particle size distribution)
[c] measured at assay deployment

Table IV – Water and organic matter content (in %) and particle size distribution (in %) of the six sediments collected at the two reference sites (R1, R2) in the Mondego River basin (Central Portugal) and at the reference site (R6) and five allegedly impacted sites (I1 to I5) in the Sado River basin (South Portugal) to evaluate the effectiveness of chambers and procedures of the proposed 48-hours *in situ* assay based on the postexposure feeding of *Theodoxus fluviatilis*. For sediment characteristics at sites R1 (medium-coarse sand) and R2 see Table II.

	R6	**I1**	**I2**	**I3**	**I4**	**I5**
Water[a]	17.5 (±1.0)	14.0 (±1.2)	24.3 (±0.14)	61.6 (±0.92)	59.3 (±0.57)	32.5 (±0.87)
Organic matter[a]	1.9 (±0.11)	3.1 (±0.42)	4.6 (±0.44)	11 (±0.45)	11 (±1.1)	4.3 (±0.19)
Particle size						
32 - 64 mm	0.00	0.00	0.00	0.00	0.00	0.00
16 - 32 mm	0.00	0.00	9.67	0.00	0.00	0.00
8 - 16 mm	0.00	0.00	5.82	2.98	6.55	4.19
4 - 8 mm	10.4	15.7	4.40	13.1	2.94	6.02
2 - 4 mm	24.2	2.40	8.53	19.1	10.1	19.8
1 - 2 mm	23.9	10.5	25.1	17.8	13.8	12.3
500 µm - 1 mm	21.3	44.6	23.8	12.5	14.8	20.2
250 - 500 µm	11.6	23.6	12.3	2.31	23.5	17.7
125 - 250 µm	5.69	2.28	5.58	17.2	14.0	11.7
63 - 125 µm	1.98	0.190	2.47	2.22	7.87	4.75
<63 µm	0.988	0,345	2.33	12.8	6.51	3.37

[a] content is presented with associated standard deviations of the mean of three subreplicates

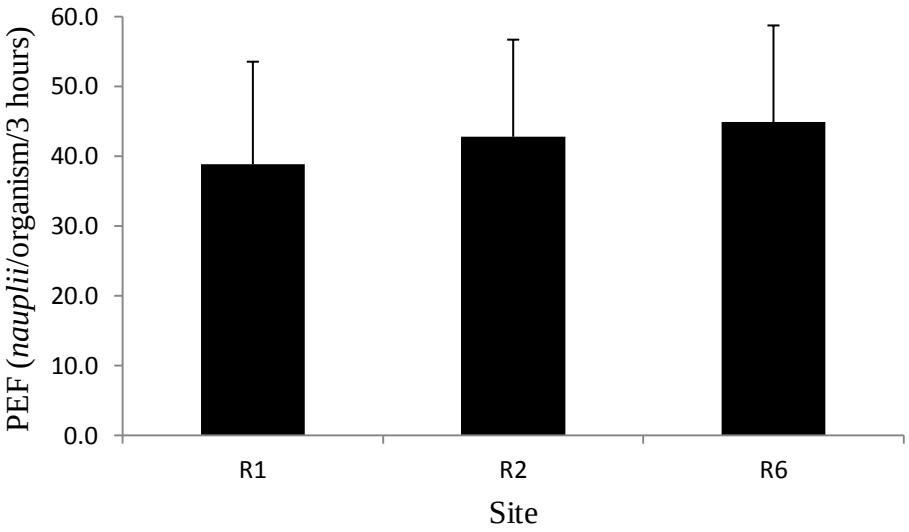

Figure 5 – Mean postexposure feeding rate of *Theodoxus fluviatilis* (number of consumed *nauplii* of *Artemia fransciscana*/organism/3 hours) after a 48 hours *in situ* exposure at the reference sites R1 and R2 (within the Mondego River basin) and R6 (within the Sado River basin). Error bars indicate + 1 standard deviation.

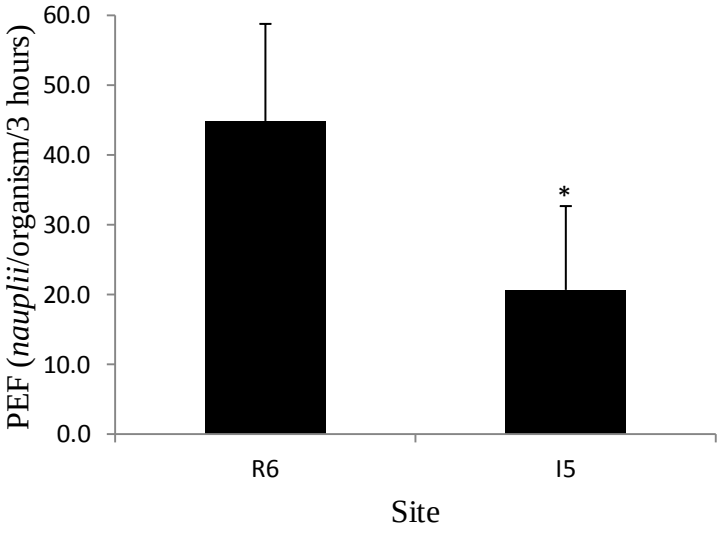

Figure 6 – Mean postexposure feeding rate of *Theodoxus fluviatilis* (number of consumed *nauplii* of *Artemia fransciscana*/organism/3 hours) after a 48 hours *in situ* exposure at the reference site R6 and at the impacted site I5 (within the Sado River basin). Error bars indicate + 1 standard deviation; * indicate mean significantly different from the reference site R6 by one-way nested ANOVA.

Chapter 4 Discussion

The present study intended to develop and evaluate a short-term sublethal *in situ* toxicity assay based on the postexposure feeding of the snail *T. fluviatilis* to be proposed to complement river quality assessment and monitoring. To achieve this purpose four steps needed to be sequentially tackled. Firstly, a method to precisely quantify the feeding rate of *T. fluviatilis* before organisms' recovery was established under laboratory conditions. Secondly, to infer upon the sensitivity of the postexposure feeding response, the later response was compared with lethality by exposing organisms to the reference toxicant cadmium. Thirdly, the influence of environmental variables on the background variability of the postexposure feeding response was evaluated to be able to discriminate toxic effects from those associated to differences in environmental factors across sites. Lastly, the effectiveness of the proposed *in situ* assay was evaluated by deploying it at several reference and allegedly impacted sites within the Mondego and Sado River basins.

4.1 - Feeding quantification

The methodology adopted in the present study to quantify feeding rates of *T. fluviatilis* was based on previously developed approaches to quantify feeding rates in the period immediately after exposure – postexposure feeding as the assay endpoint (e.g. Soares *et al*., 2005; Moreira *et al*., 2006a). Ideally, feeding rates should be measured during the exposure period to minimize an eventual physiological recovery of the organisms in the period after exposure. However, because it is difficult to quantify feeds and thus feeding rates during exposure, particularly during *in situ* testing, the use of postexposure feeding responses has been widely acknowledged as a valid approach since toxic effects generally persist in the period immediately after toxicant exposure,

before physiological recovery (McWilliam & Baird, 2002; Crichton *et al.*, 2004; Moreira *et al.*, 2005). Postexposure feeding has already been successfully used with freshwater and estuarine organisms including cladocerans (McWilliam & Baird, 2002), amphipods (Agostinho *et al.*, 2012), snails (Crichton *et al.*, 2004), mudnails (Krell *et al.*, 2011), midges (Soares *et al.*, 2005), crabs (Moreira *et al.*, 2006a), polychaetes (Moreira *et al.*, 2005, 2006b), prawns (Satapornvanit *et al.*, 2009), and small fish (Castro *et al.*, 2004). Also in accordance with various *in situ* assays previously developed (McWilliam & Baird, 2002; Moreira *et al.*, 2005; Soares *et al.*, 2005; Moreira *et al.*, 2006a,b; Krell *et al.*, 2011), the quantification of the postexposure response was performed under dark conditions. Quantifying feeding in darkness, rather than under a light regime, allows estimations to be performed in shorter periods (Krell *et al.*, 2011) as well as the establishment of more precise feeding rates (Moreira *et al.*, 2005; Soares *et al.*, 2005).

Up to present, to our knowledge, toxicity tests based on the feeding response of snails proposed as test endpoint the use of egestion rate as a surrogate to quantify feeding rates, either on spinach mats (freshwater snail *Lymnaea peregra*; Crichton *et al.*, 2004) or on sediment (mudsnail *Hydrobia ulvae*; Shipp & Grant, 2006; Krell *et al.*, 2011). In the present study, preliminary attempts to quantify feeding rates of *T. fluviatilis* indirectly through egestion rates after a feeding period on spinach mats were first carried out. However, the spinach consumption of *T. fluviatilis* was minimal, individual faecal pellets were difficult to quantify and a high variability in faecal pellets production was found. Yet, surprisingly for a scrapper, preliminary attempts also revealed that defrosted n*auplii* of *A. fransciscana* was a suitable food item to directly quantify postexposure feeding rates of *T. fluviatilis*, as they were a readily consumed food item. Using *nauplii* greatly simplified the feeding quantification procedures

turning them faster and shorter, and yielded more precise results. This finding is in agreement with previous studies using *nauplii* to successfully quantify feeding rates of midges (Soares et al., 2005), polychaetes (Moreira *et al.*, 2006b; Rosen & Miller, 2011), prawns (Satapornvanit *et al*., 2009), and amphipods (Agostinho *et al.*, 2012). Thus, for aquatic snails, the quantification of feeding rates on *nauplii* is a novel suitable approach to be used as an assay endpoint.

The methodology for assay endpoint estimation proposed in the present study requires a postexposure feeding period of 3 hours, a compromise between the estimation of precise feeding rates and an eventual physiological recovery of the organisms from the effects of the toxicants. Indeed, shorter postexposure feeding periods have preferably been established (e.g., 30 min for an amphipod; Agostinho *et al.*, 2011, 1 hour for a midge; Soares *et al.*, 2005), given that it is essential to minimize the eventual physiological recovery of the organisms from the effects during exposure (Brent & Herricks, 1998; McWilliam & Baird, 2002). Physiological recovery from toxicant effects has been reported for the cladoceran *Daphnia magna* approximately 4 hours after exposure to sublethal concentrations of copper, fluoranthene (McWilliam & Baird; 2002) and phenol (Brent & Herricks, 1998). However, Brent and Herricks (1988) did not found the amphipod *Hyalella azteca* to recover its mobility 7 days after a 10-days exposure to cadmium and zinc.

When testing the influence of food quantity on the feeding rate of *T. fluviatilis*, results from the present study showed organisms to eat significantly more when 150 rather than 100 *nauplii* were provided, even though no differences were found between the coefficients of variation of the two means. As expected, the probability of a snail to find a *nauplii* increases with an increase in food concentration, as it has been previously observed for the midge *Chironomus riparius* (Soares *et al*., 2005). Thus, 150 *nauplii*

were used in all subsequent experiments/assays because a higher consumption allows better discriminating differences in feeding rates across treatments.

In the experiment here performed to evaluate the influence of the time lag between field collection and feeding quantification, i.e., laboratory culture age (1 up to 7 days), on the feeding rate of *T. fluviatilis*, no differences in the consumption of *nauplii* were detected among the recently field collected snails (1-day old culture) and the ones kept in the laboratory up to one week. This is a very important finding indicating not only that the established laboratory culture procedures are suitable to maintain organisms under optimal conditions to be used in 48-hours postexposure feeding assays but also that there is a high flexibility in the use of the snails as test organisms.

Lastly, the influence of the organism size on the feeding rate of *T. fluviatilis* was also investigated in the present study, with organism of two different size classes. Not only no differences on *nauplii* consumption by *T. fluviatilis* were observed between the two size classes tested, but also no correlation between snail size, either shell height or length, and feeding rates was found. This result guarantees that potential differences in feeding rate are not associated with the size of the organisms, at least for organisms with a mean shell height and length of 4.02 to 4.86 mm and 5.05 to 6.12 mm, respectively.

4.2 - Endpoint sensitivity

Theodoxus fluviatilis proved to be a very sensitive organism with 48- and 96-hours LC50 values for cadmium of 208 and 141 µg/L, respectively. Indeed, this lethal toxicity parameter revealed *T. fluviatilis* to be more sensitive to cadmium, by three to 24 times, than other freshwater snail species, such as *Physa acuta* (48-hours LC50 of 1050

µg/L; Cheung and Lam, 1998), *Lymnaea stagnalis* (48-hours LC50 of 583 µg/L; Slooff, 1983) and *Filopaludina martensi* (48- and 96-hours LC50 of 5010 and 2330 µg/L, respectively; Piyatiratitivorakula & Boonchamoib, 2008), and with a similar sensitivity relatively to *Aplexa hypnorum* (96-hours LC50 of 93 µg/L; Holcombe *et al.*, 1984).

Results of the present study showed that after an exposure period of 48 hours to cadmium, the postexposure feeding and lethal responses of *T. fluviatilis* showed a similar sensitivity; the 48-hours EC50 was lower than the 48-hours LC50 by 1.2 times and higher than the 96-hours LC50 by 1.3 times, whereas the 48-hours EC20 was lower and higher than the 48- and 96-hours LC20 by 1.7 and 1.0 times, respectively. These findings were somehow unexpected given that previous studies demonstrated the postexposure feeding response to be more sensitive than lethality for different invertebrate species. For the polychaete *Hediste diversicolor*, the 48-hours EC50 for postexposure feeding was 4.6 and 2.4 more sensitive to copper than the 48- and 96-hours LC50, respectively (Moreira *et al.*, 2005). An even more sensitive postexposure feeding response was found for the crab *Carcinus maenas* after cadmium exposure, with the 48-hours postexposure feeding (EC50) more sensitive than lethality (LC50) by 6.5 and 3.2 times after a 48- and 96-hours exposure, respectively (Moreira *et al.*, 2006a). Krell *et al.* (2011) found for the mudsnail *H. ulvae* exposed to copper, a postexposure feeding response (EC50) 3 times more sensitive than lethalilty (LC50) after exposure periods of 48 and 96 hours, respectively. Yet, in a study conducted with the freshwater amphipod *Echinogammarus meridionalis* exposed to copper for 48 hours, the EC50 for postexposure feeding was only two times more sensitive than lethality (LC50) (Agostinho *et al.*, 2012). The absence of sublethal and lethal sensitivity differences found in the present study could be due to a physiological recovery of the organisms

from the cadmium effects during the 3-hours postexposure feeding period. Further studies with different toxicants should be conducted to unravel this issue.

Feeding depression and lethality appeared to contribute equally to a feeding depression at the population level since the sensitivity of both responses was similar. This means that the depression at the population level is due not only to the feeding depression of the organisms that are still alive, but also to the absence of feeding of the dead ones. Therefore, the recently proposed novel toxicity parameter (Krell *et al*., 2011; Agostinho *et al*., 2012) – immediate inhibition of population consumption (popEC50) – was calculated in order to integrate the survival and feeding depression in a single toxicity parameter to improve ecological realism. Two methods were used to calculate the popEC50, each having advantages and disadvantages. The first method calculated the popEC50 by multiplying, for each concentration, the individual feeding by the survival percentage, and thus has the advantage of allowing the computation of confidence limits. However, it increases the chance of violating the assumption of homoscedasticity in regression, since a strong association between feeding depression and mortality is expected. The second method, overcomes this drawback because it integrates the concentration-response relationships for feeding and survival after independent tests specifically designed to assess either lethality or feeding depression are carried out. However, this second technique assumes that feeding depression is constant over time and that there is no correlation between the tolerance to sublethal (feeding) and lethal levels of a toxicant. Violating this assumption may lead to an under or overestimation of risk. For instance, if organisms more sensitive to feeding depression are the ones that die faster, then the survivors will feed at a higher rate than the individual average of the initial population, partially compensating, at the population level, the loss of some individuals (the dead ones) (Krell *et al.*, 2011; Agostinho *et al.*,

2012). Both methods led to similar 48-hours popEC50 values (157 and 150 µg/L) with the second method revealing that at the 48-hours median lethal concentration (LC50), the immediate inhibition of population consumption would be 79%, which correspond to a very high disruption of the ecosystem function. Similar results through the two methods were also obtained by Krell *et al.*, (2011) (75 and 68 µg/L) and Agostinho *et al.*, (2012) (187 and 168 µg/L) for a mudsnail and an amphipod, respectively, exposed to copper.

4.3 – Influence of environmental exposure conditions on postexposure feeding

The influence of environmental conditions on the postexposure feeding rate was investigated because environmental conditions may act as confounding factors during *in situ* assays, not allowing to distinguish potential toxics effects from effects associated with the variability in environmental conditions across study sites (Moreira *et al.*, 2005; Soares *et al.*, 2005; Moreira *et al.*, 2006a; Krell *et al.*, 2012).

For all the investigated environmental exposure conditions (current velocity, hardness, conductivity, and sediment organic matter content and particle size distribution), the absence of a significant effect on the postexposure feeding rate of the freshwater snail *T. fluviatlis* indicated that the selected assay endpoint was not influenced by such variables. Therefore, sites with such different conditions can be directly compared for *in situ* evaluations with *T. fluviatilis*, but solely within the tested ranges. The temperature was not included in the model because its variation among the different sites was minimal. Therefore, caution should be taken when working with sites with different temperatures values. Significant effects of the temperature on the

postexposure feeding were found in other studies (Soares *et al.*, 2005; Moreira *et al.*, 2005, 2006a, Krell *et al.*, 2011).

4.4 – *In situ* assay effectiveness

To evaluate the effectiveness of the proposed *in situ* assay based on the postexposure feeding rate of the freshwater snail *T. fluviatilis*, organisms were deployed at two reference sites (R1 and R2) within the Mondego River basin and one reference site (R6) and five allegedly impacted sites (I1 to I5) within the Sado River basin. The eight organisms designed for postexposure feeding rate were easily recovered from the *in situ* assay chambers. This result demonstrated the suitability of the *in situ* chambers and procedures, as it has been verified for other organisms (Castro *et al.*, 2004; Soares *et al.*, 2005), enlarging thus the battery of the *in situ* assays available to perform toxicity assessments in rivers. No significant differences were found on the postexposure feeding rate of the *T. fluviatilis* across the three references sites. Therefore, the reference site selected in the Roxo River showed to be a valid reference to assess the effectiveness of the proposed *in situ* assay.

Given that only at the impacted site located furthest downstream the dilution gradient of the mine effluent (site I5 practically at the confluence of the Água Forte Stream with the Roxo River) live organisms were found (63%), only one impacted site could be used to evaluate the postexposure feeding depression of the *T. fluviatilis*. However, since a significant postexposure feeding depression of 46% was detected for the organisms that were alive, the feeding depression of the organisms exposed at site I5 demonstrated that *T. fluviatilis* postexposure feeding is a potential responsive sublethal endpoint. Moreover, the immediate effect on the consumption at the population level,

by integrating mortality and feeding inhibition at I5, would be 71%, which corresponds to a very high disruption of the snails ecosystem function.

Chapter 5 Conclusion

The short-term sublethal *in situ* assay based on the postexposure feeding of the freshwater snail *T. fluviatilis* proposed in the present study showed to be a potential valuable tool to be added to the battery of *in situ* assays developed in recent years to assess contaminant effects in freshwater aquatic systems, particularly in rivers. The species *T. fluviatilis* was easy to collect, transport, maintain under laboratory cultures, and manipulate, and has a key role in the functioning of river ecosystems, all essential characteristics for its proposal as a test species. The methodologies here proposed for the *in situ* deployment and retrieval of the organisms and to quantify postexposure feeding rates are simple and short-demanding in time and effort, and thus inexpensive, allowing a rapid estimation of the response of snails to contaminants, all critical aspects for the assessment and monitoring of environmental quality, especially now in the context of the Water Framework Directive (Allan et al., 2006). In particular, the integration of the postexposure feeding response and lethal effects in a single parameter (immediate inhibitory population consumption) may be useful to provide a more relevant evaluation on potential toxicant impacts directly at the population level and thus on key ecosystem functions.

References

REFERENCES

Adams S.M. (2003). Establishing causality between environmental stressors and effects on aquatic ecosystems. Human and Ecological Risk Assessment: An International Journal 9:17-35.

Agostinho M., Moreira-Santos M. & Ribeiro R. (2012). A freshwater amphipod toxicity test based on postexposure feeding and the population consumption inhibitory concentration. Chemosphere 87:43-48.

Allan I.J., Vrana B., Greenwood R., Mills G.A., Roig B. & Gonzalez C. (2006). A "toolbox" for biological and chemical monitoring requirements for the European Union's Water Framework Directive. Talanta 69:302-322.

Alvarenga P., Palma P., Gonçalves A.P., Fernandes R.M., Varennes A., Vallini G., Duarte E. & Cunha-Queda A.C. (2008). Evaluation of tests to assess the quality of mine-contaminated soils. Environmental Geochemistry and Health 30:95-99.

Amiard-Triquet C. (2009). Behavioral Disturbances: The missing link between sub-organismal and supra-organismal responses to stress? Prospects based on aquatic research. Human and Ecological Risk Assessment: An International Journal 15:87-110.

ASTM (American Society for Testing and Materials). (2002). Standard guide for conducting acute toxicity tests on test materials with fishes, macroinvertebrates, and amphibians. E 729-96. In: Annual Book of American Society for Testing and Materials Standards, vol. 11.05. ASTM, Philadelphia, USA.

Baird D.J., Brown S.S., Lagadic L., Liess M., Maltby L., Moreira- Santos M., Schulz R. & Scott G.I. (2007). *In situ* - based effects measures: determining the ecological relevance of measured responses. Integrated Environmental Assessment and Management 3:259-267.

Besser J.M., Hardesty D.K., Greer E.I., Mebane C.A., Mount D.R. & Ingersoll C.G. (2007). Sensitivity of freshwater snails to contaminants: chronic toxicity tests with endangered species and surrogates. Society of Environmental Toxicology and Chemistry 27th Annual Meeting Abstracts.

Brent R.N. & Herricks E. (1998). Postexposure effects of brief cadmium, zinc, and phenol exposures on freshwater organisms. Environmental Toxicology and Chemistry 17:2091-2099.

Brönmark C. (1989). Interactions between epiphytes, macrophytes and freshwater snails: a Review. Journal of Molluscan Studies 55:299-311.

Brown K.M. & DeVries D.R. (1985). Predation and the distribution and abundance of a pulmonate pond snail. Oecologia 66:93-99.

Buchanan J.B. & Kain J.M. (1971). Measurement of the physical and chemical environment. In: Holme N.A. & McIntyre A.D. (eds). Methods for the study of marine benthos—IBP handbook, 16. Blackwell Scientific Publications, Oxford, UK, pp 30-58.

Bunje P.M.E. (2005). Pan-European phylogeography of the aquatic snail *Theodoxus fluviatilis* (Gastropoda: Neritidae). Molecular Ecology 14:4323-4340.

Bunje P.M.E. & Lindberg D.R. (2007). Lineage divergence of a freshwater snail clade associated with post-Tethys marine basin development. Molecular Phylogenetics and Evolution 42:373-387.

Burke A., Meignen L., Bisson M., Pimentel N., Henriques V., Andrade C., Freitas M.C., Kageyama M., Fletcher W., Parslow C. & Guiducci D. (2011). The Palaeolithic occupation of southern Alentejo: the Sado River Drainage Survey. Trabajos de Prehistoria 68:25-49.

Burton G.A., Batley G.E., Chapman P.M., Forbes V.E., Smith E.P., Reynoldson T., Schlekat, C.E., Besten P.J., Bailer A.J., Green A.S. & Dwyer R.L. (2002). A weight-of-evidence framework for assessing sediment (or other) contamination: Improving certainty in the decision-making process. Human and Ecological Risk Assessment: An International Journal 8:1675-1696.

Burton G.A., Greenberg M.S., Rowland C., Irvine C.A., Lavoie D.R., Brooker J.A., Moore L., Raymer D.F.N. & McWilliam R.A. (2005). *In situ* exposures using caged organisms: a multi-compartment approach to detect aquatic toxicity and bioaccumulation. Environmental Pollution 134:133-144.

Carlsson R. (2000). The distribution of the gastropods *Theodoxus fluviatilis* (L.) and *Potamopyrgus antipodarum* (Gray) in Lakes on the land Islands, southwestern Finland. Boreal Environment Research 5:187-195.

Castro B.B., Guilhermino L. & Ribeiro R. (2003). *In situ* bioassay chambers and procedures for assessment of sediment toxicity with *Chironomus riparius*. Environmental Pollution 125:325-335.

Castro B.B., Sobral O., Guilhermino L. & Ribeiro R. (2004). An *in situ* bioassay integrating individual and biochemical responses using small fish species. Ecotoxicology 13:667-681.

Chapman P.M. (1996). Presentation and interpretation of Sediment Quality Triad data. Ecotoxicology 5:327-339.

Chapman P.M., Ho K.T., Munnus W.R., Solomon K. & Weinstein, M.P. (2002). Issues in sediment toxicity and ecological risk assessment. Marine Pollution Bulletin 44:271-278.

Chapman P.M., Wang F., Janssen C.R., Goulet R.R. & Kamunde C.N. (2003). Conducting ecological risk assessments of inorganic metals and metalloids:

current status. Human and Ecological Risk Assessment: An International Journal 9:641-697.

Cheung C.C.C. & Lam P.K.S. (1998). Effect of cadmium on the embryos and juveniles of a tropical freshwater snail, *Physa acuta* (Draparnaud, 1805). Water Science and Technology 38:263-270.

Connell D.W., Lam P.K.S., Richardson B.J. & Wu R.S.S. (1999). Introduction to Ecotoxicology, 1st edition, Blackwell Science, UK, 1:11 pp.

Crane M., Burton G.A., Culp J.M., Greenberg M.S., Munkittrick K.R., Ribeiro R., Salazar M.H. & St-Jean S.D. (2007). Review of aquatic *in situ* approaches for stressor and effect diagnosis. Integrated Environmental Assessment and Management 3:234-245.

Crichton C.A., Conrad A.U. & Baird D.J. (2004). Assessing stream grazer response to stress: a post-exposure feeding bioassay using the freshwater snail *Lymnaea peregra* (Müller). Bulletin of Environmental Contamination and Toxicology 72:564-570.

DeWitt T.H., Hickey C.W., Morrisey D.J., Nipper M.G., Roper D.S., Wiliamson R.B., Van Dam L. & Williams E.K. (1999). Do amphipods have the same concentration-response to contaminated sediment *in situ* as *in vitro*? Environmental Toxicology and Chemistry 18:1026-1037.

EC (European Community) (2000). Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy, The European Parliament and Council, L327/1, 2000, 72 pp.

EC (Environment Canada) (2004). Biological test method: tests for toxicity of contaminated soil to earthworms ('*Eisenia andrei*', *'Eisenia fetida'*, or *'Lumbricus terrestris'*). EPS 1/RM/43. Ottawa, Ontario, Canada.

Evans-White M.A. & Lamberti G.A. (2009). Direct and indirect effects of a potential aquatic contaminant on grazer-algae interactions. Environmental Toxicology and Chemistry 28:418.

Feio M.J., Reynoldson T.B., Ferreira V. & Graça M.A.S. (2007). A predictive model for freshwater bioassessment (Mondego River, Portugal). Hydrobiologia 589:55-68.

Feio M.J., Alves T., Boavida M., Medeiros A. & Graça M.A.S. (2010). Functional Indicators of stream health: a river-basin approach. Freshwater Biology 55:1050-1065.

Forrow D.M. & Maltby L. (2000). Toward a mechanistic understanding of contaminant-induced changes in detritus processing in streams: direct and indirect effects on detritivore feeding. Environmental Toxicology and Chemistry 19:2100-2106.

Gerhardt A. (1996). Behavioural early warning responses to polluted water. Environmental Science and Pollution Research 3:63-70.

Graça M.A.S, Serra S.R.Q. & Ferreira V. (2012). A stable temperature may favour continuous reproduction by *Theodoxus fluviatilis* and explain its high densities in some karstic springs. Limnetica 31:129–140.

Harris D.L., Lottermoser B.G. & Duchesne J. (2003). Ephemeral acid mine drainage at the Montalbion silver mine, north Queensland. Australian Journal of Earth Sciences 50:797-809.

Holcombe G.W., Phipps G.L. & Marier J.W. (1984). Methods for conducting snail (*Aplexa hypnorum*) embryo through adult exposures: effects of cadmium and

reduced pH levels. Archives of Environmental Contamination and Toxicology 13:627-634.

Islam M.S. & Tanaka M. (2004). Impacts of pollution on coastal and marine ecosystems including coastal and marine fisheries and approach for management: a review and synthesis. Marine Pollution Bulletin 48:624-649.

Kirkegaard J. (2006). Life history, growth and production of *Theodoxus fluviatilis* in Lake Esrom, Denmark. Limnologica 36:26-41.

Krell B., Moreira-Santos M. & Ribeiro R. (2011). An estuarine mudsnail *in situ* toxicity assay based on postexposure feeding. Environmental Toxicology and Chemistry 30:1935-1942.

Lefcort H., Ammann E. & Eiger S.M. (2000). Antipredatory behavior as an index of heavy-metal pollution? A test using snails and caddisflies. Archives of Environmental Contamination and Toxicology 38:311-316.

Liber K., Goodfellow W., Besten P.D., Clements W., Galloway T., Gerhardt A., Green A. & Simpson S. (2007). *In situ* - based effects measures: considerations for improving methods and approaches. Integrated Environmental Assessment and Management 3:246-258.

Lodge D.M. (1986). Selective grazing on periphyton: a determinant of freshwater gastropod microdistributions. Freshwater Biology 16:831-841.

Lopes I., Gonçalves F., Nogueira A., Soares A.M.V.M. & Ribeiro R. (2000). Field validation of specific ecotoxicological tools for aquatic systems impacted with acid mine drainage. International Journal of Environmental Studies 58:3-20.

Luís A., Teixeira P., Almeida S., Ector L., Matos J.X. & Ferreira da Silva E. (2009). Impact of acid mine drainage (AMD) on water quality, stream sediments and

periphytic diatom communities in the surrounding streams of Aljustrel mining area (Portugal). Water, Air, & Soil Pollution 200:147-167.

Maltby L. (1999). Studying stress: the importance of organism-level responses. Ecological Applications 9:431-440.

Maltby L., Clayton S.A., Yu H., McLoughlin N., Wood R.M. & Yin D. (2000). Using single-species toxicity tests, community-level responses, and toxicity identification evaluations to investigate effluent impacts. Environmental Toxicology and Chemistry 19:151-157.

Maltby L., Clayton S.A., Wood R.M. & McLoughlin N. (2002). Evaluation of the *Gammarus pulex* in situ feeding assay as a biomonitor of water quality: robustness, responsiveness, and relevance. Environmental Toxicology and Chemistry 21:361-368.

Marín-Guirao L., Cesar A., Marín A., Lloret J. & Vita R. (2005). Establishing the ecological quality status of soft-bottom mining-impacted coastal water bodies in the scope of the Water Framework Directive. Marine Pollution Bulletin 50:374-387.

Marques J.C., Graça M.A.S. & Pardal M.A. (2002). Introducing the Mondego River Basin. In: Pardal M.A., J. C. Marques & M. A. Graça (eds.). Aquatic Ecology of the Mondego River Basin: Global Importance of Local Experience, Universidade de Coimbra, Coimbra, Portugal, pp. 219-230.

McWilliam R.A. & Baird D.J. (2002). Postexposure feeding depression: a new toxicity endpoint for use in laboratory studies with *Daphnia magna*. Environmental Toxicology and Chemistry 21:1198-1205.

Mills C.L., Shukla D.H. & Compton G.J. (2006). Development of a new low cost high sensitivity system for behavioural ecotoxicity testing. Aquatic Toxicology 77:197-201.

Moreira S.M., Moreira-Santos M., Guilhermino L. & Ribeiro R. (2005). A short-term sublethal *in situ* toxicity assay with *Hediste diversicolor* (Polychaeta) for estuarine sediments based on postexposure feeding. Environmental Toxicology and Chemistry 24:2010-2018.

Moreira S.M., Moreira-Santos M., Guilhermino L. & Ribeiro R. (2006a). An *in situ* postexposure feeding assay with *Carcinus maenas* for estuarine sediment-overlying water toxicity evaluations. Environmental Pollution 139:318-329.

Moreira S.M., Lima I., Ribeiro R. & Guilhermino L. (2006b). Effects of estuarine sediment contamination on feeding and on key physiological functions of *Hediste diversicolor*: laboratory and *in situ* assays. Aquatic Toxicology 78:186-201.

Pereira A.M., Soares A.M., Gonçalves F. & Ribeiro R. (2000). Water-column, sediment, and *in situ* chronic bioassays with cladocerans. Ecotoxicology and Environmental Safety 47:27-38.

Pestana J.L.T., Nogueira A.J.A. & Soares A.M.V.M. (2007). Effects of cadmium and zinc on the feeding behaviour of two freshwater crustaceans: *Atyaephyra desmarestii* (Decapoda) and *Echinogammarus meridionalis* (Amphipoda). Chemosphere 68:1556-1562.

Piyatiratitivorakul P. & Boonchamoib P. (2008). Comparative toxicity of mercury and cadmium to the juvenile freshwater snail, *Filopaludina martensi martensi*. Science Asia 34:367-370.

Rand G.M. (1995). Fundamentals of Aquatic Toxicology: Effects, Environmental Fate and Risk Assessment. 2nd edition. CRC Press, EUA, 3-67 pp.

Reed W.L. & Janzen F.J. (1999). Natural selection by avian predators on size and colour of a freshwater snail (*Pomacea flagellata*). Biological Journal of the Linnean Society 67:331-342.

Ringwood A.H. & Keppler C.J. (2002). Comparative *in situ* and laboratory sediment bioassays with juvenile *Mercenaria mercenaria*. Environmental Toxicology and Chemistry 21:1651-1657.

Rocha C. (1998). Rhythmic ammonium regeneration and flushing in intertidal sediments of the Sado estuary. Limnology and oceanography 43:823-831.

Rodriguez-Mozaz S., Marco M.-P., Lopez de Alda M.J. & Barceló D. (2004). Biosensors for environmental applications: future development trends. Pure and Applied Chemistry 76:723-752.

Roman Y.E., Schamphelaere K.A.C., Nguyen L.T.H. & Janssen C.R. (2007). Chronic toxicity of copper to five benthic invertebrates in laboratory-formulated sediment: sensitivity comparison and preliminary risk assessment. Science of the Total Environment 387:128-140.

Rosen G. & Miller K. (2011). A postexposure feeding assay using the marine polychaete *Neanthes arenaceodentata* suitable for laboratory and *in situ* exposures. Environmental Toxicology and Chemistry 30:730-737.

Ruehl C.B. & Trexler J.C. (2011). Comparison of snail density, standing stock, and body size between Caribbean karst wetlands and other freshwater ecosystems. Hydrobiologia 665:1-13.

Sakuma M. (1998). Probit analysis of preference data. Applied Entomology and Zoology 33:339-347.

Satapornvanit K., Baird D.J. & Little D.C. (2009). Laboratory toxicity test and post-exposure feeding inhibition using the giant freshwater prawn *Macrobrachium rosenbergii*. Chemosphere 74:1209-1215.

Shipp E. & Grant A. (2006). *Hydrobia ulvae* feeding rates: a novel way to assess sediment toxicity. Environmental Toxicology and Chemistry 25:3246-3252.

Slooff W. (1983). Benthic macroinvertebrates and water quality assessment: some toxicological considerations. Aquatic Toxicology 4:73-82.

Soares S., Cativa I., Moreira-Santos M., Soares A.M.V.M. & Ribeiro R. (2005). A short-term sublethal *in situ* sediment assay with *Chironomus riparius* based on postexposure feeding. Archives of Environmental Contamination and Toxicology 49:163-172

Taylor G., Baird D.J. & Soares A.M.V.M. (1998). Surface binding of contaminants by algae: consequences for lethal toxicity and feeding to *Daphnia magna* Straus. Environmental Toxicology and Chemistry 17:412-419.

Wharfe J., Adams W., Apitz S.E., Barra R., Bridges T.S., Hickey C. & Ireland S. (2007). *In situ* methods of measurement — an important line of evidence in the environmental risk framework. Integrated Environmental Assessment and Management 3:268-274.

Zar J.H. (1996). Biostatistical Analysis. Prentice Hall, Upper Saddle River, NY, USA.

Zettler M.L., Frankowski J., Bochert R. & Röhner M. (2004). Morphological and ecological features of *Theodoxus fluviatilis* (Linnaeus, 1758) from Baltic brackish water and German freshwater populations. Journal of Conchology 38: 305-316.