Universidade de Aveiro Departamento de Biologia

Ano 2010

Henrique Miguel Veiga Simão de Azevedo Pereira

TESTES ECOTOXICOLÓGICOS COM *CHIRONOMUS RIPARIUS*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Professor Doutor Amadeu M.V.M Soares, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro.

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o júri

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palavras-chave

Mercúrio; Imidacloprid; *Chironomus riparius*; Toxicidade no desenvolvimento; Biomarcadores; Resposta comportamental

resumo

Os sistemas aquáticos naturais podem estar sujeitos frequentemente a entrada de tóxicos, quer seja através da lixiviação dos campos agrícolas ou da descarga por parte de unidades industriais. Avaliar o impacto potencial destes contaminantes nos sistemas aquáticos é muito importante, porque pode promover consequências sérias no balanço ecológico dos ecossistemas. Os efeitos de níveis sub-letais destes tóxicos nas populações aquáticas são detectados, em muitos casos, somente após diversas gerações, dependendo da espécie e do contaminante. O comportamento animal é considerado como sendo a primeira linha de defesa perante estímulos ambientais, e pode ser uma representação de alterações fisiológicas no organismo, sendo portanto um indicador excelente de alterações ambientais. O desenvolvimento dos sistemas de aviso prévio que integram parâmetros comportamentais pode ajudar a prever mais rapidamente possíveis alterações ao nível das populações naturais, do que a utilização de testes ecotoxicológicos padrão com a mesma finalidade. O conhecimento acerca de possíveis implicações devido a alterações comportamentais, em organismos bentónicos e em populações do campo sujeitas a tóxicos, é ainda escasso. Sabendo isto, neste estudo pretendeu-se investigar como o comportamento de *Chironomus riparius* – usando um biomonitor em tempo real – e outros parâmetros tais como crescimento, emergência de adultos, bioacumulação e biomarcadores, são afectados pela exposição a imidacloprid e ao mercúrio, que foram seleccionados como contaminantes. Os resultados demonstraram que a exposição às concentrações sub-letais de imidacloprid afecta o crescimento e o comportamento dos quironomídeos e que estes organismos podem recuperar de uma exposição curta ao insecticida. O comportamento que corresponde à ventilação de *C. riparius* revelou-se como um parâmetro mais sensível do que a locomoção e do que as respostas bioquímicas, quando as larvas foram sujeitas ao imidacloprid. Larvas de *C. riparius* expostas a concentrações sub-letais de mercúrio apresentaram uma tendência de diminuição de actividade comportamental, em testes com concentrações crescentes do tóxico; o crescimento das larvas foi também prejudicado, e as taxas de emergência de adultos e o tempo de desenvolvimento apresentaram retardamento. Estes organismos podem bioacumular rapidamente o mercúrio em condições de não alimentação e apresentam uma lenta depuração deste metal. Estes efeitos podem, em último caso, conduzir a prováveis repercussões ao nível da população e das comunidades. As reduções em actividades comportamentais, mesmo em concentrações baixas, podem diminuir a quantidade de tempo gasta na procura de alimento, produzindo efeitos aos níveis morfo-fisiológicos, e assim afectar severamente o desempenho dos quironomídeos no ambiente. O uso destes factores comportamentais como um parâmetro ecotoxicológico sub-letal relevante ao nível da toxicologia aumentará a versatilidade dos testes, permitindo uma resposta comportamental mensurável e quantitativa ao nível do organismo, utilizando uma avaliação não destrutiva, e assim certificando que esta aproximação pode ser usada em testes ecotoxicológicos futuros.

keywords

Mercury; Imidacloprid*; Chironomus riparius*; Developmental toxicity; Biomarker; Behavioural responses

abstract

Natural aquatic systems can be frequently subjected to toxicant inputs, either by runoff from agriculture fields or discharge from industrial plants. Assessing the potential impact of these contaminants on aquatic systems is an asset, as it can elicit serious consequences to the ecosystem balance. The effects of sub-lethal levels of these contaminants at the aquatic population levels are only detected, in many cases, after several generations, depending on the species and contaminant. Behaviour is considered to be the first line of defence towards environmental stimuli, and being a representation of physiological alterations in the organism it can be an excellent indicator of environmental changes. The development of early warning systems comprising behavioural endpoints can help to predict possible alterations at the field population levels even faster than conventional standard ecotoxicological tests.

The knowledge on the implication of behavioural disturbance by toxicants in benthonic organisms and in field populations is still scarce. Bearing this in mind, this study aimed to investigate how *Chironomus riparius*' behaviour – using an online biomonitor – and other parameters such as growth, emergence, bioaccumulation and biomarker effect, are affected by exposure to the selected toxicants imidacloprid and mercury.

Results have shown that exposure to sub-lethal concentrations of imidacloprid affects growth and behaviour of chironomids and the organisms can recover from a short exposure to the insecticide. *C. riparius* ventilation behaviour appeared as a more sensitive endpoint than locomotion and biochemical responses when larvae were subjected to imidacloprid. Sub-lethal concentrations of mercury on *C. riparius* elicited a trend of impairment in behavioural patterns with increasing concentrations of the toxicant; growth was also impaired and delayed emergence rates / development time were noticed. These organisms can also quickly bioaccumulate mercury in unfed conditions and present a slow depuration of the heavy metal. These effects may in last instance lead to probable repercussions at the population and community level. Reductions in behavioural activities even at low concentrations might decrease the amount of time spent foraging, producing effects at the morpho-physiological levels, and thus severely affecting the chironomids performance in the environment.

The use of these behavioural endpoints as a sub-lethal ecotoxicogical relevant parameter in toxicology will increase the versatility of the tests, allowing a measurable and quantitative behavioural response at the whole-organism level in a non-destructive assessment, thus certifying that this approach can be used in further assays.

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General Introduction

1. GENERAL INTRODUCTION

1.1. Preamble

Many surface water bodies are now contaminated due to the increasing usage of pesticides, mainly in agriculture, and to heavy metal contaminations from industry and/or natural sources. This contamination may cause impairment of ecological functions (Fleeger et al., 2003) and decline of non-target species (Rohr et al., 2006).

Ecotoxicology is interested in studying the effects of toxicants on the ecosystems. Pollutants matter because of their effects on populations and communities, through their effects on individual organisms (Moriarty, 1993). Since the immediate effects of pollutants are on organisms, either indirect (through habitat alterations) or direct (toxic effects of chemicals at the organismal level), one needs to assess what happens at the individual level to understand the impact on populations. The direct effect on individuals may range from rapid death through sub-lethal effects to no effects at all (Moriarty, 1993).

Ecotoxicology tests are needed to anticipate how toxicants are likely to impact ecological systems and to assess what changes are taking place in these systems under the influence of released toxic substances (Calow, 1997). When assessing the effects of a certain pollutant on a test species, the endpoints generally used are mortality (quantal type of data), and sub-lethal parameters like growth, reproduction, bioaccumulation and/or biomarker expression (continuous data), among others (Adams and Rowland, 2003). These responses can be ecologically relevant, as they are important components of fitness and determine the health, structure and dynamics of populations (Sibley et al., 1997).

In aquatic ecotoxicology the effects of anthropogenic (and natural) toxicants on aquatic biota are studied. These contaminants enter (and can be deposited) in the aquatic environment either from direct discharge from effluents, terrestrial runoff, or atmospheric deposition. Biomonitoring of these effects may be done by routine

monitoring (identify unanticipated contamination and effects) or by targeted monitoring (focused on specific, known contaminant situations) (Grue et al., 2002). In the aquatic environments, biomonitoring may involve sampling of organisms as an indication of possible contamination, and *in situ* tests by assessing acute and chronic toxicity in caged organisms exposed to either contaminated water, sediment or both. Laboratory toxicity tests are also an asset, either by transposing and assessing in the laboratory field organisms and/or contaminated water/sediment; by using test species cultured in the laboratory with field contaminated water/sediment; or by assessing test species with artificially contaminated sediment/water. One must also bear in mind the ecological relevance of the experimental approach, in order to reach a compromise between realistic exposure situations and the scientific interest of the study.

Tested species can be representatives of the studied populations or model organisms that are regularly used in toxicity tests, with well studied endpoints. In this thesis the benthonic midge larvae of *Chironomus riparius* (Meigen) were used as model organisms. Several guidelines (e.g. EPA, 2000; OECD, 2004) are in use for this species, in order to standardize ecotoxicological tests and allow the replicability, repeatability and reproducibility of the experiments, thus increasing the test precision and uniformity among laboratories.

One of the drawbacks in using benthic macroinvertebrates for biomonitoring and assessment of water quality is the amount of effort required to process the samples, either in *in situ* tests (e.g. sorting animals in the sediment, measurements at the laboratory) or in laboratory tests where, in chronic tests, quantitative results on toxicity are only available at the end of the experimental period. For instance, to assess sub-lethal toxicity of pollutants on chironomids in laboratory, results on the effects on growth are only available after several days [e.g. 10 days and larvae still need to be measured (OECD, 2004)]; on emergence, after 20-28 days (OECD, 2004); on head-capsule deformity induction after several days [e.g. 10 days and still needs to mount the larvae (Meregalli et al., 2001)]; and to assess biomarker effects one needs to process the samples and quantify biomarker activities (Domingues et al., 2007), which can be time consuming. Survival is measured daily (one need to bear in mind that dead larva may be in the

sediment, thus not visible), but only at the end of the experimental period one can have certainties about mortalities. Ecotoxicologists want to use bioassays that are quick and easy, giving valuable information readily on contaminant effects on individual organisms, in order to make predictions about long-term impacts at an ecological level. In fact, Forbes et al. (2006) referred the need to devote more effort in developing and improving methods that directly measure effects of chemical impacts on populations, communities and ecosystems, and that less effort must be invested on measures that, at best, can only ever be suggestive of risks.

Beitinger and McCauley (1990) suggested that responses to environmental changes can be divided in four categories: passive – no response, when the stimulus is not sensed or occurs too rapidly thus leading to a decrease in performance capacities or even death; behavioural reactions – when subjected to certain chemicals, animals usually react in seconds or minutes, avoiding stress and trying to obtain a favourable position relative to the level of stimulus; physiological responses – organisms suffer internal changes in various physiological processes, including adjustments in physiological rate functions and tolerance acclimation enhancement, which may occur within hours to weeks; and biochemical responses – synthesis of new molecules like “stress” proteins in response to environmental change, in order to restore homeostasis within genetic constrains, which may take from days to weeks. So, adding behaviour as an endpoint can help to formulate a quantitative minute-to-minute or hour-to-hour assessment of how tested species are (re)acting towards the toxicant concentration, bearing in mind that behaviour can be classified as the cumulative interaction of a variety of biotic and abiotic factors that represents the animal’s response to internal (physiological) and external (environmental, social) factors and that relates one organism to another (Dell’Omo, 2002). Behaviour provides an insight into various levels of biological organization, being a result and determinant of molecular, physiological, and ecological aspects of toxicology (Scott and Sloman, 2004). Therefore, behavioural responses may reflect biochemical changes in the individual organism and subsequently promote alterations in

communities, which can be translated into ecological consequences (Lagadic et al., 1994).

In former studies (e.g. with fish) behavioural parameters (considering swimming, ventilation, and foraging) have been suggested to be more sensitive than other endpoints (Beitinger, 1990; Beitinger and McCauley, 1990; Dell'Omo, 2002; Gerhardt, 2007). However, few studies have been made linking behavioural parameters to other biological (physiological, morphological) and ecological responses.

1.2. Behaviour as an endpoint

Behavioural responses comprise the first line of defence against adverse stimuli, since they can come into play within seconds after a stimulus is encountered (Beitinger, 1990). Behaviour can therefore be classified in different ways (Gerhardt, 2007): internal biochemical / physiological processes / mechanisms (neurobiological, hormonal, etc.); external ecological effects / consequences / purpose (e.g., avoidance, mating); degree of complexity such as from foraging behaviour; the distinction between individual (locomotion, foraging, learning with increasing complexity) and interactive behaviour (interspecific interactions such as predator-prey, or intraspecific interaction such as aggregation, territoriality, social interaction, reproduction related behaviours such as courtship, mating, spawning and parental care, etc.).

The study of behaviour in ecotoxicology, or behavioural ecotoxicology, is a comprehensive field which is the summation of many interactive disciplines, like ethology, ecology and toxicology (Fig. 1.1; Dell'Omo, 2002), studying how behaviour is modified by environmental pollutants.

The integrative nature of this parameter has some advantages: short response times (early warning responses), its non-invasive and non-destructive sensitiveness, and presents ecological relevance in laboratory toxicity tests (Depledge and Fossi, 1994; Gerhardt et al., 1994).

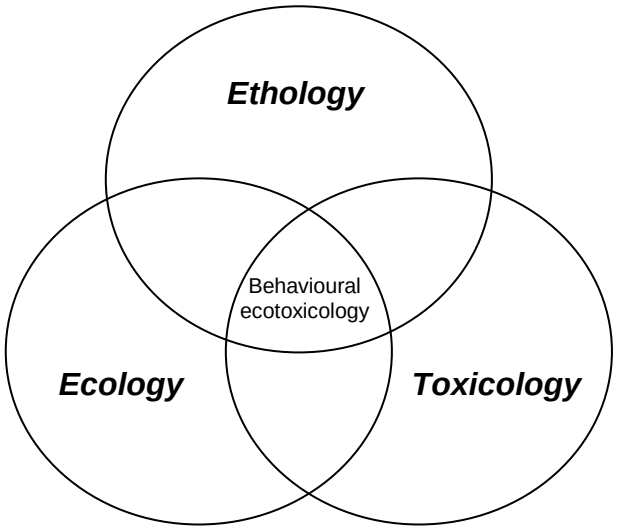

Figure 1.1 - Relationship between behavioural ecotoxicology and other disciplines [adapted from Dell'Omo (2002)].

The objective of a behavioural bioassay is to determine whether a stimulus elicits an abnormal or adverse behavioural change outside the normal range of variability in an organism, which will adversely affect its survival, growth or reproduction (Beitinger, 1990). When an organism is subjected to an adverse stimulus, if there is no immediate physiological shock, usually that organism may behaviourally avoid the stimulus and effectively reduce exposure (Beitinger, 1990). If there is no avoidance capacity, organisms may suffer impairment of physiological responses, translated into a decrease in behavioural activities, making them less fit to avoid/hide from predators and/or foraging, thus increasing the lethality probability. The development of behavioural endpoints in toxicity assessment has improved the sensitivity and versatility of these studies, providing a unique toxicological perspective because they link biochemical causes of pollutants with ecological consequences on the population and community levels (Little, 1990).

Behaviour responses can be addressed by using avoidance tests, by empirical observation or by using biomonitors. Avoidance tests are mainly focused on ecological risk assessment using soil organisms (Loureiro et al., 2005; Natal-da-Luz et al., 2008). Empirical visual observation and manual data analysis is commonly used in toxicological tests to assess location of the animals during the experimental period (Pestana et al., 2009). However, avoidance is only measured

at the end of the experiment while visual observations, although applied during the tests, do not give us a measurable and discriminated response. Besides, these observations are very time-consuming and many times even impossible (e.g. benthonic animals that are inside the sediment may exhibit deleterious behaviour and cannot be observed). Behavioural biomonitors are employed to provide a visual and, therefore, measurable and quantitative behavioural response at the whole-organism level, offering an ecologically relevant, sensitive, fast and non-destructive assessment. There are several types of biomonitors that have been employed in multiple ecology and toxicology tests in the past decade. Most frequently used biomonitors in ecotoxicology experiments are: using video/image by computer-aided video tracking system [e.g. locomotor activity of isopods, in soil (Engenheiro et al., 2005)]; or using test chambers based on quadropole impedance technique [(Gerhardt et al., 1994) e.g. behavioural activities of benthic invertebrates (Gerhardt et al., 2005; Macedo-Sousa et al., 2007), including tests with chironomids (Janssens de Bisthoven et al., 2004)].

1.3. Selection of the test species

The selection of the studied species was based on the following criteria: easiness of handling and keeping the organisms in the laboratory under controlled conditions; the organisms must live in the sediment, but need to evidence drifting/swimming behaviour; evidence of early studies comprising measured behaviour in biomonitors; and need to be sensitive towards the selected toxicants.

1.3.1. Chironomids: Ecology, biology and toxicology

Chironomidae (Insecta, Diptera), frequently referred to as *non-biting midges*, are opportunistic tube-dwelling detritivores (Pinder, 1986) that play an important part in freshwater ecosystems, as they are ubiquitous and often dominate the benthic

communities of lotic and lentic environments, preferring eutrophic and organic enriched waters (Armitage et al., 1995; Vos, 2001).

They are able to invade habitats from where other species (e.g. competitors and predators) are often excluded (Pinder, 1986) and act many times as a major food source for other animals (Armitage et al., 1995; Rieradevall et al., 1995; Garcia-Berthou, 1999), playing an important part in bioturbation and nutrient cycling (Svensson and Leonardson, 1996) in these ecosystems.

Chironomids have a short life cycle (Fig. 1.2), comprising eggs, four larval stages, pupal stage (aquatic phases), and an adult stage (terrestrial/aerial phase). Adult females after swarming and mating lay the gelatinous egg batches (arranged helicoidally) on the water surface attached to several substrates, like plants or rocks. After the hatching, first instar larvae (white/transparent) are mainly pelagic. The following instars often inhabit the upper layer of the sediment, building protecting tubes from sediment particles.

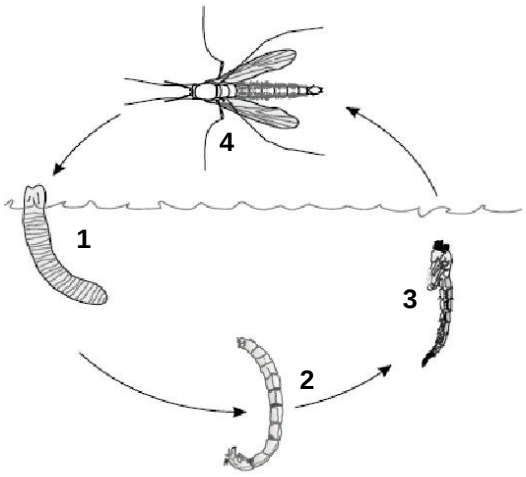

Figure 1.2 - Chironomid life cycle, displaying the egg stage (1), larval stage (2), pupal stage (3) and terrestrial imago (4) [adapted from Ristola (2000)].

The later instars are red coloured due to the presence of haemoglobin, that allows the midges to be tolerant to poorly oxygenated conditions (Ewer, 1942; Pinder, 1986) and increases the probability of a possible adaptation of the larvae to environmental changes, due to the response flexibility of haemoglobin (Ha and Choi, 2008). Larval stage can vary from few weeks to several years, being strongly related with temperature and food quality/quantity (Pinder, 1986). Pupal stage lasts for a few days, until adult emergence. Adults have a very quick existence.

Despite being benthonic organisms, larvae can be often found in the water column (Takagi et al., 2005) and can evidence drifting behaviour (Boothroyd, 1995), a probable strategy to colonize other areas, avoid predators or escape from contaminated sites. These midges exhibit characteristic motile activities like swimming, wholebody respiratory undulations and crawling. Both swimming and respiratory undulation are fast movements that involve body bending in a head-to-tail direction, while crawling combines the alternating use of the abdominal and prothoracic pseudopods as anchorage points, producing a form of locomotion analogous to caterpillar-looping (Brackenbury, 2000).

This study focused on *Chironomus riparius* that, along with *C. tentans*, are among some of the test species recognized as useful tools to study sediment toxicity (Ankley et al., 1994), being frequently used to assess the toxicity of natural (Péry et al., 2003; Faria et al., 2007) or spiked (Milani et al., 2003; Åkerblom et al., 2008) sediments. They can also be used in water only toxicity tests (Lydy et al., 2000; Stuijfzand et al., 2000) or in tests simulating contamination events either by aerial dispersion or runoff from agricultural fields (Agra and Soares, 2009; Pestana et al., 2009). For the assessment of pesticide toxic effects, several standardized methods using chironomids have been developed (ASTM, 2000; EPA, 2000; OECD, 2004). These multiple procedures and assessments are possible due to its multivoltine life cycle (Groenendijk et al., 1998); to the above mentioned widespread occurrence and ecological relevance; easiness to rear under laboratory conditions (Péry et al., 2002); and because during the larval development they are frequently in contact with the sediment (Goodyear and McNeill, 1999). Thus, these larvae have a high potential to play an important role as sentinel organisms in environmental monitoring (Choi et al., 1998).

The *C. riparius* Meigen larvae used in experiments were originated from our laboratory culture, which has been maintained for several years, genetically enriched episodically.

1.4. Tested chemicals: Insecticide (Imidacloprid) and a heavy metal (Mercury)

1.4.1. Imidacloprid

One of the most innovative and growing group of pesticides is the neonicotinoids (Tomizawa and Casida, 2003). These pesticides are chemically related to nicotine and epibatidine that are agonists of the natural nicotinic acetylcholine receptor (nAChR) (Matsuda et al. 2001). Imidacloprid (IMI) is a chloronicotinoid insecticide that belongs to this new class of pesticides and is already commonly and worldwide applied in order to control sucking insects in crops (Tomizawa and Casida, 2005; Tomlin, 2000).

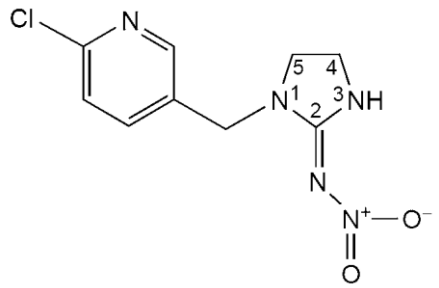

Figure 1.3 - Structure of the synthetic insecticide imidacloprid [adapted from Matsuda et al., (2001)].

Developed and patented by Bayer CropScience® AG (Monheim, Germany), IMI [1-[(6-chloro-3-pyridinyl)methyl]-*N*-nitro-2-imidazolidinimine ($C_9H_{10}ClN_5O_2$)] (Fig. 1.3) is very neurotoxic, acting via direct contact or ingestion and subsequent binding to the postsynaptic nAChRs of insects. IMI prevents acetylcholine from binding to the same receptor and, because it is not promptly degraded by acetylcholinesterase, promotes overstimulation of the insects' nervous systems, causing tremors, lack of muscular coordination, decreased activity, desensitization and blocking of the receptors leading to modified behaviours and probable death of insects (Matsuda et al., 2001; Tomizawa and Casida, 2003). Neonicotinoids present higher selectivity factors for insects versus mammals, which is attributable to both target site specificity and detoxification. Nicotinoids (e.g. nicotine) are cationic and consequently selective for the mammalian nAChR, while neonicotinoids are not protonated and selective to insect nAChR (Tomizawa and Casida, 2005).

Confidor®, Admire® and Gaucho® are some examples of commercial systemic insecticides that have IMI as the active ingredient and are used worldwide to control sucking insect pests, soil insects, termites, and some chewing insects, being also effective against adult and larval stages. In fact, IMI is used in urban areas to control turf pests in household lawns, parks, athletic fields, golf courses, etc., and this type of use appears to be increasing (CCME, 2007).

Nowadays the use of neonicotinoids is rising quicker than that of any other insecticides (e.g. organophosphates, pyrethroids) (Matsuda et al., 2001) and annual sales of neonicotinoids already account for 11%–15% of the total insecticide market (Tomizawa and Casida, 2005). This is mainly owed to their outstanding plant systemic activity and because the use of other neuroactive insecticides is declining due to selection of resistant insect strains and increasing restrictions based on human safety considerations (Tomizawa and Casida, 2005).

Due to the boost in the use of IMI, it has been frequently detected in aquatic systems (surface and groundwater), especially during rainfall events and in shallow wells (CCME, 2007), increasing the awareness on possible effects of low concentrations in aquatic life. Since it's applied in terrestrial habitats it can reach surface and ground waters via drift, leaching or dissolved runoff (Fossen, 2006; Gupta et al., 2002). IMIs' physical-chemical characteristics might promote this contamination: persistent in soils [soil photolysis half life is 38.9 days and soil aerobic half life is 997 days (ExToxNet, 1998)]; high solubility in water [0.514 g L^{-1} at 20 ºC and pH 7 (ExToxNet, 1998)]; has a long water and sediment half life [66 days (Sanchez-Bayo and Goka, 2005)], with a slow hydrolysis [half life >30 days, depending on formulation, pH and temperature (Sarkar et al., 1999), and can reach almost 1 year (CCME, 2007)], but with a fast aquatic photolysis [half life of CONFIDOR® is 2.1h at λ=280 (Wamhoff and Schneider, 1999)]; low log K_{ow} [low octanol-water partition coefficient – 0.57 (ExToxNet, 1998), with a low accumulation potential in aquatic species]. In natural field conditions, concentrations ranging from 0.13 to 11.9 µg L^{-1} of IMI have been registered (Phillips and Bode, 2004; CCME, 2007).

Nauen et al. (2002) refers that P450-monooxygenases are important in IMI detoxification and resistance development in insects, and studies with the aerial

insects *Apis mellifera* (Suchail et al., 2003) and *Musca domestica* (Nishiwaki et al., 2004) showed that IMI is metabolised very quickly and thoroughly, but its metabolites may extend the action of the insecticide.

Regarding toxicity, IMI can wield lethal and sublethal effects on non target species, being extremely toxic to aquatic invertebrates even at low concentrations. *Daphnia magna* shows a 48 h LC_{50} varying from 17.36 mg L^{-1} (Song et al., 1997) until 85 mg L^{-1} (ExToxNet, 1998); for *Lumbriculus variegatus*, a 96h EC_{50} for immobilization of 6.2 µg L^{-1} was reported by Alexander et al. (2007); for chironomids, Stoughton et al. (2008) found a 96h LC_{50} for *Chironomus tentans* using the formulated product of IMI (Admire®) of 5.40 µg L^{-1}, whilst for *Chironomus riparius* a 96h EC_{50} for mortality of 12.94 µg L^{-1} was reported by Pestana et al. (2009).

The exposure to the active ingredient compared to commercial products might yield different levels of toxicity to aquatic organisms, generating diverse results as this could vary depending on the formulation of several products, endpoints and species tested (CCME 2007; Jemec et al., 2007; Stoughton et al., 2008).

1.4.2. Mercury

The Minamata and Niigata (Japan) incidents in the 1950s and 1960s focused worldwide attention and concerns on environmental mercury pollution, when many people were poisoned by methylmercury after eating fish and shellfish highly contaminated by mercury from direct industrial sources (Wiener et al., 2003; Ekino et al., 2007). Despite the actual imposed legislations have intended to minimize and eliminate mercury discharges to the environment in the last decades, mercury legacy in sediments and soils continues to be a worldwide concern.

Mercury (Hg) is a non-essential metal (Group B), showing lack of specific binding to organic ligands, and form strong covalent bonds (Mason and Jenkins, 1995), presenting a high toxicity to all biota. As an elementary substance, mercury is persistent and cannot be degraded into harmless products. It will therefore be

permanently recycled in the physical, chemical and biological processes in the environment (OSPAR, 2000).

This heavy metal occurs naturally and is very common in the environment, deriving from both natural processes and anthropogenic activities (Wolfe et al., 1998; EPA, 2001; Wiener et al., 2003). In case of natural activities, the major occurrence is derived from fallout of atmospheric gases from volcanic activity and geothermic emissions or emissions from deep-hydrothermal vents. Anthropogenic emissions of mercury, like mine tailings or industry, have since pre-industrial times resulted in a deposition rate increase by a factor of 2-10 in the most industrialized regions (Europe, North America, south-eastern China) during the last 200 years (Hylander, 2001). Due to its high volatility, it can also be dispersed via atmospheric transportation and deposited in other regions (Morel et al. 1998; Boening 2000) mainly as Hg(II) (EPA, 1997), being in this way available to biota even in regions far away from any pollution source. As so, two cycles are believed to be involved in the environmental transport and distribution of mercury: a global atmospheric circulation of elemental mercury vapour from sources on land to the oceans, while locally transport and distribution depend on the methylation processes of inorganic mercury from mainly anthropogenic sources (Boening, 2000).

Despite declining use, mercury has many applications, like extracting gold from ores (using liquid metallic mercury); treatment of diseases such as syphilis (until the 20th century) and parasitic infections; as fungicides in agriculture (Clarkson et al., 2003); use in chlor-alkali plants [manufacture of chlorine and caustic soda from brine for, amongst other applications, use in the food industry, textile production, cleaning agents, water treatment and pharmaceuticals, as well as intermediates in manufacturing other substances (OSPAR, 2009)], industry that produces nearly 90% of European anthropogenic Hg emissions to the atmosphere (Hylander, 2001).

The extensive past industrial use of the metal and its compounds together with widespread agricultural application of organochemicals has frequently resulted in serious contamination of surface water and sediments (Ullrich et al., 2001).

Mercury can occur in three valence states (0, +1 and +2) and may be present in various physical and chemical forms in the environment. For instance, can form

salts in two ionic states: mercury (I) and mercury (II). The environmental cycle of mercury has four strongly interconnected compartments (atmospheric, terrestrial, aquatic and biotic). The atmospheric compartment is dominated by gaseous elemental mercury (Hg^0); the terrestrial compartment is dominated by Hg (II), sorbed to organic matter in soils. On the other hand, the aquatic compartment is dominated by Hg (II)-ligand pairs in water and Hg (II) in sediments, whilst the biotic compartment is dominated by methylmercury (including in the higher trophic levels of the aquatic food web). Mercury is highly reactive in the environment and cycles readily among these compartments (Wiener et al., 2003). Metallic mercury (Hg) can be oxidized to mercury ions (Hg^{2+}) which have a high affinity to sediments and which are easily transformed in the environment into mercuric ions (OSPAR, 2000). Elemental mercury (Hg^0) in surface waters occurs mainly from the reduction of Hg (II) compounds by aquatic organisms, and oxidation of Hg^0 can conversely form Hg (II) (Ullrich, 2001). Since Hg^0 is very volatile (Morel et al., 1998), it can be readily lost from the aquatic environment at normal temperatures, playing an important part in the global Hg cycle, since its atmospheric transport in the vapor phase represents one of the major pathways of global deposition. The inorganic Hg forms, then again, can be transformed into methylmercury (MeHg) by chemical processes and microorganisms like sulfate-reducing bacteria – methylation (Wiener et al., 2003). Subsequent exposure of biota to the newly formed MeHg, a potent neurotoxin that is readily accumulated by aquatic biota due to its lipophilic and protein-binding properties, may pose a threat to humans and other fish-eating (Ullrich et al., 2001).

Contamination of biota from freshwater ecosystems by this heavy-metal is therefore a chronic and widespread environmental problem (Eisler, 1987; Boening, 2000). At high exposures Hg causes behavioural modifications, growth inhibition, reproductive impairment, decreased embryo/larval survival, and a variety of neurological and enzymatic dysfunctions in aquatic species (Zillioux et al., 1993).

Mercury bioaccumulation usually starts by exposure through direct contact, breathing or by ingestion and subsequent retention on a tissue or organ (Fisher and Reinfelder, 1995) and can happen even at low concentrations, exhibiting high toxicity towards aquatic organisms (Suchanek et al., 1995; Tremblay and Lucotte,

1997). In fact, because it can be biomagnified through the trophic chain (Morel et al., 1998), mercury bioaccumulation can present an ecological risk. The majority of the toxicological studies assessing the effects of Hg in aquatic biota have, therefore, focused on bioaccumulation and trophic transfer of the heavy-metal (Mason et al., 1995; Wong et al., 1997; Vázquez-Núñez et al., 2007; Žižek et al., 2007).

Lethal and sub-lethal toxicological endpoints in freshwater organisms have also been reported. Boening (2000) describes several LC_{50} values: 96h LC_{50} for fish ranges between 33 and 400 µg L^{-1}; for *Hydropsyche betteni*, 96h LC_{50} is 2000 µg L^{-1}; for *Daphnia magna*, the 48h LC_{50} is 3610 µg L^{-1}. Vidal and Horne (2003) also reported a 96h LC_{50} of 0.18 mg L^{-1} for *Tubifex tubifex*, while Rossaro et al. (1985) accounted for a 48h LC_{50} of 750 µg L^{-1} for *C. riparius*.

So, mercury can have deleterious effects on biota, is easily bioaccumulated and very persistent in the environment, with the nature and reactions of the Hg species determining the solubility, transport and toxicity of Hg in aquatic ecosystems.

1.5. Research goals and thesis outline

The information on the implications of the disturbance of complex behaviours by toxicants in benthonic organisms and in field populations is still scarce. The development and validation of behavioural tests to provide early warning information on these organisms' behavioural reactions to the potential impact of contaminants (in this study: mercury and imidacloprid) is very important, especially during discharge or runoff periods, in order to evaluate potential effects on field communities.

This thesis aimed to investigate how *Chironomus riparius*' behaviour (a new approach, using an online biomonitor) and other endpoints (e.g. growth, emergence, bioaccumulation and biomarker effect) are affected by exposure to the selected contaminants. Results are expected to improve the knowledge of the effects of imidacloprid and mercury exposure on benthic larvae, and to assess the sensitiveness of the endpoints chosen for this study.

In this research, the null hypothesis tested was that mercury and imidacloprid do not affect the normal behaviour nor compromise the development of *Chironomus riparius*.
To address this, four separate chapters are organized in order to focus on these issues, followed by a general conclusion with final remarks.

Chapter two: "Behaviour and growth of *Chironomus riparius* Meigen (Diptera: Chironomidae) under Imidacloprid pulse and constant exposure scenarios", we focused on the effects of the insecticide on growth and behaviour of the chironomids when subjected to a constant and a pulse (followed by a recovery period) exposure to the pesticide.

Chapter three: we address the "Effects of imidacloprid exposure on *Chironomus riparius* Meigen larvae: linking acetylcholinesterase activity to behaviour". In this study we perform a link between parameters with ecological relevance at individual level (behavioural parameters) with biochemical responses, to fully understand xenobiotics' mode of action.

Chapter four: "Effects of mercury on growth, emergence and behaviour of *Chironomus riparius* Meigen (Diptera: Chironomidae)", we assessed the effects of mercury on *C. riparius*, simulating a mercury discharge. Growth was measured after 8 days exposure, while behaviour was measured at days 4 and 10, and emergence and development time were also assessed.

Chapter five: "Bioaccumulation and elimination of mercury in the midge larvae *Chironomus riparius* Meigen (Diptera: Chironomidae): a link to behaviour", mercury toxicokinetics (uptake and elimination) was evaluated using *C. riparius* under a water-only exposure. Behavioural parameters were monitored during the experimental (uptake and elimination) period.

Chapter six: general conclusions and final remarks.

References

Adams WJ, Rowland CD (2003) Aquatic toxicology test methods. In: Handbook of Ecotoxicology (2nd ed). Hoffman DJ, Rattner BA, Burton GA, Cairns J (Eds.) CRC Press, Boca Raton, Florida.

Agra AR, Soares AMVM (2009) Effects of two insecticides on survival, growth and emergence of Chironomus riparius Meigen. Bulletin of Environmental Contamination and Toxicology. 82: 501-504.

Åkerblom N, Arbjörk C, Hedlund M, Goedkoop W (2008) Deltamethrin toxicity to the midge Chironomus riparius Meigen—Effects of exposure scenario and sediment quality Ecotoxicology and Environmental Safety. 70: 53-60.

Alexander AC, Culp JM, Liber K, Cessna AJ (2007) Effects of insecticide exposure on feeding inhibition in mayflies and oligochaetes. Environmental Toxicology and Chemistry. 26: 1726-1732.

Ankley GT, Benoit DA, Balogh JC, Reynoldson TB, Day KE, Hoke RA (1994) Evaluation of potential confounding factors in sediment toxicity tests with three freshwater benthic invertebrates. Environmental Toxicology and Chemistry. 13: 627-635.

Armitage PD, Cranston PS, Pinder LCV (1995) The Chironomidae: The biology and ecology of non-biting midges. Armitage PD, Cranston PS, Pinder LCV (Eds.) Chapman & Hall, London, UK.

ASTM (2000) Standard test methods for measuring the toxicity of sediment-associated contaminants with freshwater invertebrates (E 1706). In: Annual Book of ASTM standards, vol 11.05, Philadelphia.

Beitinger TL (1990): Behavioural reactions for the assessment of stress in fishes. Journal of Great Lakes Research. 16: 495–528.

Beitinger TL, McCauley RW (1990) Whole-animal physiological processes for the assessment of stress in fishes. Journal of Great Lakes Research. 16: 542–575.

Boothroyd IKG (1995) Temporal and diel drift of Chironomidae (Diptera: Insecta) larvae in a Northern New Zealand stream. In: Cranston P (Ed.). Chironomids: from genes to ecosystems. Melbourne, Australia: CSIRO Australia.

Boening DW (2000) Ecological effects, transport, and fate of mercury: a general review, Chemosphere. 40: 1335-1351.

Brackenbury J (2000) Locomotory modes in the larva and pupa of *Chironomus plumosus* (Diptera, Chironomidae). Journal of Insect Physiology. 46: 1517-1527.

Calow P (1997) Handbook of ecotoxicology. Calow P (ed.) Blackwell Science, Oxford, Uk. 885pp.

Clarkson TW, Magos L, Myers GJ (2003) Human Exposure to Mercury: The Three Modern Dilemmas. The Journal of Trace Elements in Experimental Medicine. 16: 321–343.

CCME (2007) Canadian water quality guidelines: imidacloprid scientific supporting document. Winnipeg, Canadian Council of Ministers of the Environment.

Choi J, Rivoal F, Roche H, Caquet T (1998) Identification de biomarqueurs d'ecotoxicite chez deux organismes sentinelles potentials, le chironome (*Chironomus riparius* (Mg.)) et la lymnee (Lymnaea palustris (Muller)). Ichtyophysiologica Acta. 21: 89-106.

Dell'Omo (2002) Behavioural Ecotoxicology. Dell'Omo G (Ed.) John Wiley & Sons, Inc., Hoboken, New Jersey, USA.

Depledge MH, Fossi MC (1994) The role of biomarkers in environmental assessment (2). Invertebrates. Ecotoxicology. 3: 161-172.

Domingues I, Guilhermino L, Soares AMVM, Nogueira, AJA (2007) Assessing dimethoate contamination in temperate and tropical climates: Potential use of biomarkers in bioassays with two chironomid species. Chemosphere. 69: 145-154.

Eisler R (1987) Mercury hazards to fish, wildlife, and invertebrates: a synoptic review. U.S. Fish and Wildlife Service Biological Report 85 (1.10).

Ekino S, Susa M, Ninomiya T, Imamura K, Kitamura T (2007) Minamata disease revisited: An update on acute and chronic manifestations of mercury poisoning. Journal of the Neurological Sciences. 262: 131-144.

Engenheiro EL, Hankard PK, Sousa JP, Lemos MF, Weeks JM, Soares AMVM (2005) Influence of dimethoate on acetylcholinesterase activity and locomotor function in terrestrial isopods. Environmental Toxicology and Chemistry. 24: 603-609.

EPA – United States Environmental Protection Agency (1997) Fate and transport of mercury in the environment. In: Mercury study report to congress (Vol. III). EPA- 452/R-97-005.

EPA – United States Environmental Protection Agency (2000) Methods for Measuring the Toxicity and Bioaccumulation of Sediment-associated Contaminants with Freshwater Invertebrates - Second Edition. EPA/600/R-99/064.

EPA – Unites States Environmental Protection Agency (2001) Mercury update: Impact on fish advisories. Washington DC. USEPA.

Ewer RF (1942) On the function of haemoglobin in *Chironomus*. Journal of Experimental Biology. 18: 197-205.

Extension Toxicology Network – ExToxNet (1998) Imidacloprid Pesticide Information Profile. [Online]. Available at http://extoxnet.orst.edu/pips/imidaclo.htm. (Verified March 2010). Oregon State University, Corvallis, OR.

Faria MS, Nogueira AJA, Soares AMVM (2007) The use of Chironomus riparius larvae to assess effects of pesticides from rice fields in adjacent freshwater ecosystems. Ecotoxicology and Environmental Safety. 67: 218-226.

Fisher N, Reinfelder, J (1995) The trophic transfer of metals in marine systems. In: Tessier A, Turner DR (eds). Metal speciation and bioavailability in aquatic systems. John Wiley & Sons.

Fleeger JW, Carman KR, Nisbet RM (2003) Indirect effects of contaminants in aquatic ecosystems. Science of the Total Environment. 317: 207-233.

Forbes, V. E., Palmqvist A, Bach L (2006) The use and misuse of biomarkers in ecotoxicology. Environmental Toxicology and Chemistry. 25: 272-280.

Fossen M (2006). Environmental fate of imidacloprid. Environmental Monitoring, Department of Pesticide Regulation, Sacramento, CA.

Garcia-Berthou E (1999) Food of introduced mosquito fish: ontogenetic diet shift and prey selection. Journal of Fish Biology. 55: 135-147.

Gerhardt A, Svensson E, Clostermann M, Fridlund B (1994) Monitoring of behavioral patterns of aquatic organisms with an impedance conversion technique. Environment international. 20: 209-219.

Gerhardt A, Janssens de Bisthoven L, Soares AMVM (2005) Effects of acid mine drainage and acidity on the activity of *Choroterpes picteti* (Ephemeroptera: Leptophlebiidae). Archives of Environmental Contamination and Toxicology. 48: 450-458.

Gerhardt A (2007) Importance of exposure route for behavioural responses in Lumbriculus variegatus Müller (Oligochaeta: Lumbriculida) in short-term exposures to Pb. Environmental Science and Pollution Research. 14: 430-434.

Goodyear KL, McNeill S (1999) Bioaccumulation of heavy metals by aquatic macro-invertebrates of different feeding guilds: a review. The Science of the Total Environment. 229: 1-19.

Groenendijk D, Postma JF, Kraak MHS, Admiraal W (1998) Seasonal dynamics and larval drift of Chironomus riparius (Diptera) in a metal contaminated lowland river. Aquatic Toxicology. 38: 341-351.

Grue CE, Gardner SC, Gilbert PL (2002) On the significance of pollutant-induced alterations in the behaviour of fish and wildlife. In: Behavioural Ecotoxicology. Dell'Omo G (Ed.) John Wiley & Sons, Inc., Hoboken, New Jersey, USA.

Gupta S, Gajbhiye VT, Kalpana, Agnihotri NP (2002) Leaching behavior of imidacloprid formulations in soil. Bulletin of Environmental Contamination and Toxicology. 68: 502-508.

Ha M, Choi J (2008) Effects of environmental contaminants on hemoglobin of larvae of aquatic midge, *Chironomus riparius* (Diptera: Chironomidae): A potential biomarker for ecotoxicity monitoring. Chemosphere 71: 1928-1936.

Hylander LD (2001) Global mercury pollution and its expected decrease after a mercury trade ban. Water, Air and Soil Pollution. 125: 331-344.

Janssens de Bisthoven L, Gerhardt A, Soares AMVM (2004) Effects of acid mine drainage on larval *Chironomus* (Diptera, Chironomidae) measured with the Multispecies Freshwater Biomonitor™. Environmental Toxicology and Chemistry. 23: 1123-1128.

Jemec A, Tisler T, Drobne D, Sepcic K, Fournier D, Trebse P (2007) Comparative toxicity of imidacloprid, of its commercial liquid formulation and of diazinon to a non-target arthropod, the microcrustacean *Daphnia magna*. Chemosphere. 68: 1408-1418.

Lagadic L, Caquet T, Ramade F (1994) The role of biomarkers in environmental assessment (5). Invertebrate populations and communities. Ecotoxicology. 3: 193-208.

Little EE (1990) Behavioural toxicology: Stimulating challenges for a growing discipline. Environmental Toxicology and Chemistry. 9: 1-2.

Loureiro S, Soares AMVM, Nogueira AJA (2005) Terrestrial avoidance behaviour tests as screening tool to assess soil contamination. Environmental Pollution. 138: 121-131.

Lydy MJ, Lasater JL, Landrum PF (2000) Toxicokinetics of DDE and 2-Chlorobiphenyl in *Chironomus tentans*. Archives of Environmental Contamination and Toxicology. 38: 163-168.

Macedo-Sousa JA, Pestana JLT, Gerhardt A, Nogueira AJA, Soares AMVM (2007) Behavioural and feeding responses of *Echinogammarus meridionalis* (Crustacea, Amphipoda) to acid mine drainage. Chemosphere. 67: 1663-1670.

Mason AZ, Jenkins KD (1995) Metal detoxification in aquatic organisms. In: Metal Speciation and Bioavailability in Aquatic Systems. Tessier A, Turner DR (Eds). John Wiley & Sons, West Sussex, UK, 479–609.

Mason RP, Reinfelder JR, Morel FMM (1995) Bioaccumulation of mercury and methylmercury. Water, Air and Soil Pollution. 80: 915-921.

Matsuda K, Buckingham SD, Kleier D, Rauh JJ, Grauso M, Sattelle DB (2001) Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. Trends in Pharmacological Sciences. 22: 573-580.

Meregalli G, Bettinetti R, Pluymers L, Vermeulen AC, Rossaro B, Ollevier F (2002) Mouthpart Deformities and Nucleolus Activity in Field-Collected *Chironomus riparius* Larvae. Archives of Environmental Contamination and Toxicology 42: 405-409.

Milani D, Reynoldson TB, Borgmann U, Kolasa J (2003) "The relative sensitivity of four benthic invertebrates to metals in spiked-sediment exposures and application to contaminated field sediment. Environmental Toxicology and Chemistry. 22: 845-854.

Morel FMM, Kraepiel AML, Amyot M (1998) The chemical cycle and bioaccumulation of mercury. Annual Review of Ecology and Systematics. 29: 543-566.

Moriarty F (1993) Ecotoxicology, The study of pollutants in ecosystems. 2nd Ed. Academic Press, London, UK.

Natal-da-Luz T, Amorim MJM, Römbke J, Sousa JP (2008) Avoidance tests with earthworms and springtails: Defining the minimum exposure time to observe a significant response. Ecotoxicology and Environmental Safety. 71: 545–551.

Nauen R, Stumpf N, Elbert A (2002) Toxicological and mechanistic studies on neonicotinoid cross resistance in Q-type *Bemisia tabaci* (Hemiptera : Aleyrodidae). Pest Management Science. 58: 868-875.

Nishiwaki N, Sato K, Nakagawa Y, Miyaishita M, Miyagawa H (2004) Meatbolism of imidacloprid in houseflies. Journal of Pesticide Science. 29: 110-116.

OECD (2004) Guideline 219 - Sediment-water chironomid toxicity test using spiked water.

OSPAR – The Convention for the Protection of the Marine Environment of the North-East Atlantic (2000) OSPAR Background document on mercury and organic mercury compounds. London, OSPAR Commission.

Péry ARR, Mons R, Flammarion P, Lagadic L, Garric J (2002) A modeling approach to link food availability, growth, emergence, and reproduction for the midge *Chironomus riparius*. Environmental Toxicology and Chemistry. 21: 2507-2513.

Péry ARR, Sulmon V, Mons R, Flammarion P, Lagadic L, Garric J (2003). A model to understand the confounding effects of natural sediments in toxicity tests with *Chironomus riparius*. Environmental Toxicology and Chemistry. 22: 2476-2481.

Pestana JL, Loureiro S, Baird DJ, Soares AMVM (2009) Fear and loathing in the benthos: Responses of aquatic insect larvae to the pesticide imidacloprid in the presence of chemical signals of predation risk. Aquatic Toxicology. 93: 138-49.

Phillips PJ, Bode RW (2004) Pesticides in surface water runoff in south-eastern New York State, USA: seasonal and stormflow effects on concentrations. Pest Management Science. 60: 531-543.

Pinder LCV (1986) Biology of Freshwater Chironomidae. Annual Review of Entomology. 31: 1-23.

Rieradevall M, García-Berthou E, Prat N (1995) Chironomids in the diet of fish in Lake Banyoles (Catalonia, Spain). In: Cranston P (Ed.). Chironomids: from genes to ecosystems. Melbourne, Australia: CSIRO Australia.

Ristola T (2000). Assessment of sediment toxicity using the midge *Chironomus riparius* (Diptera: Chironomidae). PhD Thesis. University of Joensuu, Joensuu, Finland.

Rohr JR, Kerby JL, Sih A (2006) Community ecology as a framework for predicting contaminant effects. Trends in Ecology & Evolution. 21: 606-613.

Rossaro B, Gagging GF, Marchetti R (1986) Accumulation of mercury in larvae and adults, *Chironomus riparius* (Meigen). Bulletin of Environmental Contamination and Toxicology. 37: 402-406.

Sanchez-Bayo F, Goka K (2005) Unexpected effects of zinc pyrithione and imidacloprid on Japanese medaka fish (*Oryzias latipes*). Aquatic Toxicology. 74: 285-293.

Sarkar MA, Biswas PK, Roy S, Kole RK, Chowdhury A (1999) Effect of pH and type of formulation on the persistence of imidacloprid in water. Bulletin of Environmental Contamination and Toxicology. 63: 604-609.

Scott GR, Sloman KA (2004) The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. Aquatic Toxicology. 68: 369-392.

Sibley PK, Benoit DA, Ankley GT (1997) The significance of growth in *Chironomus tentans* sediment toxicity tests: relationship to reproduction and demographic endpoints. Environmental Toxicology and Chemistry. 16: 336-345.

Song MY, Stark JD, Brown JJ (1997) Comparative toxicity of four insecticides, including imidacloprid and tebufenozide, to four aquatic arthropods. Environmental Toxicology and Chemistry. 16: 2494-2500.

Stoughton SJ, Liber K, Culp J, Cessna A (2008) Acute and Chronic Toxicity of Imidacloprid to the Aquatic Invertebrates *Chironomus tentans* and *Hyalella azteca* under Constant- and Pulse-Exposure Condition. Archives of Environmental Contamination and Toxicology. 54: 662-673.

Stuijfzand SC, Helms M, Kraak MHS, Admiraal W (2000) Interacting effects of toxicants and organic matter on the midge *Chironomus riparius* in polluted river water. Ecotoxicology and Environmental Safety. 46: 351-356.

Suchail S, Debrauwer L, Belzunces LP (2003) Metabolism of Imidacloprid in *Apis mellifera*. Pest Management Science. 60: 291-296.

Suchanek TH, Richerson PJ, Holts LJ, Lamphere BA, Woodmansee CE, Slotton DG, Harner EJ, Woodward, LA (1995) Impacts of mercury on benthic invertebrate populations and communities within the aquatic ecosystem of Clear Lake, California. Water, Air and Soil Pollution. 80: 951-960.

Svensson JM, Leonardson L (1996). "ffects of bioturbation by tube-dwelling chironomid larvae on oxygen uptake and denitrification in eutrophic lake sediments. Freshwater Biology. 35: 289-300.

Takagi S, Kikuchi E, Doi H, Shikano S (2005) Swimming behaviour of *Chironomus acerbiphilus* larvae in Lake Katanuma. Hydrobiologia. 548: 153-165.

Tomizawa M, Casida JE (2003) Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. Annual Review of Entomology. 48: 339-364.

Tomizawa M, Casida JE (2005) Neonicotinoid insecticide toxicology: mechanisms of selective action. Annual Review of Pharmacology and Toxicology. 45: 247-268.

Tomlin CDS (2000) The Pesticide Manual. Berkshire, U.K., BCPC Publications.

Tremblay A, Cloutier L, Lucotte M (1998) Total mercury and methylmercury fluxes via emerging insects in recently flooded hydroelectric reservoirs and a natural lake. Science of The Total Environment. 219: 209-211.

Ullrich SM, Tanton TW, Abdrashitova SA (2001) Mercury in the Aquatic Environment: A Review of Factors Affecting Methylation. Critical Reviews in Environmental Science and Technology. 31: 241-293.

Vázquez-Núñez R, Méndez N, Green-Ruíz C (2007) Bioaccumulation and Elimination of Hg in the Fireworm *Eurythoe complanata* (Annelida: Polychaeta) from Mazatlan, Mexico. Archives of Environmental Contamination and Toxicology. 52: 541-548.

Vidal DE, Horne AJ (2003) Mercury Toxicity in the Aquatic Oligochaete *Sparganophilus pearsei*: I. Variation in Resistance Among Populations. Archives of Environmental Contamination and Toxicology. 45: 184-189.

Vos JH (2001) Feeding of detritivores in freshwater sediments. PhD Thesis. University of Amsterdam, Amsterdam, The Netherlands.

Wamhoff H, Schneider V (1999) Photodegradation of imidacloprid. Journal of Agricultural and Food Chemistry. 47: 1730-1734.

Wiener JG, Krabbenhoft DP, Heinz GH, Scheuhammer AM (2003) Ecotoxicology of mercury. In: Handbook of Ecotoxicology (2nd ed). Hoffman DJ, Rattner BA, Burton GA, Cairns J (Eds.) CRC Press, Boca Raton, Florida.

Wolfe MF, Schwarzbach S, Sulaiman RA (1998) Effects of mercury on wildlife: a comprehensive review. Environmental Toxicology and Chemistry. 17: 146-160.

Wong AHK, McQueen DJ, Williams DD, Demers E (1997) Transfer of mercury from benthic invertebrates to fishes in lakes with contrasting fish community structures. Canadian Journal of Fisheries and Aquatic Science. 54: 1320-1330.

Zillioux EJ Porcella DB, Benoit JM (1993) Mercury cycling and effects in freshwater wetland ecosystems. Environmental Toxicology and Chemistry. 12: 2245-2264.

Žižek S, Horvat M, Gibičar D, Fajon V, Toman MJ (2007) Bioaccumulation of mercury in benthic communities of a river ecosystem affected by mercury mining. The Science of The Total Environment. 377: 407-415.

Chapter 2

Behaviour and growth of *Chironomus riparius* Meigen (Diptera: Chironomidae) under Imidacloprid pulse and constant exposure scenarios

2. BEHAVIOUR AND GROWTH OF *CHIRONOMUS RIPARIUS* MEIGEN (DIPTERA: CHIRONOMIDAE) UNDER IMIDACLOPRID PULSE AND CONSTANT EXPOSURE SCENARIOS

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Abstract

Imidacloprid is a new insecticide that mimics nicotine, combining its insecticidal activity with a reduced persistence in the environment. The toxicity of imidacloprid to *Chironomus riparius* Meigen using the formulated product Confidor® from Bayer®, in pulse and continuous exposure was evaluated in this study. The behavioural response of the midge after toxicant exposure using an online biomonitor was also investigated. Early second instar *C. riparius* larvae were exposed in either constant (10 days) or pulse (4 days, followed by 6 days post exposure in clean medium) conditions. Imidacloprid constant exposure resulted in a decrease in growth and impairment of the behavioural pattern of the midge larvae. Pulsed exposure followed by a recovery period revealed a recovery of midge physiological conditions, by reaching a stabilization of normal behavioural activities and growth among treatments. Moreover, ventilation showed to be a more sensitive parameter by revealing a faster recovery than locomotion. Behaviour alterations may weaken the ability to escape from predators, and reduce food acquisition with consequent growth impairment. These effects may have an impact at the population and community level.

Keywords Insecticide; Biomonitor; Confidor; Behavioral Response; Post-exposure; Recovery

2.1. Introduction

The application of pesticides in agriculture fields can frequently contaminate nearby freshwater ecosystems (Crane et al., 1995). Usually they occur in short time spans either by field runoff, ground water flows, spray drift or accidental spillage (Liess et al., 1999). Therefore, aquatic macroinvertebrates present in those systems can be continuously or episodically exposed to inputs of lethal or sub-lethal concentrations of pesticides that might cause a loss of biodiversity and impair ecosystem function.. Several efforts have been carried out to reduce the effects of pesticides to non-target organisms, such as the development of new chemicals that do not require airborne spraying and that are specific for a certain plague. One of the most common and worldwide used pesticides in the control of sucking insects is imidacloprid [IMI; 1-[(6-chloro-3-pyridinyl)methyl]-*N*-nitro-2-imidazolidinimine ($C_9H_{10}ClN_5O_2$)], developed by Bayer CropScience® AG (Monheim, Germany). This insecticide shows selective toxicity for insects since it binds to their postsynaptic nicotinic acetylcholine receptors, causing its overstimulation by mimicking acetylcholine action in the central nervous system (Tomizawa and Casida, 2005). IMI might also be toxic towards non-target insects, such as *Apis mellifera* [LD_{50} of IMi at 24 and 48 h were about 5ng/bee (Suchail et al., 2000)], *Daphnia magna* [LC_{50} of IMI at 48 h was 10.44 mg L^{-1} (Song et al., 1997)], *Hyallela azteca* [LC_{50} of IMI at 96 h was 0.526 mg L^{-1} (SERA, 2005)], *Simulium vittatum* [LC_{50} of IMI at 48 h was 6.74 – 9.45 µg L^{-1} (Overmyer et al., 2005)], or even chironomids [LC_{50} of IMI at 96 h for *Chironomus tentans*, using the technical Imidacloprid and the formulated product Admire® were 5.75 and 5.40 µg L^{-1}, respectively (Stoughton et al., 2008); 96 h EC_{50} of IMI for *C. riparius* was 12.94 µg L^{-1} (Pestana et al., 2009a)]; and also toxic to other macroinvertebrates [LC_{50} of IMI after 14 days were 3.74 mg kg^{-1} dry soil for *Aporrectodea nocturna* and 2.81 mg kg^{-1} dry soil for *A. icterica* (Capowiez et al., 2005)]. IMI is typically applied in agricultural fields only once or twice in a season, so any transport to surface waters is likely to occur in short-duration pulses, followed by dissipation and biodegradation (CCME, 2007). In samples collected in natural field conditions, concentrations ranging from 0.5 to 11.9 µg L^{-1} of IMI have been measured in

agricultural runoff; and concentrations of 6.4 μg L^{-1} were detected in groundwater (CCME, 2007). A maximum of 0.13 μg L^{-1} of IMI was detected in river water in summer months and during the growing season, which could reflect a nearly constant application of these in a variety of settings (Phillips and Bode, 2004). The persistence of this chemical in aquatic systems is also dependent of several factors such as light, temperature, and microbial communities (Liess et al., 1999). IMI is highly soluble in water [0.514 g L^{-1} at 20 ºC and pH 7 (ExToxNet, 1998)]; with a fast aquatic photolysis [half life of CONFIDOR® is 2.1h at λ=280 (Wamhoff and Schneider, 1999)]; with a low log K_{ow} [low octanol-water partition coefficient – 0.57 (ExToxNet, 1998), with a low accumulation potential in aquatic species].The majority of laboratory toxicity studies assessing the effects of pesticides use constant concentrations and measure effects after a short period of exposure (Pestana et al., 2009a; Agra and Soares, 2009; Stoughton et al., 2008). They do not take into consideration more realistic and relevant exposure scenarios, as pulse exposures, thus do not allow a real understanding of when the toxic starts to act on the organism and what is happening to the organism throughout the experimental period.

To assess the effects of the exposure to a toxicant, one may recur to organisms' behavioural responses, which has been the subject of an increasing body of work (Natal-da-Luz et al., 2004; Loureiro et al., 2005; Pestana et al., 2009a). Being the result of the interaction of an organism with the surrounding environment, behaviour integrates the complexity of individual physiological processes and mechanisms with the environmental stimuli that cause them (Dell'Omo, 2002). Behaviour can thus be considered the first line of defence to environmental stimuli (Beitinger, 1990), and it is regarded as one of the most sensitive indicators of chemical stress (Gerhardt et al., 1994). Behavioural biomonitors are used to record animal activities and are, therefore, employed to provide a measurable response at the organism level (e.g. Engenheiro et al., 2005; Macedo-Sousa et al., 2007; Azevedo-Pereira and Soares, 2010 – Chapter 4). In fact, online biomonitors can generate quick and early-stage data that can be linked to higher levels of biological organization, even at the population and community level (Gerhardt, 1995).

Chironomids are opportunistic tube-dwelling detritivores that play an important role in aquatic ecosystems, not only due to its predominance on benthic communities of almost all freshwater environments (Vos, 2001; Péry et al., 2002) but also because they are a major food source for fish and aquatic birds (Rieradevall et al., 1995). For the above mentioned and because of the easiness to rear under laboratory conditions, the larvae of the midge *Chironomus riparius* Meigen are widely used test organisms in acute and chronic toxicity tests (e.g. Faria et al., 2006, 2007; Agra and Soares, 2009), For the assessment of pesticide toxic effects, several standardized methods using sediment-dwelling organisms such as chironomids have been developed (e.g. ASTM – E1706, 2000; EPA – EPA/600/R-99/064, 2000; OECD – guideline 219, 2004).

Here, *C. riparius*' behaviour and growth were studied after different IMI exposure regimes by exposing the organisms in a 10 days constant exposure and 4 days exposure followed by 6 days in clean medium, where the organisms' recovery after the exposure was also assessed.

2.2. Material and Methods

2.2.1. Test organisms

C. riparius midges were obtained from our laboratory culture established for 3 years. The cultures were maintained in standard conditions at 20 ± 2 ºC, in a 16 h light-8 h dark cycle, in an enclosed transparent acrylic box that contains several plastic beakers. Each beaker contained a 2 cm layer of acid-washed burned commercial river sand (< 1 mm), approximately 2.5 L of reconstituted hard water ASTM (ASTM – E1706, 2000), and a constant and gentle inflow of oxygen. This system allows the whole life cycle of the chironomids to occur, including swarming and copulation of emerged adults (OECD, 2004). Freshly laid egg masses are transferred onto crystallizing dishes with culture medium until hatching occurs, after approximately 2–3 days, and the hatched F1 larvae were used either to start

a new culture or to use in bioassays. Water and sediment were renewed every week and larvae were fed (1 mg animal^{-1} day^{-1}) twice a week with a suspension of ground Tetramin® (Tetrawerke, Germany).

2.2.2. Imidacloprid

Confidor® 200 SL was acquired from Bayer CropScience® AG (Monheim, Germany) and was used to prepare the stock solution of imidacloprid with Milli-Q water, which was stored at 4 ºC and protected from light. The concentration determined for the stock solution was 494 µg of IMI L^{-1}. Tests solutions used in the bioassays were prepared by adding the relevant amount of stock solution in ASTM hard water.

The nominal concentrations used for both presented bioassays were 0.00 (control) 0.50, 1.50 and 4.50 µg of IMI L^{-1}, selected after previous results from an acute testing [48h LC50 (95.0 % Confidence Interval) was 19.90 µg of IMI L^{-1} (14.64 – 27.16 µg of IMI L^{-1})] and bibliography (Stoughton et al., 2008; Pestana et al., 2009a). Chemical analyses of the IMI samples from the stock solution and bioassays were conducted by Terracon Laboratorium für Umwelt- und Pestizidanalytik GmbH (Jütterborg, Germany), using a HPLC-PDA-System equipped with 2 HPLC pumps Model LC-10ADvp, Autosampler SIL-10ADvp, column oven CTO-10ASvp, and a photodiodenarray-detector (PDA) SPD-M10Avp (Shimadzu, Japan). Procedure consisted in: all samples containing high IMI concentrations (e.g. stock solutions) were diluted with deionised water, while samples with lower concentrations were extracted from 100–200 mL water samples (flow of 0.5 mL min^{-1}) using solid phase extraction (SPE cartridges Supelclean ENVI-18, Supelco, Schnelldorf, Germany) and acetonitrile (1:1 v:v) for elution. 10 µL acetonitrile-extracts were then applied to a chromatography column (LUNA C18, Phenomenex, Aschaffenburg, Germany), at a flow rate of 0.4 mL min^{-1} using water, 0.1 % formic acid and acetonitrile as eluents. Detection was carried out at 270 nm with a limit of quantification of 0.1 µg L^{-1}.

2.2.3. Acute toxic experiments

Mortality of *C. riparius* exposed to the pesticide was estimated to establish a range of sub-lethal concentrations to be used in chronic bioassays and to estimate the sensitivity of this species to IMI. The solutions of imidacloprid used in the test were prepared in ASTM hard water and comprised nominal concentrations of 1.25, 2.50, 5.00, 10.00, 15.00, 20.00, 30.00 and 40.00 µg L^{-1}. Twenty-five 7 days old larvae divided by 5 replicates were exposed in glass beakers containing 150 mL of pesticide solutions with no addition of food. After 48 h exposure, mortality was determined by mechanical stimulation. Animals that did not respond to this stimulation were considered dead.

2.2.4. Organisms' exposure

To simulate an insecticide contamination by runoff from agricultural fields, experiments were performed as an adaptation of the OECD guideline sediment-water chironomid toxicity test using spiked water (OECD – guideline 219, 2004). Two bioassays were performed: one comprising 10 days of exposure to a gradient of pesticide concentrations, and the other with 4 days of pesticide exposure followed by 6 days of exposure to clean medium (sediment and water). The experiments were performed using 200 mL glass vials (10 replicates per treatment), with five 2^{nd} instar larvae (four days old) per replicate. Each replicate contained 40 g of acid-washed inorganic fine sediment (granulometry <1 mm) and 150 mL of test solution.

Every 48 h, 75 mL of the test solution was renewed and organisms were fed with ground Tetramin® (0.5 mg $animal^{-1}$ day^{-1}). Tests were performed in same conditions as described for the cultures. In the recovery test, after 4 days of exposure the larvae were gently removed from the test vessels and placed in beakers with clean medium and sediment, until the end of the experiment.

2.2.5. Chronic toxicity assessment

Behaviour and total length of larvae were used as the response parameters. Replicates were examined daily. Growth was calculated as the difference between the final and the initial size of the larvae divided by the number of test days. In the post exposure test, additional replicates were used to measure growth on day four. Five replicates were used to measure larvae body length using a stereo dissecting microscope (MS5, Leica Microsystems, Houston, USA) with a built-in calibrated eye-piece micrometer.

Behaviour patterns were measured as a function of animal movements recorded by an online biomonitor (Multispecies Freshwater Biomonitor, MFB). The MFB was developed by Gerhardt et al. (1994) and is based on a quadropole impedance technique where the organism moves freely inside a chamber that contains two pairs of stainless steel plates attached to the inner walls that serve as electrodes. One pair of electrodes generates a high frequency alternating current perturbation while the other pair measures changes of the impedance and its frequency within the chamber due to the organism movements (Gerhardt, 2000). The data generated is registered in the measuring device and processed in the equipment software, and presenting the result as the percentage of time spent in each activity (Gerhardt, 2000). The MFB allows 4 minutes recordings every 10 minutes (equivalent to 6 recordings per hour). Preliminary studies (unpublished data) showed that *C. riparius* exhibits a regular behavioural pattern in water: a lower frequency behaviour (0.5-2.5 Hz) – the larvae generally exhibit locomotion and other low frequency movements, and higher frequency behaviour (3.0-8.0 Hz) – the larvae presented faster movements such as undulating with the body in a regular pattern (ventilation). These frequencies are in accordance with the ones described for the same species by Janssens de Bisthoven et al. (2004). For this study, five larvae from each concentration and from the control were chosen randomly, placed individually in the MFB chambers with ASTM hard water, and behaviour was recorded during 2 h (n=12 recordings per replicate), every 48 h after the initial 96 h exposure and until the end of the test.

2.2.6. Statistics

LC50 was determined using the PROBIT method (Finney, 1971). All data were checked for normality and homoscedascity. Two-way ANOVA's were performed, using IMI concentrations and days as factors. Data from behavioural experiments were arcsin square root transformed to stabilise variances across treatments (Zar, 1996). Whenever significant differences were observed, a Tukey post hoc test was used for multiple pairwise comparisons to assess which treatments were significantly different. Where applicable, results are presented as mean ±SE. For all statistical tests the significance level was set at $p \leq 0.05$. All calculations were performed with SigmaStat software package (Systat Software Inc., 2006).

2.3. Results

Despite the replacement of 50% of the test medium every 48 hours, at the end of the constant exposure test there was a 22%, 51% and 52% decrease in the first, second and third concentrations, respectively, when compared with the nominal concentrations used. For a better understanding, the analytical concentrations detected are used in graphs, results and discussion (0.39, 0.74 and 2.15 µg of IMI L^{-1}).

In the acute experiments, conducted without sediment or food, the larvae did not present any sign of cannibalism and no mortality was observed in the control treatments.

More than 80% survival was registered in the controls for all the exposure periods, thus validating the experiments. In acute tests, the 48 h LC50 (95.0 % Confidence Interval) was 19.90 µg of IMI L^{-1} (14.64 – 27.16 µg of IMI L^{-1}). *C. riparius* growth decreased with higher concentrations of IMI, being statistically significantly different for 2.15 µg of IMI L^{-1} - 43 and 34% less growth after 96 and 240 h of exposure respectively [ANOVA, Tukey's test: $q= 14.726$, $p<0.05$ (for 96 h); and $q=6.120$, $p<0.05$, (for 240 h); Fig. 2.1].

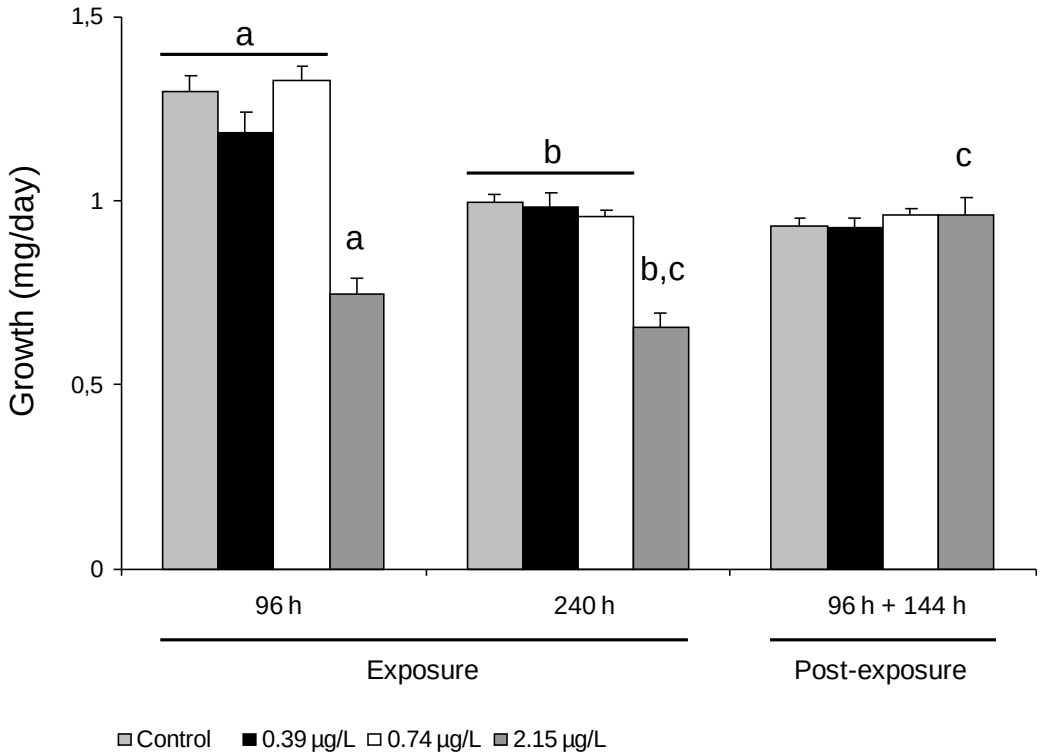

Figure 2.1 - Average growth of *Chironomus riparius* when exposed to imidacloprid for a period of 96 and 240 h and when exposed for a period of 96 hours followed by a post-exposure period of 144 h in clean water. (a,b,c) same letters represent differences between treatments at a significance level $p<0.05$ (ANOVA, Tukey's test).

Contrastly, when exposed for 4 days and transferred to clean medium for 6 days, the larvae showed no differences in growth compared to control (ANOVA, Tukey's test: $q=0.549$, $p=0.980$; Fig. 2.1) showing a recovery in growth compared to those exposed to the constant 10 day exposure, which revealed statistically significant differences for concentration of 2.15 µg of IMI L^{-1} (ANOVA, Tukey's test: $q=4.375$, $p=0.006$; Fig. 2.1).

When exposed for 96 h to IMI, behavioural patterns of the larvae were affected - reduced, being statistically significantly different for 2.15 µg of IMI L^{-1} in both locomotion and ventilation frequencies (ANOVA, Tukey's test: $q=5.710$, $p<0.001$; and $q= 6.255$, $p<0.001$, respectively; Fig. 2.2A and 2.3A). This reduction in behavioural patterns lasts even after the 10 day exposure period being statistically significant for concentration 2.15 µg of IMI L^{-1} in both locomotion and ventilation

frequencies (ANOVA, Tukey's test: q=11.524, p<0.001; and q=8.252, p<0.001, respectively; Fig. 2.2A and 2.3A).

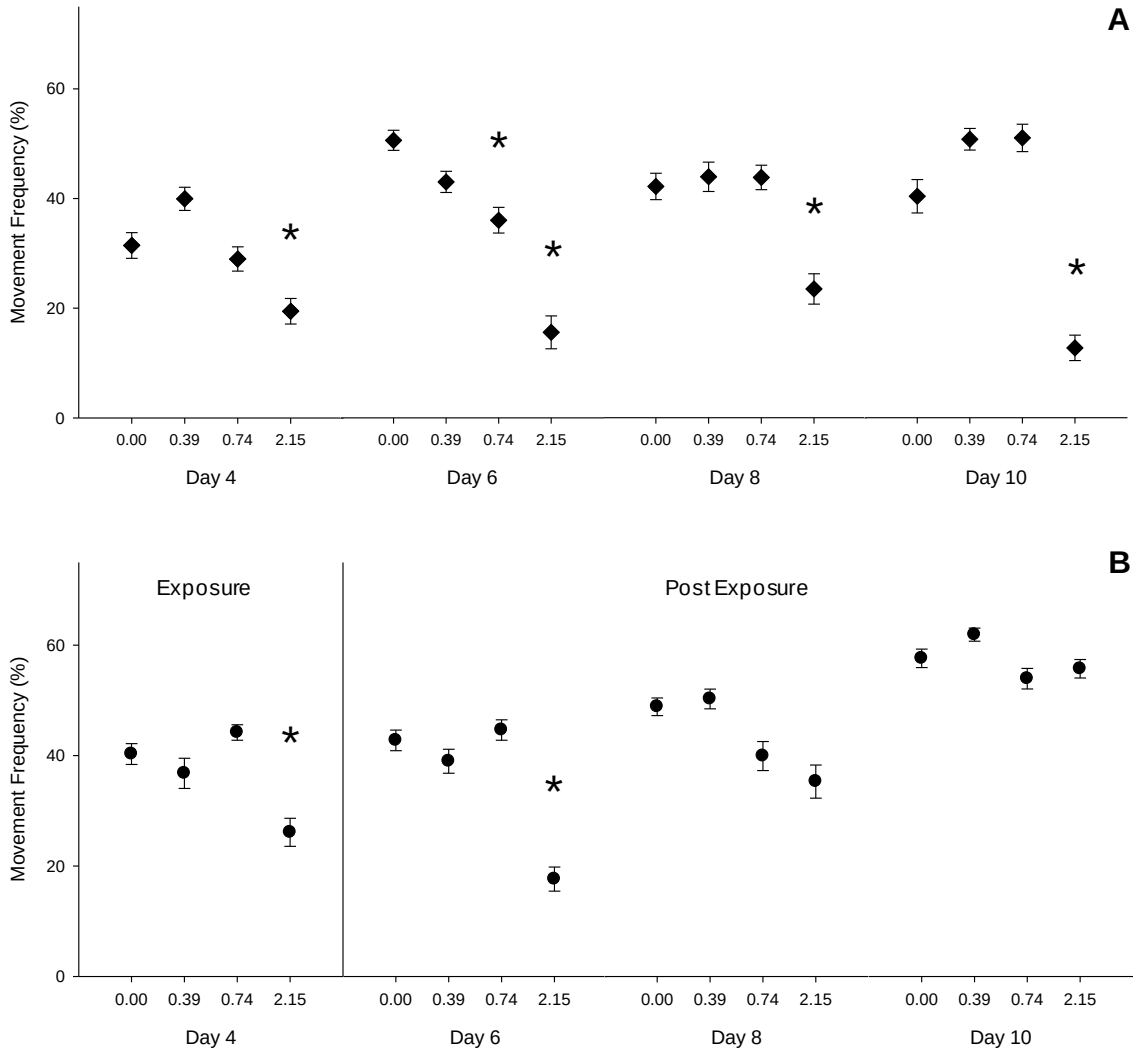

Figure 2.2 - Average activity frequencies of locomotion of *Chironomus riparius* when exposed for a period of 4, 6, 8, and 10 days to imidacloprid (A), and when exposed for a period of four days to imidacloprid, followed by a post-exposure period of 2, 4, and 6 days in clean water (B). Concentrations used are expressed in the XX axis, as well as the days of recording in the Multispecies Freshwater Biomonitor. (*) represent significance level p<0.001 (ANOVA, Tukey's test).

Despite the inhibition of the behavioural patterns after 4 days exposure to 2.15 µg of IMI L^{-1} in locomotion frequencies, at the end of the 6 day post-exposure period in clean medium, larvae showed a tendency to recover in this concentration when

comparing to control – no statistically significant differences (ANOVA, Tukey's test: q=0.691, p=0.962; Fig. 2.2B). As for the larvae's ventilation frequencies, no statistically significant differences from control were found, hence recovery occurred, from post-exposure day 8 onward (ANOVA, Tukey's test: q=3.294, p=0.229; Fig. 2.3B).

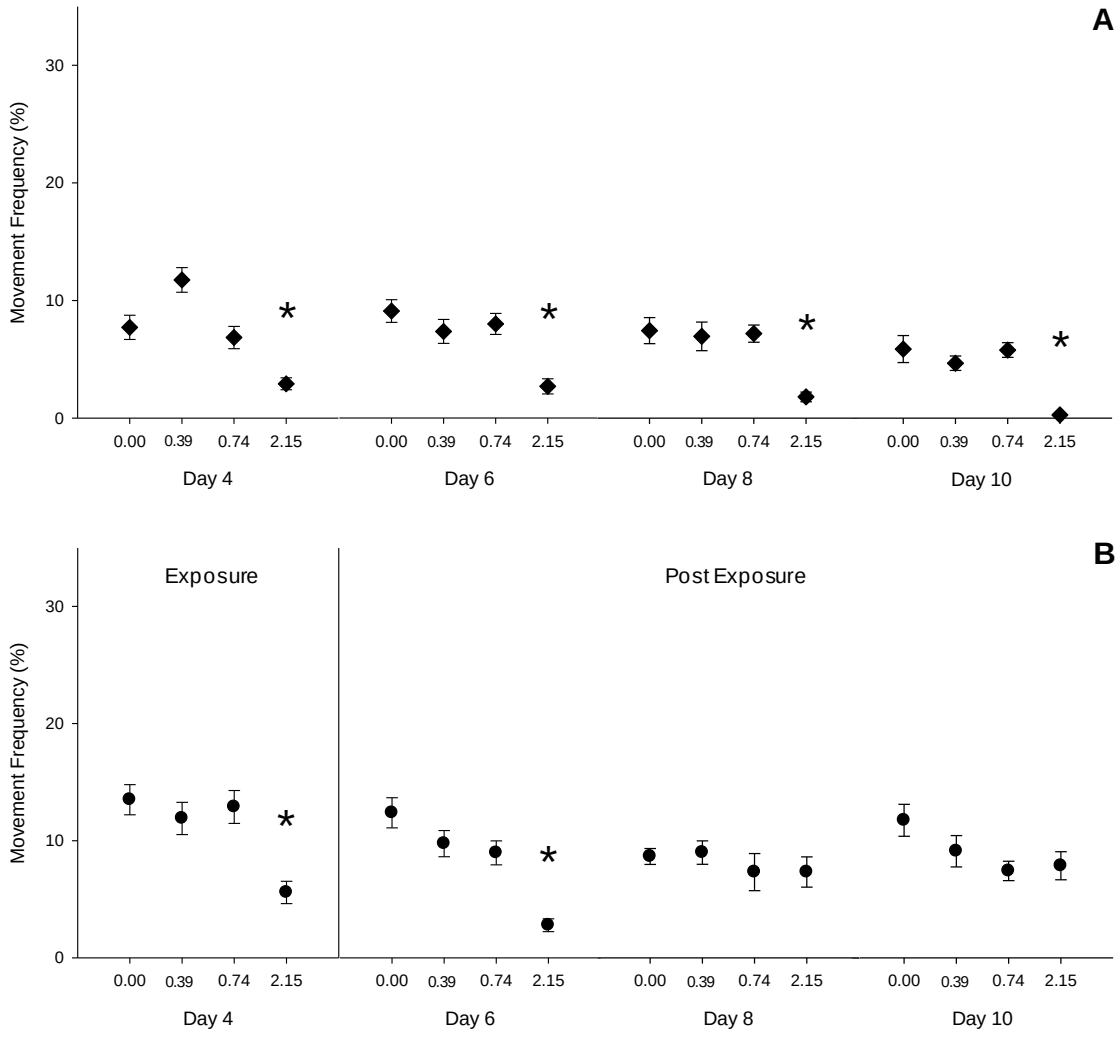

Figure 2.3 - Average activity frequencies of ventilation of *Chironomus riparius* when exposed for a period of 4, 6, 8, and 10 days to imidacloprid (A), and when exposed for a period of four days to imidacloprid, followed by a post-exposure period of 2, 4, and 6 days in clean water (B). Concentrations used are expressed in the XX axis, as well as the days of recording in the Multispecies Freshwater Biomonitor. (*) represent significance level p<0.001 (ANOVA, Tukey's test).

2.4. Discussion

The effects of sub-lethal concentrations of IMI on growth and behaviour of *C. riparius* were assessed under two different exposure scenarios occurring in natural conditions. The experimental design employed in this study aimed to assess the effects of formulated IMI on organisms, closely matching methods commonly used in ecotoxicological hazard assessment. The concentrations used are environmentally relevant, but one could use a broader range of concentrations, in order to reach a more comprehensive assessment on the effects of IMI in these organisms.

Previous studies have shown effects of sub-lethal concentrations of IMI in aquatic non-target macroinvertebrates, such as *Daphnia magna*, *Simulium vittatum*, *Lumbriculus variegatus*, *Chironomus tentans* and *C. riparius* (Song et al., 1997; Overmyer et al., 2005; Alexander et al., 2007; Jemec et al., 2007; Stoughton et al., 2008; Pestana et al., 2009b). Experiments with *C. riparius* exposed to the same compound report a 96 h LC50 of 12.94 µg of IMI L^{-1} (Pestana et al., 2009a). A 96 h LC50 of 5.40 µg of IMI L^{-1} for *C. tentans* exposed to Admire® (Stoughton et al., 2008) and a 48 h LC50 of 9.45 – 6.74 µg of IMI L^{-1} for *Simulium vittatum* exposed to technical IMI (Overmyer et al., 2005) are also described. These results are consistent with our acute responses.

The lower concentrations of IMI detected in the experimental period are probably due to bacterial growth in the beakers due to continuous food addition, which can increase the degradation rate of imidacloprid (CCME, 2007).

Although it cannot be completely excluded that IMI toxicity is also due to unknown compounds included in the formulation of Confidor®, previous studies have confirmed that both commercial formulation (Confidor® 200 SL) and its active ingredient (IMI) show similar toxicity to other organisms (e.g., *Daphnia magna*; Jemec et al., 2007).

Neonicotinoids, such as IMI, act as agonists at the postsynaptic nicotinic acetylcholine receptors (Matsuda et al., 2001) disrupting the neural processes of the organisms. Even low doses of IMI can provoke alterations in behaviour activity, and if concentrations are high enough they can cause uncontrolled muscular

tremors, which can limit foraging and subsequent growth of aquatic insects (Alexander et al., 2007; Pestana et al., 2009b), with probable consequences at the population level – emergence / reproduction (Alexander et al., 2008). Exposing *C. riparius* larvae for a 10 day period to IMI significantly reduced growth, which is in accordance with literature for the terrestrial pea aphid, *Acyrthosiphon pisum* (Laskowski, 2001), and the aquatic insects *C. tentans, Hyalella azteca*, and *C. riparius* (Stoughton et al., 2008; Pestana et al., 2009a). After 4 days exposure the results were similar, with midges presenting a decrease in total length (for the same concentrations; see Fig. 2.1). After being transferred to a clean medium for 6 days, larvae were able to recover from the pulse exposure, exhibiting a similar growth to the control. Stoughton et al. (2008) found similar results in a 96 h pulse exposure with *C. tentans* subject to 3.5 µg of IMI L^{-1}, with a recovery of the midge's growth after the subsequent 6 days in clean medium.

To evaluate the physiological effects of the insecticide, midge behaviour was measured throughout the experimental period, giving a sensitive representation of the organisms' physiological response to environmental factors (Dell'Omo, 2002). Although chironomids are mainly benthic organisms, they can frequently travel considerable distances by drifting, exhibiting a characteristic swimming behaviour (Armitage et al., 1995; Brackenbury, 2000). Chironomids' behavioural patterns indicated a clear sub-lethal toxic stress, which may be due to an overstimulation of larvae neural activities caused by IMI exposure (Buckingham et al., 1997). During the exposure experiment, larvae showed lower locomotion and ventilation activities at the highest IMI concentration throughout the test. Using a more classical visual methodology, Pestana et al. (2009a) also evidenced a significant impairment of the burrowing behaviour of the midge larvae exposed to IMI. This response may be linked with the uncontrolled muscular activity provoked by the insecticide that would diminish the animals' ability to burrow and dislocate, either by abdominal and prothoracic pseudopod movements or by undulated body movements. In fact, locomotion impairment was recorded throughout all the exposure period. Moreover, ventilation at day 10 was almost inexistent. This is probably due to the continuous stimulation of the nervous system, which in turn might reduce the ability and time spent in foraging, and therefore reduced intake of

energy to supply a highly energetic demanding type of behaviour, as discussed in Penttinen and Holopainen (1995). Studies concerning behaviour with other aquatic macroinvertebrates have also described an impairment of behavioural activity when the organisms where under other toxic sources such as metals or pharmaceuticals (Gerhardt et al., 2005; De Lange et al., 2006). Organisms can also present other behavioural responses, such as an increase in activity, usually linked with attempts to escape from contaminated areas (Janssens de Bisthoven et al., 2004; 2006). These different responses might be related not only to the toxicant concentrations tested but also to its mode of action and surely to the fact that different species were used.

After the 4 day exposure and removal to clean medium for 6 days, larvae that were exposed to 2.15 µg of IMI L^{-1} exhibited an increase of locomotory and ventilatory movements when compared to the larvae that were continuously exposed to 2.15 µg of IMI L^{-1} for 10 days. Moreover, a complete recovery of both locomotion and ventilation parameters at the last day of post-exposure was found, indicating that at day 10 the larvae fully recovered from the initial 4 days exposure to the insecticide - ventilation revealed this recovery sooner, by day 8. These behavioural post-exposure data are thus in accordance with the growth parameter data. This recovery might be linked to the resuming of normal neural activities and therefore picking up the normal foraging activity and subsequent energy uptake that enables the restart of high energy costly behaviours such as those described here. Behavioural effects can be related to fitness of individuals and therefore have population-level impacts (Capowiez et al., 2003). To enlighten the above mentioned wider picture of the effects of the pesticide, further testing should consider both the energy intake and metabolic and fitness costs.

Our results are in agreement with the work of Sanchez-Bayo and Goka (2006) that assessed the ecological changes caused by IMI in experimental paddy fields. Although referring the need for further studies, they suggested that IMI toxicity towards aquatic arthropods is reversible and may not affect the long-term ecology of those ecosystems.

In natural conditions, concentrations ranging from 0.13 to 12 µg of IMI L^{-1} have been measured in freshwaters (Phillips and Bode, 2004; CCME, 2007; Jemec et

al., 2007). The concentrations used in this study are thus conservative and ecologically relevant, and together with the exposures regimes assessed, might represent real environmental scenarios. The results support that *C. riparius* larvae can recover if the exposure to IMI is short. However, one also needs to take into consideration that IMI has negative effects on aquatic insects, especially in the case of high concentrations or even repeated pulses of contamination (Pestana et al. 2009b). Exposure to IMI affects the growth and behaviour of the midge larvae, and organisms can in fact recover from the exposure to the insecticide. Thus, it can be added that carefully planned pesticide application intervals should be considered in order to give aquatic organisms the possibility to recover from these pulses, contrary to continuous applications that might have more severe population implications. Furthermore, it is also shown the reliability of using behavioural endpoints and online biomonitoring as a sub-lethal ecotoxicogical relevant parameter.

References

Agra, A.R. and Soares, A.M.V.M., 2009. Effects of Two Insecticides on Survival, Growth and Emergence of *Chironomus riparius* Meigen. *Bull. Environ. Contam. Toxicol.* 82, 501-504.

Alexander, A.C., Culp, J.M., Liber, K. and Cessna, A.J., 2007. Effects of insecticide exposure on feeding inhibition in mayflies and oligochaetes. *Environ. Toxicol. Chem*. 26, 1726-1732.

Alexander, A.C., Heard, K.S. and Culp, J.M., 2008. Emergent body size of mayfly survivors. *Freshwat. Biol.* 53, 171–180.

Armitage, P.D., Cranston, P.S. and Pinder, L.C.V., 1995. *The Chironomidae: The biology and ecology of non-biting midges*. Armitage, P.D., Cranston, P.S., Pinder, L.C.V. (Eds.) Chapman & Hall, London, UK.

ASTM, 2000. *Standard test methods for measuring the toxicity of sediment-associated contaminants with freshwater invertebrates (*E1706). In: Annual Book of ASTM standards, vol 11.05, Philadelphia

Azevedo-Pereira, H.M.V.S. and A.M.V.M. Soares, 2010. Effects of Mercury on Growth, Emergence, and Behavior of *Chironomus riparius* Meigen (Diptera: Chironomidae). *Arch. Environ. Contam. Toxicol.*. 59, 216-224.

Beitinger, T.L., 1990. Behavioral reactions for the assessment of stress in fishes. *J. Great Lakes Res.* 16**,** 495–528.

Brackenbury, J., 2000. Locomotory modes in the larva and pupa of *Chironomus plumosus* (Diptera, Chironomidae). *J. Insect Physiol.* 46, 1517-1527.

Buckingham, S., Lapied, B., Corronc, H. and Sattelle, F., 1997. Imidacloprid actions on insect neuronal acetylcholine receptors. *J. Evol. Biol.* 200, 2685-2692.

Capowiez, Y., Rault, M., Mazzia, C. and Belzunces, L., 2003. Earthworm behaviour as a biomarker - a case study using Imidacloprid. *Pedobiologia* 47, 542–547.

Capowiez, Y., Rault, M., Costagliola, G. and Mazzia, C., 2005. Lethal and sublethal effects of Imidacloprid on two earthworm species (*Aporrectodea nocturna* and *Allolobophora icterica*). *Biol. Fert. Soils*. 41, 135-143.

CCME. 2007. Canadian Water Quality Guidelines: Imidacloprid. Scientific Supporting Document. Canadian Council of Ministers of the Environment, Winnipeg.

Crane, M., Delaney, P., Mainstone, C. and Clarke, S., 1995. Measurement by in situ bioassay of water quality in an agricultural catchment. *Water Res*. 29, 2441-2448.

De Lange, H.J., Noordoven, W., Murk, A.J., Lurling, M. and Peeters, E.T.H.M., 2006. Behavioural responses of *Gammarus pulex* (Crustacea, Amphipoda) to low concentrations of pharmaceuticals. *Aquat. Toxicol.* 78, 209-216.

Dell'Omo, G., 2002. *Behavioural Ecotoxicology*. Dell'Omo, G. (Eds.) John Wiley & Sons, Inc., Hoboken, New Jersey, USA.

Drobne, D., Blazic, M., Van Gestel C.A.M., Leser, V., Zidar, P., Jemec, A. and Trebse, P., 2008**.** Toxicity of imidacloprid to the terrestrial isopod *Porcellio scaber* (Isopoda, Crustacea). *Chemosphere* 71, 1326-1334.

Engenheiro, E.L., Hankard, P.K., Sousa, J.P., Lemos, M., Weeks, J.M. and Soares, A.M.V.M., 2005. Influence of dimethoate on acetylcholinesterase activity and locomotor function in terrestrial isopods. *Environ. Toxicol. Chem*. 24, 603-609.

EPA - United States Environmental Protection Agency, 2000, Methods for Measuring the Toxicity and Bioaccumulation of Sediment-associated Contaminants with Freshwater Invertebrates - Second Edition. EPA/600/R-99/064.

ExToxNet – Extension Toxicology Network (1998) Imidacloprid Pesticide Information Profile. [Online]. Available at http://extoxnet.orst.edu/pips/imidaclo.htm. (Verified March 2010). Oregon State University, Corvallis, OR

Faria, M.S., Ré, A., Malcato, J., Silva, P.CL.D., Pestana, J., Agra, A.R., Nogueira, A.J.A. and Soares, A.M.V.M., 2006. Biological and functional responses of in situ bioassays with Chironomus riparius larvae to assess river water quality and contamination. *Sci. Total Environ.* 371, 125-137.

Faria, M.S., Nogueira, A.J.A. and Soares, A.M.V.M., 2007. The use of *Chironomus riparius* larvae to assess effects of pesticides from rice fields in adjacent freshwater ecosystems. *Ecotoxicol. Environ. Saf.* 67, 218-226.

Finney, D.J., 1971. Probit Analysis. Cambridge University Press, London.

Gerhardt, A., Svensson, E., Clostermann, M. and Fridlund, B., 1994. Monitoring of behavioral patterns of aquatic organisms with an impedance conversion technique. *Environ. Int.* 20, 209-219.

Gerhardt, A., 1995. Monitoring behavioral responses to and effects of metals in *Gammarus pulex* (Crustacea) with impedance conversion. *Environ. Sci. Pollut. Res.* 2, 15–23.

Gerhardt, A., 2000. *Recent trends in biomonitoring for water quality control*. In: Gerhardt, A. (Ed.), Biomonitoring of Polluted Water. Trans Tech Publications Ltd., Zürich.

Gerhardt, A., Janssens de Bisthoven, L. and Soares, A.M.V.M., 2005. Effects of acid mine drainage and acidity on the activity of *Choroterpes picteti* (Ephemeroptera: Leptophlebiidae). *Arch. Environ. Contam. Toxicol.* 48, 450-458.

Janssens de Bisthoven, L., Gerhardt, A. and Soares, A.M.V.M., 2004. Effects of acid mine drainage on larval *Chironomus* (Diptera, Chironomidae) measured with the Multispecies Freshwater Biomonitor. *Environ. Toxicol. Chem*. 23, 1123-1128.

Janssens de Bisthoven, L., Gerhardt, A., Guhr, K. and Soares, A.M.V.M., 2006. Behavioral changes and acute toxicity to the freshwater shrimp *Atyaephyra desmaresti* Millet (Decapoda: Natantia) from exposure to acid mine drainage. *Ecotoxicology* 15, 215-227.

Jemec, A., Tišler, T., Drobne, D., Sepčić, K., Fournier, D. and Trebše, P., 2007. Comparative toxicity of imidacloprid, of its commercial liquid formulation and of diazinon to a non-target arthropod, the microcrustacean *Daphnia magna*. *Chemosphere* 68, 1408-1418.

Laskowski, R., 2001. Why short-term bioassays are not meaningful—effects of a pesticide (Imidacloprid) and a metal (Cadmium) on pea aphids (*Acyrthosiphon pisum* Harris). *Ecotoxicology* **10,** 177-183.

Liess, M., Schulz, R., Liess, M.H.D., Rother, B. and Kreuzig, R., 1999. Determination of insecticide contamination in agricultural headwater streams. *Water Res.* 33, 239-247.

Loureiro, S., Soares, A.M.V.M. and Nogueira, A.J.A., 2005. Terrestrial avoidance behaviour test as screening tools to assess soil contamination. *Environ. Pollut.* 138, 121-131

Macedo-Sousa, J.A., Pestana, J.L.T., Gerhardt, A., Nogueira, A.J.A. and Soares, A.M.V.M., 2007. Behavioural and feeding responses of *Echinogammarus meridionalis* (Crustacea, Amphipoda) to acid mine drainage. *Chemosphere* 67, 1663-1670.

Matsuda, K., Buckingham, S.D., Kleier, D., Rauh, J.J., Grauso, M. and Sattelle, D.B., 2001. Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. *Trends Pharmacol. Sci.* 22, 573-580.

Natal-da-Luz, T., Ribeiro, R. and Sousa, J.P., 2004. Avoidance tests with collembola and earthworms as early screening tools for site specific assessment of polluted soils. *Environ. Toxicol. Chem*. 23, 2188-2193.

OECD, 2004. Guideline 219 - Sediment-water chironomid toxicity test using spiked water (Paris, France).

Overmyer, J.P., Mason, B.N. and Armbrust, K.L., 2005. Acute Toxicity of Imidacloprid and Fipronil to a Nontarget Aquatic Insect, *Simulium vittatum* Zetterstedt cytospecies IS-7. *Bull. Environ. Contam. Toxicol.* 74, 872-879.

Penttinen, O.P. and I.J. Holopainen, , 1995. Physiological energetics of a midge, *Chironomus riparius* Meigen (Insecta, Diptera): normoxic heat output over the whole life cycle and response of larva to hypoxia and anoxia. *Oecologia* 103, 419-424.

Péry, A.R.R., Mons, R., Flammarion, P., Lagadic, L. and Garric, J., 2002. A modeling approach to link food availability, growth, emergence, and reproduction for the midge *Chironomus riparius*. *Environ. Toxicol. Chem*. 21, 2507-2513.

Pestana, J.L.T., Loureiro, S., Baird, D.J. and Soares, A.M.V.M., 2009a. Fear and loathing in the benthos: Responses of aquatic insect larvae to the pesticide imidacloprid in the presence of chemical signals of predation risk. *Aquat. Toxicol*. 93, 138-149.

Pestana, J.L.T., Alexander, A.C., Baird, D.J., Cessna, A. and Soares, A.M.V.M., 2009b. Structural and functional responses of benthic invertebrates to Imidacloprid in outdoor stream mesocosms. *Environ. Pollut.* 157, 2328-2334.

Phillips, P. J. and R.W. Bode, 2004. Pesticides in surface water runoff in south-eastern New York State, USA: seasonal and stormflow effects on concentrations. *Pest Manag. Sci*. 60, 531-543.

Rieradevall, M., García-Berthou, E, and Prat, N., 1995. Chironomids in the diet of fish in Lake Banyoles (Catalonia, Spain). Cranston, P. (ed). *Chironomids: from genes to ecosystems*. CSIRO, Melbourne, Australia.

Sanchez-Bayo, F. and Goka, K., 2006. Ecological effects of the insecticide imidacloprid and a pollutant from antidandruff shampoo in experimental rice fields. *Environ. Toxicol. Chem.* 25, 1677-1687.

SERA (Syracuse Environmental Research Associates, Inc.), 2005. Imidacloprid – human health and ecological risk assessment – final report; prepared for USDA, Forest Service, USA (SERA TR 05-43-24-03a). http://www.fs.fed.us/foresthealth/pesticide/pdfs/122805_Imidacloprid.pdf

Song M.Y., Stark J.D. and Brown J.J., 1997. Comparative toxicity of four insecticides, including imidacloprid and tebufenozide, to four aquatic arthropods. *Environ. Toxicol. Chem*. 16, 2494-2500.

Stoughton, S., Liber, K., Culp, J. and Cessna, A., 2008. Acute and Chronic Toxicity of Imidacloprid to the Aquatic Invertebrates *Chironomus tentans* and *Hyalella azteca* under Constant- and Pulse-Exposure Conditions. *Arch. Environ. Contam. Toxicol.* 54, 662-673.

Suchail, S., Guez, D. and Belzunces, L.P., 2000. Characteristics of imidacloprid toxicity in two *Apis mellifera* subspecies. *Environ. Toxicol. Chem.* 19, 1901-1905.

Systat Software Inc., 2006. SigmaStat for Windows (version 3.5). Chicago, IL, USA.

Tomizawa, M. and J.E. Casida, 2005. Neonicotinoid insecticide toxicology: Mechanisms of Selective Action. *Annu. Rev. Pharmacol. Toxicol*. 45, 247-268.

Vos, J.H., 2001. Feeding of detritivores in freshwater sediments. PhD Thesis. University of Amsterdam, Amsterdam, The Netherlands.

Wamhoff, H. and Schneider, V., 1999. Photodegradation of imidacloprid. *Journal of Agricultural and Food Chemistry*. 47: 1730-1734.

Zar, J.H., 1996. *Biostatistical Analysis.* Prentice-Hall International, Inc., New Jersey.

Chapter 3

Effects of imidacloprid exposure on *Chironomus riparius* Meigen larvae: linking acetylcholinesterase activity to behaviour

3. EFFECTS OF IMIDACLOPRID EXPOSURE ON *CHIRONOMUS RIPARIUS* MEIGEN LARVAE: LINKING ACETYLCHOLINESTERASE ACTIVITY TO BEHAVIOUR

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Abstract

Imidacloprid (IMI) is an insecticide that interferes with the transmission of stimuli in the nervous system of insects. It is neurotoxic by mimicking nicotine through its binding to the nicotinic acetylcholine receptor. In this work, experiments comprising 96 hours exposure followed by 48 hours in clean medium were conducted to evaluate the toxicity of IMI to *Chironomus riparius* and its potential recovery. Behavioural parameters and AChE activity were assessed. After 96 hours exposure to IMI, AChE activity, and the behaviour parameters ventilation and locomotion were reduced. There were no signs of recovery after removal to clean water for 48 hours. Ventilation behaviour was the most sensitive parameter and the one with the highest correlation to AChE activity. Despite the possibility that IMI might be having an indirect effect on AChE activity, the behavioural endpoint showed a higher sensitivity than the biochemical response itself. This work highlights the importance of linking parameters with ecological relevance at individual level (behavioural parameters) with biochemical responses, to unravel xenobiotics' mode of action.

Keywords Neonicotinoids; neurotoxicity; behavioural parameters; ecotoxicology; linking biomarkers

3.1. Introduction

Pesticides used in agriculture are designed to affect target organisms – plagues – but due to their nature they may also affect non-target organisms present in the application site or even in nearby freshwater ecosystems (Crane et al. 1995). To reduce the pesticide's impact in these non-target species, new chemicals have been developed that minimize the compounds' resilience time and maintain the pest control effectiveness. One of these innovative and fastest growing groups of pesticides is the neonicotinoids (Tomizawa and Casida 2003). Imidacloprid [IMI; 1-[(6-chloro-3-pyridinyl)methyl]-*N*-nitro-2-imidazolidinimine], developed by Bayer CropScience® AG, is a very common and worldwide used neonicotinoid employed in the control of sucking insects in crops (Tomizawa and Casida 2005; Tomlin 2000). IMI was designed to act as an agonist of the post-synaptic nicotinic acetylcholine receptors (Buckingham et al. 1997; Matsuda et al. 2001), causing their overstimulation and therefore affecting neuronal processes which may lead to overall impairment and even death. This neurotoxicity is produced through the binding or partial binding to specific sub-sites or protein subunits of the nicotinic acetylcholine receptor (nAChR), which in turn activates nAChR activity (SERA, 2005). IMI can enter freshwater bodies by leach or runoff from agricultural fields and can lead to local point-source contaminations (Fossen 2006; Gupta et al. 2002). Concentrations ranging from 0.13 to 12 µg IMI L^{-1} have been reported for natural field scenarios (CCME 2007; Phillips and Bode 2004). Pesticide persistence in aquatic systems is very variable and can occur in short time spans, depending on several abiotic (e.g. light) and biotic (e.g. microbial communities) factors (Liess et al. 1999). Research has been carried out to assess the toxicity of IMI to non-target aquatic macroinvertebrates, focusing not only on ecological relevant pulse exposure scenarios (Stoughton et al. 2008) but also on constant exposure scenarios (Pestana et al. 2009a; Pestana et al. 2009b). These studies highlighted the morphophysiological effects (survival, growth, emergence and behaviour) of IMI on the tested species, while few studies have assessed the biochemical/molecular toxic effects of this insecticide on aquatic macroinvertebrates (Jemec et al. 2007). Molecular biomarkers are being

increasingly used as early warning tools in laboratory and field experiments (e.g. Domingues et al. 2007; Lemos et al. 2009; Lemos et al. 2010a), since they allow the detection of effects at the subcellular level before they are apparent at higher levels of biological organization (Lemos et al. 2010b). To more accurately predict the direct consequences, to an organism or population, of the exposure to a known amount of a toxicant, a particular biomarker response should be related to impairment of growth, reproduction or metabolic function directly related to the survival of the organism (Depledge and Fossi 1994). One of the most employed biochemical biomarkers is cholinesterase (ChE) activity. Cholinesterases are nervous system enzymes that have a key role in the maintenance of the normal nerve functions. Acetylcholinesterase (AChE) is the enzyme responsible for the hydrolysis of the neurotransmitter acetylcholine that generates postsynaptic potentials. In its absence, acetylcholine continues to stimulate the postsynaptic neuron, leading to uncoordinated movements. Several studies have assessed the inhibition of AChE activity by several toxicants in aquatic organisms (Beauvais et al. 1999; Kallander et al. 1997; Rakotondravelo et al. 2006), linking this biomarker to other endpoints such as behaviour, feeding rate or larval emergence (Domingues et al. 2007; García-de la Parra et al. 2006).

At the individual level, behaviour is considered an early warning tool in ecotoxicology since it is one of the most sensitive indicators of chemical stress (Gerhardt et al. 1994) and the first line of defence to environmental stimuli (Beitinger 1990). Assessing behaviour alterations allows the integration of individual physiological processes and mechanisms with the environmental stimuli that are causing them (Dell'Omo 2002). Some authors have linked behavioural endpoints (like locomotion) to biochemical biomarkers (such as ACHE), on edaphic (Capowiez et al. 2003; Engenheiro et al. 2005; Jensen et al. 1997) and aquatic organisms (García-de la Parra et al. 2006).

Chironomids are an ecologically diverse group and are one of the most ubiquitous insects within freshwater ecosystems, dominating (in number and biomass) the benthic communities of lotic and lentic environments (Péry et al. 2002), and are being a major food source for other animals (García-Berthou 1999). They are

easily maintained in the laboratory and are commonly used as model organisms for sediment toxicity tests (EPA 2000; OECD 1984).

These organisms depend mostly on locomotion and ventilation (whole-body undulation in the water column) to perform all activities that enable them to dislocate, find food, emerge and avoid predators. Due to the above mentioned relation of AChE activity and behaviour, it is especially important to assess the effects of toxicants on AChE activity that might lead to the disruption of vital behavioural parameters. Despite AChE activity being usually used for organophosphate and carbamate exposure, previous studies using IMI and AChE activity have showed that AChE activity can be a sensitive biomarker, but not an early, sensitive biomarker of stress, (Jemec et al. 2007).

The aim of this study was to assess how sublethal exposure to IMI affects both *Chironomus riparius*' behaviour and AChE activity, in different periods throughout the exposure and even after the exposure episode.

3.2. Material and Methods

3.2.1. Test organism

C. riparius midges were obtained from laboratory cultures established at the University of Aveiro for 3 years. The cultures were maintained in an enclosed transparent acrylic box containing several plastic beakers holding a 2cm layer of acid-washed and burned commercial sand (< 1mm), and approximately 2.5 L of reconstituted hard water ASTM (ASTM 2000). A gentle aeration was provided in each beaker. This system permits the occurrence of the whole life cycle of the chironomids, by allowing the swarming and copulation of emerged adults (OECD 1984). The culture was maintained in standard conditions, at 20±2ºC and with a 16h-8h light-dark photoperiod. Freshly laid egg masses are transferred onto crystallizing dishes with culture medium until hatching, and the first instar larvae are used either to start a new culture or in bioassays. Water and sediment were

renewed every week and larvae were fed (1 mg animal^{-1} day^{-1}) twice a week with a suspension of ground Tetramin® (Tetrawerke, Germany).

3.2.2. Imidacloprid

Confidor® 200 SL (Bayer CropScience AG, Monheim, Germany) was used to prepare the stock solution of IMI dissolved in ultra-pure water. The analytical concentration of the stock solution was 0.725 mg L^{-1}. From this stock solution, several nominal concentrations of IMI were prepared: 0.00 (control) 0.50, 1.50 and 3.00 µg of IMI L^{-1} (nominal concentrations). Chemical analyses of the IMI samples from the stock solution and bioassays were conducted by Terracon Laboratorium für Umwelt- und Pestizidanalytik GmbH (Jütterborg, Germany), using a HPLC-PDA-System equipped with 2 HPLC pumps Model LC-10ADvp, Autosampler SIL-10ADvp, column oven CTO-10ASvp, and a photodiodenarray-detector (PDA) SPD-M10Avp (Shimadzu, Japan). Procedure consisted in: all samples containing high IMI concentrations (e.g. stock solutions) were diluted with deionised water, while samples with lower concentrations were extracted from 100–200 mL water samples (flow of 0.5 mL min^{-1}) using solid phase extraction (SPE cartridges Supelclean ENVI-18, Supelco, Schnelldorf, Germany) and acetonitrile (1:1 v:v) for elution. 10 µL acetonitrile-extracts were then applied to a chromatography column (LUNA C18, Phenomenex, Aschaffenburg, Germany), at a flow rate of 0.4 mL min^{-1} using water, 0.1 % formic acid and acetonitrile as eluents. Detection was carried out at 270 nm with a limit of quantification of 0.1 µg L^{-1}.

3.2.3. Organisms' exposure

The bioassay comprised 96 hours exposure to IMI and subsequent 48 hours in clean medium. The experiments were performed in the same temperature and photoperiod conditions as in the cultures, using 200 mL glass beakers (10

replicates per treatment) with five late 3rd instar larvae (ten days old) per replicate. Each beaker contained 40g of acid-washed inorganic fine sand (<1 mm) and 150 mL of test solution. Organisms were fed with macerated Tetramin® (0.5 mg animal^{-1} day^{-1}) every 48 hours until the end of the experiment. Replicates were examined daily for mortality. Forty eight hours after the beginning of the experiment, and before feeding, half of the test solution in each beaker was renewed. After 96 hours of exposure, larvae were removed from the test beakers and transferred to beakers with clean medium, sediment and food as described above, until the end of the experiment. Acetylcholine activity and behaviour of larvae were measured as response parameters at 48h exposure, 96h exposure and 48h after removing to clean medium.

3.2.4. Behaviour

Behavioural patterns were recorded by the Multispecies Freshwater Biomonitor (MFB) that was developed by Gerhardt et al. (1994). Description of the technology can be found in Azevedo-Pereira et al. (2010 – Chapter 4). Five larvae from each concentration and from the control were randomly chosen and placed individually in the MFB chambers with ASTM hard water. Behaviour was recorded during 2 hours (n=12 recordings per chamber) every 48h until the end of the test.

3.2.5. Biochemical analysis

Parallel to behaviour recording, a set of organisms were withdrawn from the experience and frozen in liquid nitrogen (*n* between 8 and 12). Within two weeks, the entire frozen animal was used to determine AChE activity. Each *C. riparius* was homogenised with 500 µL of phosphate buffer (0.1M, pH 7.2), using an electrical homogeniser and then centrifuged for 3 minutes at 5000 rpm and pellet discarded. AChE analyses were performed following Ellmans' method (Ellman et

al. 1961) adapted to a microplate reader, and absorbance values read at 404 nm (Guilhermino et al. 1996; Ribeiro et al. 1999) (Labsystems Multiskan EX plate reader - Helsinki, Finland). The enzyme activity was expressed as nmol ml^{-1} mg $prot^{-1}$ min^{-1}. The total quantity of protein was determined following the method of Bradford (1976), adapted to microplate reader (Ribeiro et al. 1999) and absorption measured at 595 nm (Labsystems Multiskan EX plate reader - Helsinki, Finland).

3.2.6. Statistics

For behavioural experiments, one-way ANOVA's were calculated for each recording period and a two-way ANOVA was used to compare data throughout the experimental period, using IMI concentrations and days as factors for both enzyme activity and behavioural data, testing for IMI concentrations, exposure and recovery periods, and their interactions.. Data from behavioural experiments were arcsine square root transformed to stabilise variances across treatments (Zar 1996). The Spearman Rank Correlation was calculated between AChE activity and each behavioural parameter for all sampling periods. Whenever significant differences were observed, a Tukey post hoc test was used for multiple pairwise comparisons to assess which treatments were significantly different. For all statistical tests, the significance level was set at $p \leq 0.05$. All calculations were performed with SigmaStat (2006).

3.3. Results

Less than 20% mortality was found for the controls for all the exposure periods, thus validating the experiments (EPA 2000).

IMI degraded throughout the 96h experimental period. At the end of the exposure period there was 40 (0.30 µg of IMI L^{-1}), 63 (0.55 µg of IMI L^{-1}) and 60 (1.20 µg of IMI L^{-1}) % less compound in the first, second and third concentrations,

respectively, when compared with the nominal concentrations used. For a better understanding, the analytical concentrations detected are used in figures, results and discussion (0.30, 0.55 and 1.20 µg of IMI L^{-1}). The behavioural response patterns of the midges were affected by the presence of the toxicant (Fig. 3.1 and 3.2). After 48h exposure, the larvae exposed to 0.55 µg of IMI L^{-1} showed a statistically significant increase in the locomotory activity, when compared to the control (Tukey's test: q= 4.685, p=0.005; Fig. 3.1), while animals exposed to the higher two concentrations suffered statistically significant changes in ventilation, when comparing to control [Tukey's test: q= 5.450, p<0.001 (0.55 µg of IMI L^{-1}); q= 7.698, p<0.001 (1.20 µg of IMI L^{-1}); Fig. 3.2]. After 96h of exposure, the ventilation activities decreased with increasing concentrations of IMI, with a LOEC of 0.55 µg of IMI L^{-1} [Tukey's test: q= 6.949, p<0.001 (0.55 µg of IMI L^{-1}); q= 18.675, p<0.001 (1.20 µg of IMI L^{-1}); Fig. 3.2]. For the 96h exposure period, there was a trend to increase locomotion activity in the lowest concentrations tested although without statistically significant differences to the control [Tukey's test: q= 3.257, p=0.097 (0.30 µg of IMI L^{-1}); q= 3.554, p=0.058 (0.55 µg of IMI L^{-1}); Fig. 3.1], whereas in animals exposed to 1.20 µg of IMI L^{-1} locomotion was impaired in the midges, being statistically significant when compared to the control (Tukey's test: q= 9.244, p<0.001; Fig. 3.1).

After the 48 hours post-exposure period (144 hours of total time), behavioural activities demonstrated that the organisms where still affected by the stress agent. Locomotory activity at 1.20 µg of IMI L^{-1} was still significantly reduced when compared to the control (Tukey's test: q= 6.200, p<0.001; Fig. 3.1). *C. riparius* ventilation activity was still impaired after this post-exposure period, with statistically significant differences for all concentrations tested when compared to the control [Tukey's test: q= 4.580, p=0.007 (0.30 µg of IMI L^{-1}); q= 5.333, p<0.001 (0.55 µg of IMI L^{-1}); q= 8.744, p<0.001 (1.20 µg of IMI L^{-1}); Fig. 3.2].

No differences were found in the control animals between ventilation activity at the end of the exposure period and the post-exposure recording period (Tukey's test: q=1.254, p=0.649). Nevertheless, although still being statistically different from control, animals exposed to 1.20 µg of IMI L^{-1} had an increase of ventilation

activities comparing the exposure and post-exposure periods (Tukey's test: q=8.678, p<0.001).

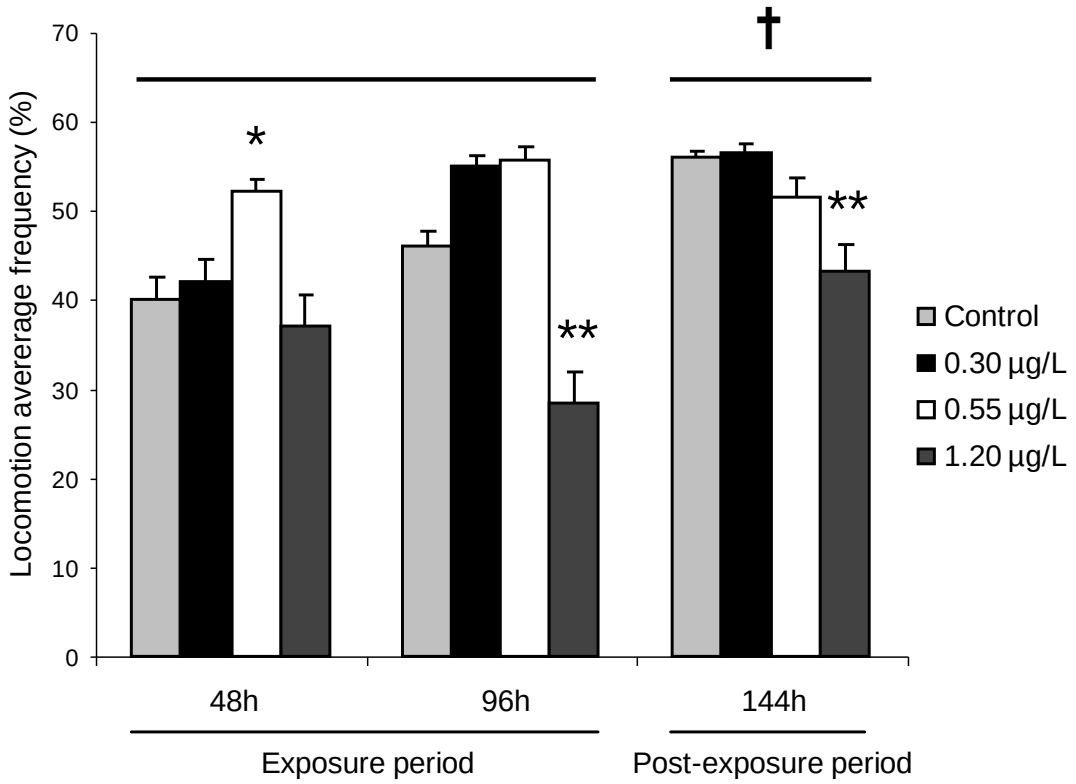

Figure 3.1 - Average activity frequencies of locomotion of *Chironomus riparius* exposed to imidacloprid for a period of two and four days, followed by a post exposure period of two days in clean water. (*) represents significance level p<0.01 and (**) represents significance level p<0.001 in comparison with the control (ANOVA, Tukey's test). (†) represents significance level p<0.01 for the comparison between times of exposure (ANOVA, Tukey's test).

When comparing the post-exposure with the exposure period, an increase of locomotion frequencies was found being statistically significantly different [Tukey's test: q= 7.113, p<0.001 (48h); q= 4.467, p=0.005 (96h); Fig. 3.1]. No statistically significant differences for ventilation frequencies were found when comparing exposure and post-exposure periods – between 96 and 144h (Tukey's test: q= 1.933, p=0.358; Fig. 3.2).

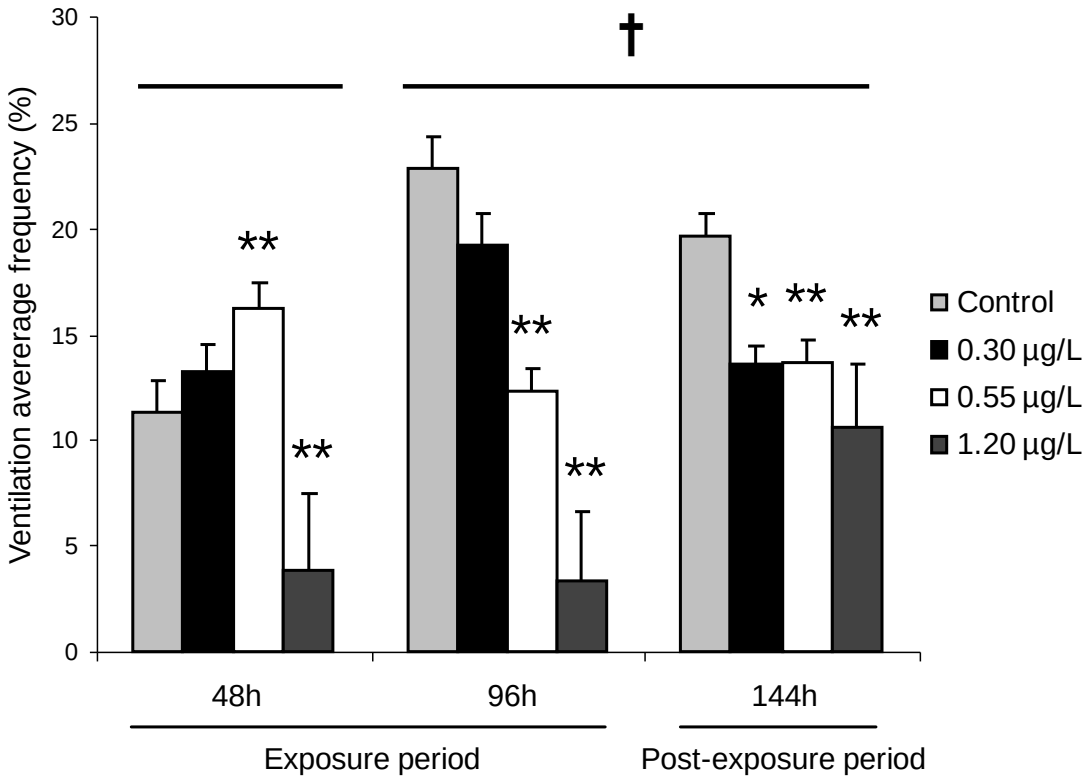

Figure 3.2 - Average activity frequencies of ventilation of *Chironomus riparius* exposed to imidacloprid for a period of two and four days, followed by a post exposure period of two days in clean water. (*) represents significance level p<0.01 and (**) represents significance level p<0.001 in comparison with the control (ANOVA, Tukey's test). (†) represents significance level p<0.01 for the comparison between times of exposure (ANOVA, Tukey's test).

Acetylcholinresterase activity in chironomids exposed for 96h to IMI was reduced, being statistically significant at the highest concentration tested (Tukey's test: q= 4.607, p=0.008; Fig. 3.3). When transferred to clean water, for the 48h post-exposure period a decrease of AChE activity was also observed, being statistically significant for all treatments tested when compared to the control group [Tukey's test: q= 8.281, p<0.001 (0.30 µg of IMI L^{-1}); q=9.098, p<0.001 (0.55 µg of IMI L^{-1}); and q= 12.445, p<0.001 (1.20 µg of IMI L^{-1}); Fig. 3.3]. Statistically significant differences for AChE activity during exposure (48 and 96h) were found (Tukey's test: q= 5.169, p<0.001; Fig. 3.3), while no statistically significant differences for AChE activity were found when comparing exposure and post-exposure periods – between 96 and 144h (Tukey's test: q= 1.562, p=0.514; Fig. 3.3).

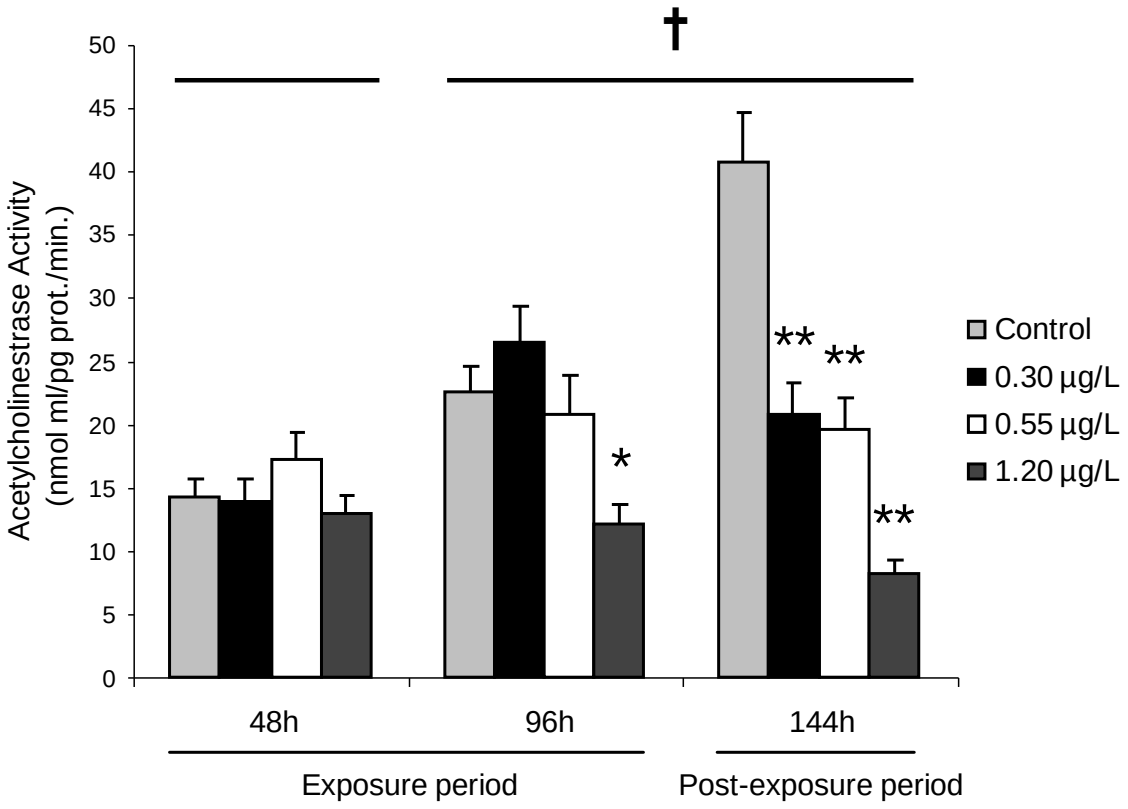

Figure 3.3 - Average acetylcholinesterase activity, of *Chironomus riparius* exposed to imidacloprid for a period of two and four days, followed by a post exposure period of two days in clean water. (*) represents significance level p<0.01 and (**) represents significance level p<0.001 in comparison with the control (ANOVA, Tukey's test). (†) represents significance level p<0.01 for the comparison between times of exposure (ANOVA, Tukey's test).

The relation between AChE activity and the behavioural parameters were also assessed. Despite AChE activity did not show any significant relation with locomotion [r=-0.034, P=0.889 (48h); r=0.105, P=0.662 (96h); r=0.196, P=0.403 (144h)], a strong and significant correlation with ventilation was found for 96 and 144h [r=-0.164, P=0.508 (48h); r=0.546, P=0.016 (96h); r=0.553, P=0.011 (144h)].

3.4. Discussion

In the present study laboratory ecotoxicology tests are encouraged, as aquatic organisms can be subjected to exposure to contaminants in levels depending not only on natural conditions but also on pesticide persistence in the environment, in order to apply an environmentally relevant procedure for pesticides testing. One should bear in mind that contamination of aquatic environments by pesticides is often due to agricultural fields' runoff, spray drift or even ground water flows (Liess et al. 1999); and that these inflows of pesticide to aquatic systems can be variable according to their application in farming soils and might be followed by periods of long-term exposure to low concentrations of the pesticides (Naddy et al. 2000). Previous acute tests (Chapter 2), showed a 48h LC50 (95.0% Confidence Interval) of 19.90 µg of IMI L^{-1} (14.64 – 27.16 µg L^{-1}), and Pestana et al (2009b) also reports a 96-h LC50 (95% CI) for *C. riparius* of 12.94 µg of IMI L^{-1} (9.74–18.22) under same exposure conditions. The values here obtained are in accordance with the previous studies The lower concentrations of IMI when compared with the nominal concentrations, detected at the end experimental period, were probably due to bacterial growth in the beakers due to food addition, which can increase the degradation rate of imidacloprid (CCME 2007).

The chironomids' behaviour was affected by exposure to sublethal doses of IMI. During the exposure period, animals exposed to the highest concentration exhibited a decrease in locomotory activity, while those exposed to the lower concentrations exhibited a small increase in locomotion in the first 48 hours of exposure (Fig. 3.1). This non-monotonic dose-response behaviour is probably due to the lower toxicity in lower concentrations not impairing the larvae's attempt to escape from the contaminated medium. The increased mobility thus probably reflects avoidance behaviour, as stated by De Bisthoven et al. (2004), while in higher concentrations the compounds' toxicity weakens the larvae's ability to respond and escape.

Similarly to locomotion, ventilation involves characteristic swimming movements (Brackenbury 2000) that allows the larvae to travel considerable distances by drifting (e.g. to escape from contaminated sites), thus the same non-monotonic

trend observed in the ventilatory activities may be explained by the same rationale. This increased mobility to avoid toxicant in the lowest concentration was then reduced as toxicity increased, i.e., longer exposure period.

Forty-eight hours after the exposure period, the animals did not show signs of recovery. Although in clean water, it is possible that the IMI presence (or its metabolites) within the midge is still sufficient to be mimicking acetylcholine at the postsynaptic nicotinic acetylcholine receptors (nAChR), thus affecting the larvae, but no assessment of internal IMI body burdens were made to fully understand this. Nevertheless, in another study (Chapter 2), midges subjected to the same exposure period revealed a behavioural recovery after 6 days of post-exposure. This period might be what it takes for the excretion of residual IMI and its metabolites. Although in some insects (e.g. houseflies) IMI may readily be excreted, it can also be metabolised and its main metabolites can also present insecticidal activity and therefore extend the toxicity of the pesticide (Nishiwaki et al. 2004; Suchail et al. 2004). The chironomids exposed to the highest concentration, although not fully recovering to values compared to the control, have a significant increase of AChE activity after transferring to clean water. A longer post-exposure period could allow for the organism to fully recover, as seen with the behaviour recovery after 6 days of post-exposure in clean water (Chapter 2).

In this study, the AChE activity of the larvae decreased with increasing concentrations of IMI from the 96h exposure onward (including after the short post-exposure period; Fig. 3.3). To our knowledge, few data about AChE activity related with IMI are available in the literature: acute testing with the earthworms *Aporrectodea nocturna* and *Allolobophora icterica* (Capowiez et al. 2003) showed no effects on AChE activity. On the other hand, chronic testing with the daphnid *Daphnia magna* (Jemec et al. 2007) reported a clear impairment of the enzyme activity with increasing concentrations of IMI.

In uncontamined conditions acetylcholine (ACh) binds its receptor (AChR), leading to the activation of the ion channel, and afterwards is hydrolyzed by AChE (Tomizawa and Casida 2003). The neonicotinoid-binding site in AChR is the same as or closely coupled to that of ACh, and displays saturable and reversible binding

with fast kinetics (Tomizawa and Casida 2003). This way it is possible that the binding of the neonicotinoid to the receptor in the AChR and subsequent non-connection of the neurotransmitter ACh to the nicotinic receptor will cause the inhibition of AChEs' activity, as seen here.

This constant stimulation of the nicotinic AChR receptors by this agonist (IMI) incites the general physiological impairment of endpoints related to the nervous function, thus leading to a decrease of both ventilation and locomotion as well as AChE activities. In this work, a high correlation between AChE and ventilation activity was found which would strength the reasoning of the link of this enzyme activity and behavioural patterns and thus with more ecological relevant levels. Nevertheless, this decrease of AChE activity is most probably a consequence of the agonistic activity of IMI to the receptor, and the ventilation impairment is probably due to the continuous stimulation of the receptor, conferring it an independent relation.

Changes in behaviour due to the continuous stimulation of the nervous system by xenobiotic provokes uncontrolled muscular tremors that by reducing locomotion will most probably interfere with foraging activities, reducing the input for the high energy demanding ventilation activities (Penttinen and Holopainen 1995), as well as the energy budget needed for the overall physiological processes such as growth and emergence (Alexander et al. 2008). Moreover, the ability to drift or escape from predators which in turn are due to have important impacts at the population and community level will also be affected (Alexander et al. 2008; Engenheiro et al. 2005, Pestana et al. 2009b).

3.5. Conclusion

Our results suggest that ventilation is a more sensitive endpoint than locomotion. Furthermore, this work highlights the understanding of the behaviour responses (as an early warning system) in relation with biochemical responses, giving a sensitive representation of the organism physiological response to environmental factors. Despite AChE activities give us a picture of IMI's mode of action in the

organism, this work suggests that behaviour, and more specifically ventilation, is a more sensitive parameter than biochemical responses. Added to its higher ecological relevance, ventilation be used as a relevant endpoint for ecotoxicology testing.

References

Alexander, A.C., Heard, K.S., Culp, J.M.,2008. Emergent body size of mayfly survivors. Freshw. Biol. 53, 171-180.

ASTM, 2000. Annual Book of ASTM Standards. Philadelphia, American Society for Testing and Materials. 11.05.

Azevedo-Pereira, H.M.V.S., Soares, A.M.V.M., 2010. Effects of Mercury on Growth, Emergence, and Behavior of *Chironomus riparius* Meigen (Diptera: Chironomidae). Arch. Environ. Contam. Toxicol. 59, 216-224

Beauvais, S.L., Atchison, G.J., Stenback, J.Z., Crumpton, W.G., 1999. Use of cholinesterase activity to monitor exposure of *Chironomus riparius* (Diptera: Chironomidae) to a pesticide mixture in hypoxic wetland mesocosms. Hydrobiologia. 416, 163-170.

Beitinger, T.L., (1990. Behavioral reactions for the assessment of stress in fishes. J. Great Lakes Res. 16, 495-528

Brackenbury, J., 2000. Locomotory modes in the larva and pupa of *Chironomus plumosus* (Diptera, Chironomidae). J. Insect. Physiol. 46, 1517-1527.

Bradford, M.M., 1976. Rapid and sensitive method for quantification of microgram quantities of protein utilizing principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Buckingham, S.D., Lapied, B., LeCorronc, H., Grolleau, F., Sattelle, D.B., 1997. Imidacloprid actions on insect neuronal acetylcholine receptors. J. Exp. Biol. 200, 2685-2692.

Capowiez, Y., Rault, M., Mazzia, C., Belzunces, L., 2003. Earthworm behaviour as a biomarker - a case study using imidacloprid. Pedobiologia. 47, 542-547.

CCME, 2007. Canadian water quality guidelines: imidacloprid scientific supporting document. Winnipeg, Canadian Council of Ministers of the Environment.

Crane, M., Delaney, P., Mainstone, C., Clarke, S., 1995. Measurement by *in-situ* bioassay of water-quality in an agricultural catchment. Water. Res. 29, 2441-2448.

De Bisthoven, L.J., Gerhardt, A., Soares, A.M.V.M., 2004. Effects of acid mine drainage on larval *Chironomus* (diptera, chironomidae) measured with the multispecies freshwater biomonitor. Environ. Toxicol. Chem. 23, 1123-1128.

Dell'Omo, G., 2002. Behavioural Ecotoxicology, Wiley and Sons, West Sussex.

Depledge, M.H., Fossi, M.C., 1994. The role of biomarkers in environmental assessment. 2. Invertebrates. Ecotoxicology. 3, 161-172.

Domingues, I., Guilhermino, L., Soares, A.M.V.M., Nogueira, A.J.A., 2007. Assessing dimethoate contamination in temperate and tropical climates: potential use of biomarkers in bioassays with two chironomid species. Chemosphere. 69, 145-154.

Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88-95.

Engenheiro, E.L., Hankard, P.K., Sousa, J.P., Lemos, M.F., Weeks, J.M., Soares, A.M.V.M., 2005. Influence of dimethoate on acetylcholinesterase activity and locomotor function in terrestrial isopods. Environ. Toxicol. Chem. 24, 603-609.

EPA, 2000. Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates [electronic resource]. [Washington, D.C.]: United States Environmental Protection Agency, Office of Research and Development, Mid-Continent Ecology Division; Office of Science and Technology, Office of Water.

Fossen, M., 2006. Environmental fate of imidacloprid. Environmental Monitoring, Department of Pesticide Regulation, Sacramento, CA, pp 1–16.

García-Berthou, E., 1999. Food of introduced mosquitofish: ontogenetic diet shift and prey selection. J. Fish. Biol. 55, 135-147.

García-de la Parra, L.M., Bautista-Covarrubias, J.C., Rivera-de la Rosa, N., Betancourt-Lozano, M., Guilhermino, L., 2006. Effects of methamidophos on acetylcholinesterase activity, behavior, and feeding rate of the white shrimp (*Litopenaeus vannamei*). Ecotoxicol. Environ. Saf. 65, 372-380.

Gerhardt, A., Svensson, E., Clostermann, M., Fridlund, B., 1994. Monitoring of behavioral patterns of aquatic organisms with an impedance conversion technique. Environ. Int. 20, 209-219.

Gerhardt, A., 2000. Recent trends in biomonitoring for water quality control, in: Gerhardt, A. (Ed.), Biomonitoring of Polluted Water..Trans Tech Publications Ltd., Zürich, pp. 95-118.

Guilhermino, L., Lopes, M.C., Carvalho, A.P., Soares, A.M.V.M., 1996. Inhibition of acetylcholinesterase activity as effect criterion in acute tests with juvenile *Daphnia Magna*. Chemosphere. 32, 727-738.

Gupta, S., Gajbhiye, V.T., Kalpana, Agnihotri, N.P., 2002. Leaching behavior of imidacloprid formulations in soil. Bull Environ. Contam. Toxicol. 68, 502-508.

Jemec, A., Tisler, T., Drobne, D., Sepcic, K., Fournier, D., Trebse, P., 2007. Comparative toxicity of imidacloprid, of its commercial liquid formulation and of diazinon to a non-target arthropod, the microcrustacean *Daphnia magna*. Chemosphere. 68, 1408-1418.

Jensen, C.S., Garsdal, L., Baatrup, E., 1997. Acetylcholinesterase inhibition and altered locomotor behavior in the carabid beetle *Pterostichus cupreus*. A linkage between biomarkers at two levels of biological complexity. Environ. Toxicol. Chem. 16, 1727-1732.

Kallander, D.B., Fisher, S.W., Lydy, M.J., 1997. Recovery following pulsed exposure to organophosphorus and carbamate insecticides in the midge, *Chironomus riparius*. Arch. Environ. Contam. Toxicol. 33, 29-33.

Lemos, M.F.L., van Gestel, C.A.M., Soares, A.M.V.M., 2009. Endocrine disruption in a terrestrial isopod under exposure to bisphenol A and vinclozolin. J. Soils. Sediments. 9, 492-500.

Lemos, M.F.L., Esteves, A.C., Samyn, B., Timperman, I., van Beeumen, J., Correia, A.C., van Gestel, C.A.M., Soares, A.M.V.M., 2010a. Protein differential expression induced by endocrine disrupting compounds in a terrestrial isopod. Chemosphere. 79, 570-576.

Lemos, M.F.L., Soares, A.M.V.M., Correia, A.C., Esteves, A.C., 2010b. Proteins in ecotoxicology-how, why and why not? Proteomics. 10, 873-887.

Liess, M., Schulz, R., Liess, M.H.D., Rother, B., Kreuzig, R., 1999. Determination of insecticide contamination in agricultural headwater streams. Water Res. 33, 239-247.

Matsuda, K., Buckingham, S.D., Kleier, D., Rauh, J.J., Grauso, M., Sattelle, D.B., 2001. Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. Trends Pharmacol. Sci. 22, 573-580.

Minitab, 2006. Minitab Statistical Software 14.20, Minitab Inc., State College.

Naddy, R.B., Johnson, K.A., Klaine, S.J., 2000. Response of *Daphnia magna* to pulsed exposures of chlorpyrifos. Environ. Toxicol. Chem. 19, 423-431.

Nishiwaki, H., Sato, K., Nakagawa, Y., Miyashita, M., Miyagawa, H., 2004. Metabolism of imidacloprid in houseflies. J. Pestic. Sci. 29, 110-116.

OECD, 1984. Test No. 219: Sediment-water chironomid toxicity using spiked water. OECD Guidelines for the Testing of Chemicals 1: 1-21

Penttinen, O.P., Holopainen, I.J., 1995. Physiological energetics of a midge, *Chironomus riparius meigen* (Insecta, Diptera) - normoxic heat output over the whole life-cycle and response of larva to hypoxia and anoxia. Oecologia. 103, 419-424.

Pery, A.R.R., Mons, R., Flammarion, P., Lagadic, L., Garric, J., 2002. A modeling approach to link food availability, growth, emergence, and reproduction for the midge *Chironomus riparius*. Environ. Toxicol. Chem. 21, 2507-2513.

Pestana, J.L.T., Alexander, A.C., Culp, J.M., Baird, D.J., Cessna, A.J., Soares, A.M.V.M., 2009a. Structural and functional responses of benthic invertebrates to imidacloprid in outdoor stream mesocosms. Environ. Pollut. 157, 2328-2334.

Pestana, J.L.T., Loureiro, S., Baird, D.J., Soares, A.M.V.M., 2009b. Fear and loathing in the benthos: responses of aquatic insect larvae to the pesticide imidacloprid in the presence of chemical signals of predation risk. Aquat. Toxicol. 93, 138-149.

Phillips, P.J., Bode, R.W., 2004. Pesticides in surface water runoff in south-eastern New York State, USA: seasonal and stormflow effects on concentrations. Pest. Manag. Sci. 60, 531-543.

Rakotondravelo, M.L., Anderson, T.D., Charlton, R.E., Zhu, K.Y., 2006. Sublethal effects of three pesticides on activities of selected target and detoxification enzymes in the aquatic midge, *Chironomus tentans* (Diptera: Chironomidae). Arch. Environ. Contam. Toxicol. 51, 360-366.

Ribeiro, S., Guilhermino, L., Sousa, J.P., Soares, A.M.V.M., 1999. Novel bioassay based on acetylcholinesterase and lactate dehydrogenase activities to evaluate the toxicity of chemicals to soil isopods. Ecotoxicol. Environ. Saf. 44, 287-293.

SERA (Syracuse Environmental Research Associates, Inc.), 2005. Imidacloprid – human health and ecological risk assessment – final report; prepared for USDA, Forest Service, USA (SERA TR 05-43-24-03a). http://www.fs.fed.us/foresthealth/pesticide/pdfs/122805_Imidacloprid.pdf.

SigmaStat, 2006. SigmaStat 3.5, Systat Software Inc., Chicago.

Stoughton, S.J., Liber, K., Culp, J., Cessna, A., 2008. Acute and chronic toxicity of imidacloprid to the aquatic invertebrates *Chironomus tentans* and *Hyalella azteca* under constant- and pulse-exposure conditions. Arch. Environ. Contam. Toxicol. 54, 662-673.

Suchail, S., Debrauwer, L., Belzunces, L.P., 2004. Metabolism of imidacloprid in *Apis mellifera*. Pest. Manag. Sci. 60, 291-296.

Tomizawa, M., Casida, J.E., 2003. Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. Annu. Rev. Entomol. 48, 339-364.

Tomizawa, M., Casida, J.E., 2005. Neonicotinoid insecticide toxicology: mechanisms of selective action. Annu Rev. Pharmacol. Toxicol. 45, 247-268.

Tomlin, C.D.S., 2000. The Pesticide Manual. BCPC Publications, Berkshire.

Zar, J.H., 1996. Biostatistical Analysis. Prentice-Hall International, Inc., New Jersey.

Chapter 4

Effects of mercury on growth, emergence and behaviour of *Chironomus riparius* Meigen (Diptera: Chironomidae)

4. EFFECTS OF MERCURY ON GROWTH, EMERGENCE AND BEHAVIOUR OF *CHIRONOMUS RIPARIUS* MEIGEN (DIPTERA: CHIRONOMIDAE)

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Abstract

Mercury is a pervasive toxicant that can be found in the environment due to anthropogenic activity as well as natural sources. The majority of studies in freshwater environments focus mainly on bioaccumulation, population dynamics and biomagnification. Here, we study the effects of mercuric chloride on *Chironomus riparius* Meigen, simulating a mercury discharge on a freshwater ecosystem. Growth, emergence, development time and behaviour were the endpoints assessed. Growth was measured after 8 days exposure and behaviour was recorded at day 4 and day 10 of the experimental period. The behavioural responses of *C. riparius* to different mercury treatments were recorded with an online biomonitor that allows a more objective and precise behavioural understanding than visual observation. Mercury exposure resulted in reductions in growth, emergence, delayed development time and a decrease in locomotory activity of the larvae. Our results demonstrate that mercury exposure can impair life-history responses of chironomids.

Keywords Mercury; *Chironomus riparius*; Growth; Emergence; Behavioural Response

4.1. Introduction

In aquatic ecosystems, most toxicants accumulate in aquatic sediments (Ingersoll, 1995; Burton and Landrum, 2003) and organisms that live in close contact with these sediments are exposed to the chemicals either directly or by food intake. Mercury (Hg) is a non-essential metal (Group B), highly reactive, showing lack of specific binding to organic ligands, and form strong covalent bonds (Mason and Jenkins, 1995) such as cadmium (Walker, Hopkin, Sibly and Peakall, 2006). Hg is a pervasive and neurotoxic contaminant that can induce deficiencies of essential elements through competition at active sites in biologically important molecules (Walker, Hopkin, Sibly and Peakall, 2006) affecting the central nervous system of biota (Wolfe, 1998), and can be found in the environment due to anthropogenic activity as well as natural sources such as volcanic eruptions and forest fires (Eisler, 1987; Rasmussen, 1994; EPA, 1997; Weiner, Krabbenhoft, Heinz and Scheuhammer, 2003; Hammerschmidt and Fitzgerald, 2005). Due to its high volatilization properties (Schroeder et al., 1989; Morel et al., 1998; Wallschläger et al., 2000) mercury can appear in sites remotely located from any point source through long-range atmospheric transport, affecting the biotic freshwater communities (Evans et al., 2005; Chételat et al., 2008).

Contamination of biota from freshwater ecosystems by this heavy-metal is therefore a chronic and widespread environmental problem (Eisler, 1987; Boening, 2000). Several studies have assessed the ecotoxicological effects of Hg on biota resultant from anthropogenic activities such as mining (Žižek et al., 2007; Chibunda et al., 2008) or chlor-alkali plants (Pereira et al., 2008).

It has become important to understand not only the chemical cycle and the environmental dynamic of mercury, but also to assess the effects of this heavy metal in the biota that is subjected to its exposure. The majority of the studies assessing the effects of Hg in aquatic biota have focused on bioaccumulation and trophic transfer of the heavy-metal (Mason et al., 1995; Wong et al., 1997; Vázquez-Núñez et al., 2007; Žižek et al., 2007), while others have focused on lethal and sub-lethal toxicological endpoints in fish (Dave and Xiu, 1991; Samson

and Shenker, 2000) and macroinvertebrates (Vidal and Horne, 2003; Jensen et al., 2007).

Among freshwater benthonic macroinvertebrates, *Chironomus riparius* (*C. riparius*) are commonly used as test species to assess the toxicity of sediments because of its widespread occurrence and ecological relevance (Armitage et al., 1995), easiness of culture under laboratory conditions (Péry et al., 2002) and because during the larval development they are in contact with the sediment (Goodyear and McNeill, 1999). Few studies have assessed the acute and sub-lethal toxicity effects of Hg on *C. riparius* (Rossaro et al., 1986; Vermeulen et al., 2000; Chibunda et al., 2008).

Several tests can be used to assess both lethal and sub-lethal toxicity effects of prolonged exposure of chemicals that usually persist in this compartment over long time periods to these sediment-dwelling larvae. One of the most commonly used tests is the OECD Guideline 219 (OECDa, 2004) in which the exposure scenario is water spiking. Here, we intended to simulate an effluent drift event containing Hg, trying to reproduce a peak of concentrations in pore water, and understand its sub-lethal toxicity effects on *C. riparius* larvae, using growth, behaviour and emergence as endpoints. Growth is commonly used to study the chronic toxicity of contaminants, and is considered to be a very sensitive parameter regarding midges (Sibley et al. 1997). Adult emergence of chironomids is also a successful endpoint to be used in toxicity tests (Péry et al., 2002) for the assessment of pesticides (Sibley et al., 1997) and heavy-metals (Sildanchandra and Crane, 2000) toxicity.

Behavioural parameters have been reported as an alternative endpoint to assess toxic effects of contaminants on aquatic and soil organisms, being regarded as one of the most sensitive indicators of chemical stress (Dell'Omo, 2000). Behaviour is considered as an integration of the physiological processes and mechanisms with the environmental stimuli that cause them (Dell'Omo, 2000). Behaviour analysis as early screening tools allows obtaining quick and sub-lethal answers which can be assessed by using avoidance tests, by empirical observation or by using biomonitors. Avoidance tests are mainly focused on ecological risk assessment using soil organisms (Loureiro et al., 2005; Natal-da-

Luz et al., 2008). Empirical visual observation is commonly used in toxicological tests to assess location of the animals during the experimental period (Pestana et al., 2009). However, avoidance is only measured at the end of the experiment while visual observations, although applied during the tests, do not give us a measurable and discriminated response. Behavioural biomonitors are employed to provide a visual and measurable behavioural response at the whole-organism level. They have been used in multiple ecology and toxicology tests in the past decade (Engenheiro et al., 2005; Gerhardt et al., 2005; Macedo-Sousa et al., 2007), including tests with chironomids (Janssens de Bisthoven et al., 2004).
In this context, the aim of this study was to assess the effects of mercury exposure on *C. riparius* larvae in environmentally realistic concentrations. Effects of mercury on growth, emergence ratio, development time, and behaviour were assessed.

4.2. Material and Methods

4.2.1. Test organism

The midges used in the experiments were collected from our laboratory cultures. Larvae are kept in various small 4L aquaria that contain a layer of inorganic acid-washed fine sediment (< 1mm) as substrate and ASTM (1980) hard water, provided with aeration. Water and sediment are renewed on a weekly basis. In the culture, the midges are separated according to their life stage, thus facilitating the sampling for new tests. Sediment was bought as commercial sand, being subjected to a 24h acid wash (10% HNO_3) in order to remove any heavy metal ions, after which it was rinsed thoroughly with distilled water and the organic matter was removed by loss-on-ignition combustion for 8h at 450ºC. Organisms were fed twice a week *ad libitum* with macerated fish flakes, Tetramin® (Tetrawerke, Melle, Germany).
Prior to the experiment, two egg ropes were removed from the culture and transferred into a crystallizing dish with ASTM hard water, and placed at 20ºC.

When the larvae ecloded they were transferred into a beaker containing acid-washed inorganic fine sediment as substrate and ASTM hard water until they reached the size needed for the tests.

4.2.2. Test chemical

Mercuric (II) Chloride ($HgCl_2$) was purchased from Merck KGaA (Darmstadt, Germany) and was used to prepare the appropriate stock solutions of mercury with Milli-Q water. The actual concentration for the stock solution was 8,946 mg L^{-1}. Stock solution was stored at room temperature, protected from light and periodically analysed. Tests solutions were prepared by adding the appropriate amount of stock solution in ASTM hard water, in order to reach the pre-established concentrations.

4.2.3. Water-only exposures: Range finding test / LC50 determination

LC50 was estimated through the natural sensitivity of *C. riparius* to the metal, to establish a range of sub-lethal concentrations to be used in the experiment. We used five replicates with one organism (3rd instar - 8 days old) per treatment. Test solutions of mercury were prepared in ASTM hard water as mentioned above. Eight treatments and a control were used. Organisms were exposed individually in glass beakers containing 40 ml of test solutions and no food. After 48 h exposure, mortality was determined by mechanical stimulation, and animals that did not respond to this stimulation were considered dead. All tests were conducted at 20 ± 1 ºC, with a photoperiod of 16 h light: 8 h dark. Concentrations to be used in the chronic experiments derived from the results of this acute assay combined with the LC50 results from earlier studies with the same species and contaminant (Rossaro *et al.*, 1986).

4.2.4. Chronic experiments

To simulate an effluent discharge from a chlor-alkali plant some modifications were done to the procedures of the OECD Guideline 219 (OECDa, 2004). All replicates (16 per concentration) were prepared in 200-ml glass beakers containing 40 gr. of inorganic acid-washed fine sediment (<1mm; Table 4.1) and 150 ml of test solution (prepared in ASTM hard water as previously mentioned). One control and five concentrations (initial water concentrations were 12.65; 21.20; 40.88; 78.41 and 148.35 µg L^{-1} Hg) were used.

Table 4.1 - Grain size fractions of the inorganic acid-washed fine sediment.

Fraction (mm)	***%***
1.000 > 0.500	59
0.500 > 0.250	37
0.250 > 0.125	2
0.125 > 0.063	2
0.063 > 0.000	< 0.1
Total	100

Throughout the paper we established these initial concentrations as the comparable measurements between treatments. Medium and substrate were allowed to pre-stabilise for 24h, to allow some binding of the metal species to sediment particles. During this period, sediment and water samples were analysed to check the concentrations in each compartment before adding the animals, using additional replicates. After this, we carefully transferred five 2nd instar larvae into each test vessel using a plastic pipette. Thirty of these 2nd instar larvae obtained from our culture were preserved in 70% Ethanol and its body length was measured as the initial body length of the test animals. Each beaker was supplied with ground Tetramin® as food every 48h, at a ration of 0.5 mg $larvae^{-1}$ day^{-1}. We considered the start of the experimental period when at least 50% of the test organisms were placed in the beakers. All replicates received gentle aeration 12 hours after placing the animals, to minimize disturbance of the sediment and to

allow for a constant input of oxygen in the system. These bioassays were conducted at 20 ± 1 ºC, with a photoperiod of 16 h: 8 h, light: dark. All replicates where examined daily and any evaporated water was replaced with Milli-Q water. Physical-chemical parameters were measured at the beginning and every 48h until the end of test.

Growth was estimated after 8 days of exposure, using 7 replicates per concentration, by measuring the total body length of each larva using a stereo microscope (MS5, Leica Microsystems, Houston, USA) fitted with a calibrated eye-piece micrometer. Midges were removed after 8 days exposure and preserved in 70% Ethanol before growth measurements, instead of the usual 10 days, because larvae in the control were at this point already late fourth instar, thus pupal stage could start before day 10. Larval growth was calculated by subtracting the average initial length from each individual final length.

A transparent plastic paper cup with a 0.5mm mesh net was attached to the top of 5 other beakers, in order to capture emerged midges. Afterwards, the number of emerged adults was recorded on a daily basis. Emergence ratio (number of midges emerged per vessel / number of larvae introduced per vessel) and development time (1 / development rate) were calculated according to the formulas suggested by the OECD Guideline 219 (OECDa, 2004). For the calculation of the development time, the age of the larvae at the time of their introduction in the experiment was also considered.

Behaviour patterns were recorded at day 4 and day 10 of the experimental period, using the Multispecies Freshwater Biomonitor (MFB) (Gerhardt et al., 1994, 1998). This equipment allows the organism to move freely inside a chamber that contains two pairs of electrodes attached to the inner walls. One of the pairs generates a high frequency alternating current generated and the other pair detects and measures the subsequent current changes and frequency due to the organism's movements (Gerhardt, 2000). The MFB allows 4 minutes recordings every 10 minutes (equivalent to 6 recordings per hour) and the data generated is therefore created from the percentage of time that the organism spends on each activity. For this study, three larvae from each concentration and control were chosen randomly from the replicates, placed individually in the MFB chambers with ASTM

hard water and behaviour was recorded during 2 hours (n=12 recordings per replicate). After the recordings, larvae were replaced in the respective beakers from which they were removed. For *C. riparius*, regular movement patterns in water were summarized in two types of behaviour: locomotion – corresponding to lower frequency behaviour (0.5-2.5 Hz), and ventilation – higher frequency behaviour (3.0-8.0 Hz). These frequencies are in accordance with the ones described for the same species by Janssens de Bisthoven et al. (2004).

4.2.5. Mercury analysis

All test solutions were analysed before being added to the test vessels. After 24 hours the water and sediment from one beaker of each concentration were analysed, prior to the addition of the biota. Before placing the larvae in the test beakers we also analysed 25 unexposed midges in order to determine the initial Hg concentration in biota. At 36, 96 and 192 hours exposure, water (40 µl), biota (compound sample of 5 organisms) and sediment (10 mg) from one randomly chosen replicate of the lowest, middle and higher treatments were analysed for determination of mercury concentration.

All samples were analysed directly by atomic absorption spectrometry (AAS) with thermal decomposition of the sample and collection of the mercury vapour on a gold amalgamator, using an Advanced Mercury Analyser (AMA254 – Mercury Analyser) from LECO (St. Joseph, Michigan, USA), as described by Hall and Pelchat, 1997. We, however, changed the operational conditions, by applying a drying time of 60 seconds, a decomposition time of 150 seconds and a waiting time of 45 seconds, in accordance with the volume and weight of the material we used. The accuracy of the data was carried out using the reference materials: DORM-3 (fish protein certified reference material for trace metals) from the National Research Council Canada.

4.2.6. Statistical analysis

LC50 values were calculated using the probit method (Minitab, 2006). The EC50 and EC20 determination was calculate also by the probit method, but in this case we used the ToxRat Software (2003). For all other tests, one-way ANOVAs were performed using mercuric chloride concentrations as treatments. Whenever significant differences were observed, Dunnett post hoc test was used for multiple comparisons to determine which treatments were significantly different from the control. All statistical analyses were performed using the Minitab 14.0 statistical package (Minitab, 2006). Data from emergence ratio and behavioural patterns were arcsine square root transformed to stabilise variances across treatments (Zar 1996).

4.3. Results

In the chronic experiments, the physical parameters pH, dissolved oxygen and conductivity averaged 7.61 ± 0.16, 7.99 mg L^{-1} ± 0.9, 528 µS cm^{-1} ± 46, respectively, during the whole test duration.

In the acute experiments, the 48h LC50 (95.0% Confidence Interval) was 3.26 mg L^{-1} (2.17 – 6.10). Based on these results and on bibliography (Rossaro *et al.*, 1986), we selected the above mentioned concentrations to be used on the chronic experiments.

The 24h resting period allowed a mercury chemical interaction between glassware, water and sediment. After this period, mercury was found in water (concentration averaged approximately 45% of the initial concentration for all treatments) and sediment (concentration averaged approximately 35% of the initial concentration for all treatments) (Fig. 4.1A,B). Until the end of the experiment, almost all Hg in water was adsorbed to the sediment or was accumulated by the animals (Fig. 4.1A-C). A large loss of Hg from the test beakers was also reported.

Table 4.2 – ANOVA results

Factor	df	F	p	LOEC
Growth				
8 days exposure				
[Hg]	5	70.74	< 0.001	40.88 µg/L
Emergence ratio				
18 days exposure				
[Hg]	4	3.85	< 0.05	78.41 µg/L
Development time				
18 days exposure				
[Hg]	4	11.75	< 0.001	40.88 µg/L
Locomotion				
4 days exposure				
[Hg]	5	11.00	< 0.001	149.35 µg/L
10 days exposure				
[Hg]	5	10.66	< 0.001	40.88 µg/L
Ventilation				
4 days exposure				
[Hg]	5	9.06	< 0.001	21.20 µg/L
10 days exposure				
[Hg]	5	1.67	> 0.05	- - -

In the chronic experiments, survival averaged 82% in the controls. Growth was impaired when larvae were exposed to increasing sub-lethal concentrations of the heavy metal (Table 4.2, Fig. 4.2), with significant effects at 40.88 µg L^{-1} (LOEC) and succeeding concentrations. The EC50 and EC20 (95.0% Confidence Interval) for growth after 8 days exposure were, respectively, 87.66 µg L^{-1} (56.87 - 184.79) and 31.77 µg L^{-1} (7.50 - 50.30).

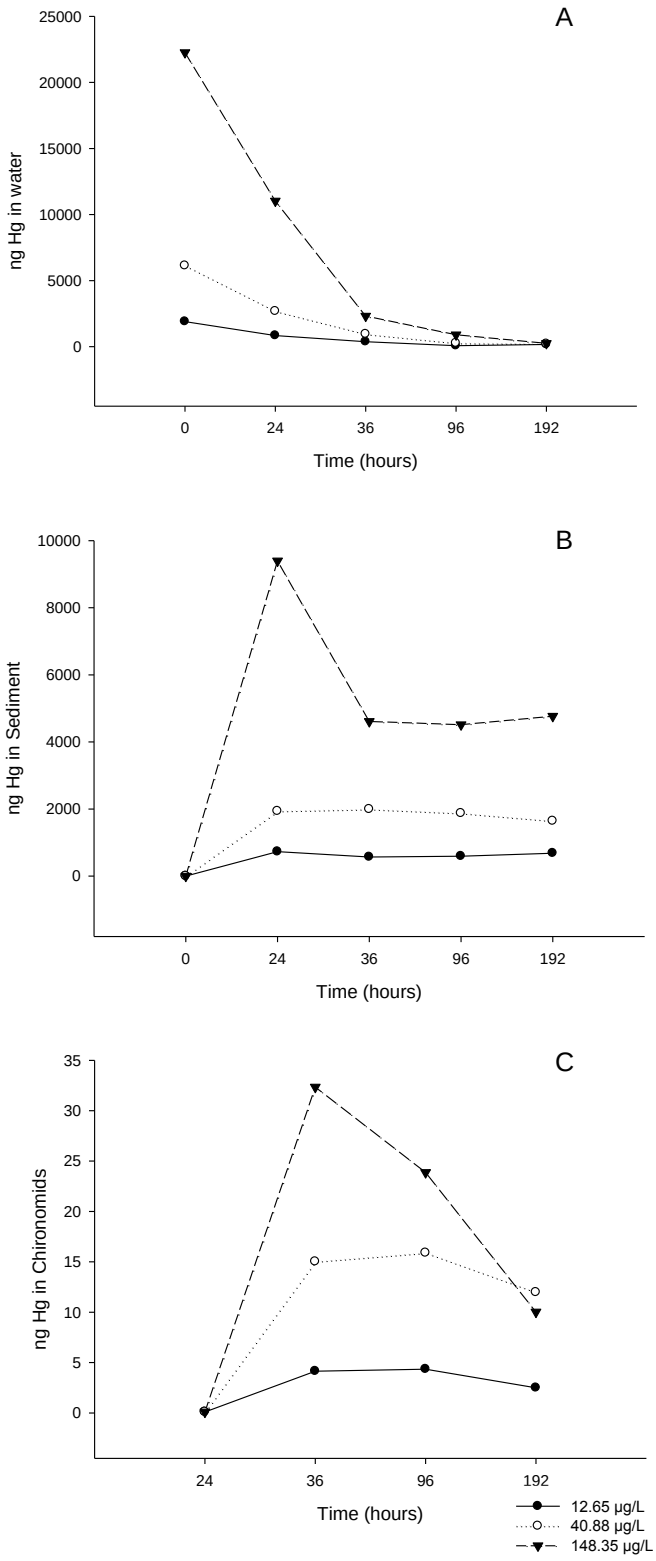

Figure 4.1 - Mercury (ng Hg) fluctuation throughout the experimental period. **A** – ng Hg dynamic in water; **B** – ng Hg dynamic in sediment; **C** – ng Hg dynamic in biota.

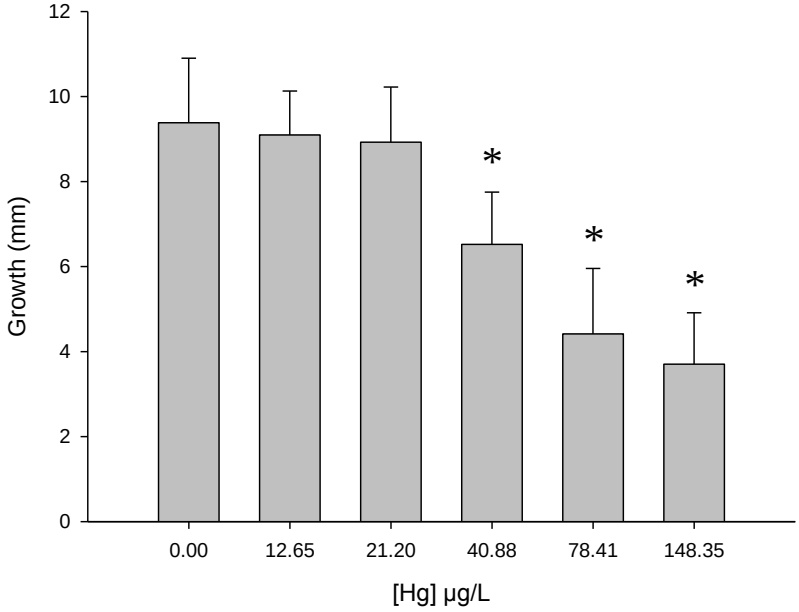

Figure 4.2 - *C. riparius* growth measurements represented by body length at day 8 subtracted by the initial body length (mean + SD) after exposure to mercury chloride. Asterisks highlight treatments that are significantly different from the control ($p<0.05$).

Midge adult emergence started at day 12 for the controls, and animals exposed to the toxicant emerged in the subsequent days. No emergence was recorded in the 148.35 µg L^{-1}, therefore this treatment was excluded from the statistical analysis of emergence. The results reveal a significant delay in development time, relative to controls, for larvae exposed to 40.88 µg L^{-1} (LOEC) and 78.41 µg L^{-1} (Table 4.2, Fig. 4.3).

Effects on emergence ratio for the tested concentrations were also found for larvae exposed to 78.41 µg L^{-1} (LOEC; Table 4.2, Fig. 4.4). In fact, the total adult emergence was reduced in 75% for animals exposed to 78.41 µg L^{-1}, when compared with the emerged adults from the control.

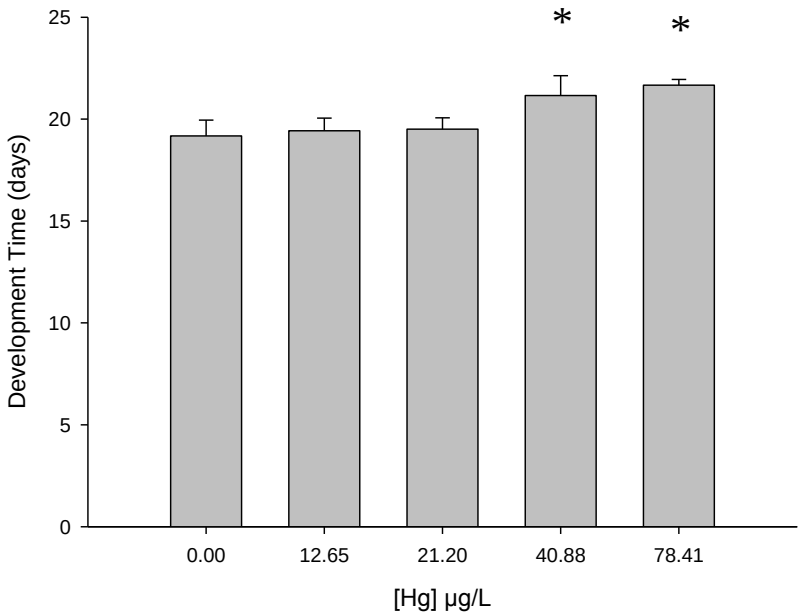

Figure 4.3 - *C. riparius* average development time (mean ± SD). Asterisks highlight treatments that are significantly different from the control ($p<0.05$)

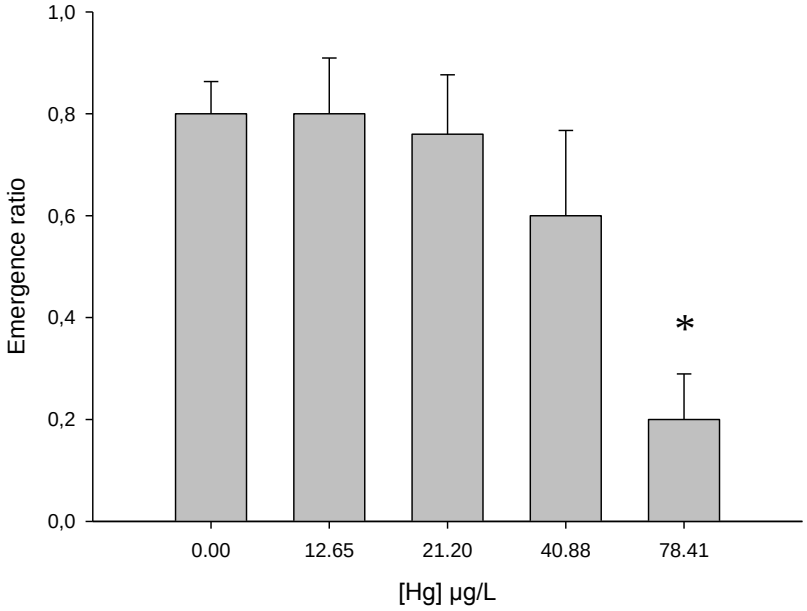

Figure 4.4 - *C. riparius* mean emergence ratio (Mean ± SE). Asterisks highlight treatments that are significantly different from the control ($p<0.05$).

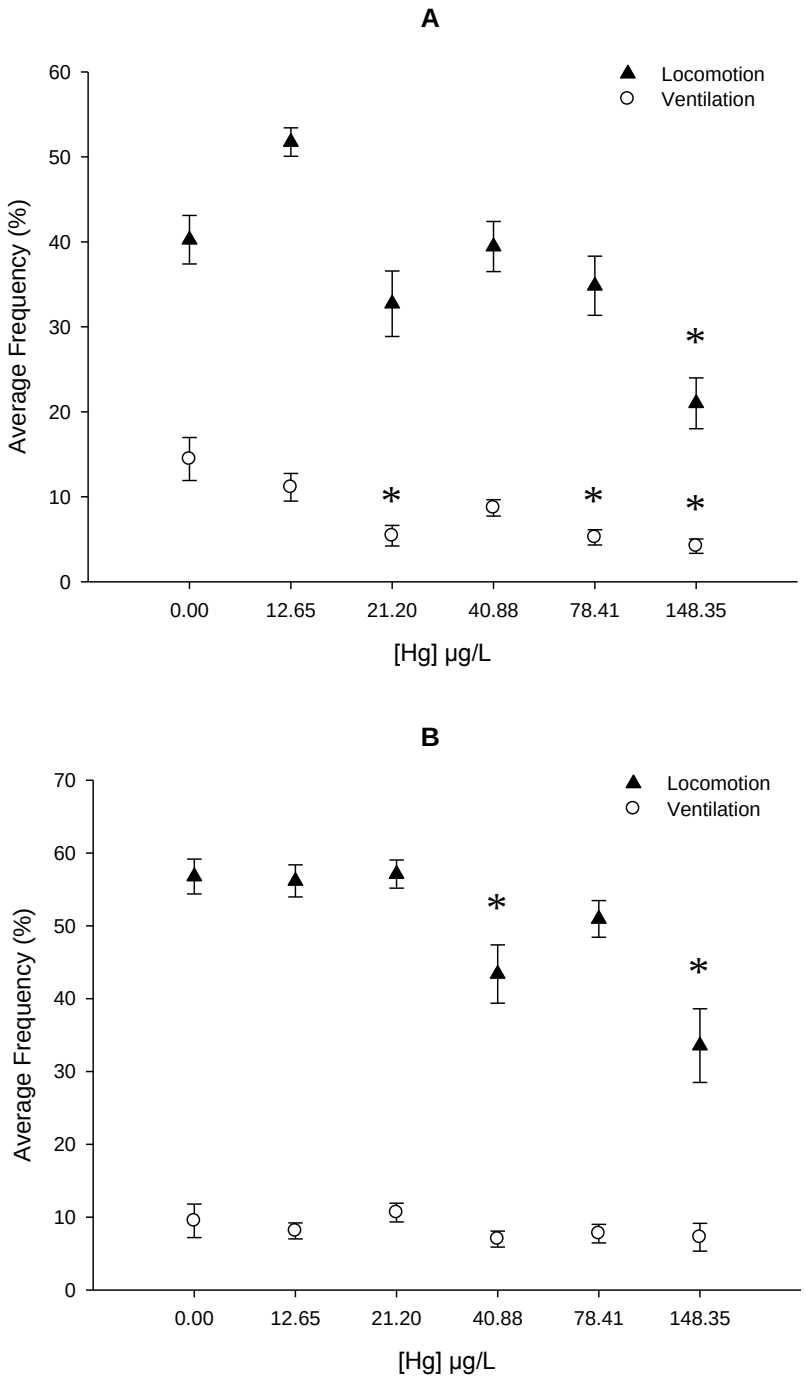

Figure 4.5 - Average activity frequencies of locomotion and ventilation of *C. riparius* when exposed to mercury for a period of 4 days (A) and 10 days (B). Vertical bars represent Standard Error (SE). Asterisks highlight treatments that are significantly different from the control ($p<0.05$).

Animals that were subjected to a 10 day mercury exposure presented a decrease in their behavioural activities with increasing concentrations. Behavioural

recordings made after 4 days exposure showed a significant decrease of the locomotory activity of the larvae at 148.35 µg L^{-1}, while ventilatory activities suffered a trend of impairment with increasing concentrations, with statistically significant differences after 21.20 µg L^{-1} when compared with the control (Table 4.2, Fig. 4.5A).

At day 10, ventilatory frequencies stabilize among treatments, showing no significant differences with the control. Locomotion is significantly affected by mercury exposure, with an impairment trend in animals exposed to concentrations higher than 40.88 µg L^{-1} (Fig. 4.5B).

4.4. Discussion

Our results indicate that *C. riparius* development can be severely impaired by exposure to mercuric chloride. The 48h LC50 was higher than the reported in the bibliography for *C. riparius* (IV instar): values of 750 µg L^{-1} and 1800 µg L^{-1} were reported by Rossaro el al. (1986) and Qureshi et al. (1980), respectively. This result might be due to the sensitivity of the species in our culture or due to the low number of organisms that was used in this experiment experiment (since the aim was to do a range finding test), where only five replicates per concentration, with one organism each, were used. Hence, when selecting the concentrations to be used in the sub-lethal experiments, the 48h LC50 values reported in the bibliography (as shown above) were also taken into account.

In the sub-lethal experiments, midges responded to mercury exposure in a concentration-dependent manner, i.e., responses were stronger when exposed to higher concentrations of the toxicant. Performing constant exposure scenarios using spiked sediments is a common procedure in laboratory toxicity experiments (Martinez et al., 2004; OECDb, 2004; Servia et al., 2006). These tests give us an understanding of how sediment-associated metals can be toxic to benthic organisms, by assessing the effects of prolonged exposure of the contaminants to the select species, intended to reproduce accumulated levels of chemicals persisting in the sediment. In this study, a mercury discharge was simulated to

understand how chironomids were affected throughout the exposure route. Further tests should consider a shorter pre-stabilization period, in order to have a more accurate understanding of the exposure route, since in our test after 24 hours around 50% of the metal was already adsorbed to the sediment.

The Hg from the test solutions accumulated in the sediment and organisms during the experimental period, but a large portion was also volatized (by evaporation and due to the aeration) and a smaller portion is considered to be adsorbed to the glassware, as we did not covered the top of the beakers, thus allowing for some evaporation (unaccounted). These findings were also reported by Vázquez-Núñez, R. et al., (2007). The selected concentrations were environmentally relevant, as they are comparable to those found in sediments from contaminated sites (Eisler, 1987; Chibunda et al., 2008) and in water and sediment from non-polluted sites (Eisler, 1987).

Information regarding the effects of mercury in invertebrates, especially benthic organisms, is scarce. Most literature related with mercury and chironomids is focused mainly in bioaccumulation (Qureshi et al., 1980; Rossaro et al., 1986), trophic transfer (Tremblay et al., 1998; Chételat et al., 2008; Eagles-Smith et al., 2008), deformities (Vermeulen et al., 2000) and population impact (Suchanek et al., 1995), so it does not assess the main physiological endpoints.

Here, *C. riparius* showed a significant impairment of growth when exposed to mercuric chloride for 8 days. These results were consistent with other tests using mercury contaminated sediment (Chibunda et al., 2008). Previous studies comprising toxic effects of other heavy metals (cadmium, zinc and/or copper) on Chironomids have also reported their negative effects on larval growth (e.g. Timmermmans et al., 1992; Postma et al., 1995; Gillis et al., 2002; Milani et al., 2003; Faria et al., 2006). Growth is a very important ecological endpoint, an important component of fitness and determinant of population health (Sibley et al., 1997), therefore any individual effect that can be translated on population decline or viability will affect the freshwater ecosystem.

Besides growth, other physiological parameters were affected by mercury exposure. The percentage of emergence found in the controls met the OECD criterion of 50% of adult emergence for acceptability of the test (OECDa, 2004).

Significantly delayed emergence rates and a delay in development time were found after exposure to the toxicant. Previous studies comprising mercury and other group B metals physiological effects also reported the same delay in development time and emergence (Timmermans et al., 1992; Vermeulen et al., 2000; Chibunda et al., 2008).

Behaviour can be referred to be the sensitive response of the organism to physiological and environmental factors (Dell'Omo, 2000). We used behavioural analysis as an endpoint to infer how the heavy metal exposure was physiologically affecting the midge larvae. Behaviour was assessed at two different periods throughout the experimental procedure, in order to have a sensitive representation and understanding of how animals were responding to different heavy metal concentrations. The MFB allows the recording of these parameters online, providing a measurable, replicable and non-destructive sub-lethal response of the organisms (Gerhardt *et al*., 2006), giving a representation of how physiological functions are affected. No sediments were used inside the test chambers because previous unpublished experimental data from our laboratory revealed confounding results upon which one could not distinguish the type of behaviour that the animal was undertaking at the measured time. So, here only water was used inside the chambers. Although the chironomids are mainly benthonic organisms they can be found in the water column, exhibiting whipping movements (Armitage et al., 1995; Gerhardt and Janssens de Bisthoven, 1995; Brackenbury, 2000). A trend of impairment in behavioural patterns with increasing concentrations of mercury was also found in this study. Although only three animals per concentration and time were used, results clearly show an impairment trend in locomotion and ventilation patterns after 4 days exposure to the toxicant. At lower concentrations (12.65 µg L^{-1}) the locomotion of the animals is higher than the control, probably due to a need of the animal to escape from the contaminant or to a need of increase the foraging activity to supply any metabolic costs. After 10 days the ventilation patterns reflect an adaptation of the animals to the toxicant, despite the locomotion patterns at higher concentrations point to a trend of activity impairment. Therefore, continuous mercury exposure can affect behaviour of *C. riparius*. Previous studies using the same equipment and chironomids, subjected to acid mine drainage, but with

different exposure and recording periods, also reported an impairment of the organism locomotion patterns (Janssens de Bisthoven et al., 2004). For a more precise assessment of how mercury affects the behaviour of chironomids, further tests should take into account more replicates (with less treatments) and shorter time gaps, each 24 hours.

After 8 days exposure to high concentrations of mercury *C. riparius* were significantly smaller, probably due to the reported reductions in behavioural activities that can impair the time spent foraging, particularly important for insects with short adult stages because fecundity is determined by the size of larva upon metamorphosis and especially by female size (Sibley et al. 2001). Thus, lack of activity might impair foraging, leading to decreased growth and consequently to a delay of the development rates and emergence. Mercury, especially its organic form methylmercury (MeHg), has been shown to induce several neurotoxic effects that might translate in behavioural responses (Eisler, 1987; Gilbert and Grant-Webster, 1995). MeHg content, that can be synthesised by bacteria from inorganic Hg compounds present in the water or in the sediments (Eisler, 1987), was not measured (but only the total Hg present in each compartment) and the influence of MeHg in the organisms physiological status could not be determined. Therefore, our data point for effects of the total Hg (mercuric chloride) present in the compartments.

Due to the fact that chironomids are one of the most widespread and abundant macroinvertebrates in freshwaters ecosystems (Ristola, 2000; Péry et al., 2002), and are also important preys for other species like fish and aquatic birds (Rieradevall et al., 1995), any effect that can affect the population (growth reduction or a delay in development time) may produce a negative impact on the freshwater ecosystem. Here, the effects of sublethal concentrations of mercury on *Chironomus riparius* by using an experimental design that intends to simulate a mercury discharge. In our opinion, this provides a more holistic view of the midge life cycle, when comparing with the standard ecotoxicological tests, by adding behaviour and a more environmentally relevant exposure route. We recommend that further tests take into account a shorter pre-stabilisation period and that MeHg

concentration and effects should be accounted for if one aims to assess the link between neurotoxic effects and behaviour.

References

Armitage PD, Cranston PS, Pinder, LCV (1995) *The Chironomidae: The biology and ecology of non-biting midges*. Armitage PD, Cranston PS, Pinder LCV (Eds.) Chapman & Hall, London, UK.

ASTM (1980), Standard practise for conducting acute toxicity tests with fishes, macroinvertebrates and amphibians. E-729-80, ASTM Philadelphia, P.A.

Boening DW (2000) Ecological effects, transport, and fate of mercury: a general review, *Chemosphere*. 40:1335-1351.

Brackenbury J (2000) Locomotory modes in the larva and pupa of *Chironomus plumosus* (Diptera, Chironomidae). *J Insect Physiol*. 46:1517-1527.

Burton GA, Landrum P (2003) Toxicity of sediments. In: *Encyclopedia of sediments and sedimentary rocks*, Middleton GV, Church MJ., Corigilo M, Hardie LA, Longstaffe FJ (Eds.). Kluwer Academic Publishers, Dordrecht, 748-751.

Chételat J, Amyot M, Cloutier L, Poulain A (2008) Metamorphosis in Chironomids, More than Mercury Supply, Controls Methylmercury Transfer to Fish in High Arctic Lakes. *Environ Sci Technol.* 42:9110-9115.

Chibunda RTP, Pereka AE, Tungaraza C (2008) Effects of sediment contamination by artisanal gold mining on *Chironomus riparius* in Mabubi River, Tanzania. *Phys Chem Earth*. 33:738-743.

Dave G, Xiu R (1991) Toxicity of mercury, copper, nickel, lead, and cobalt to embryos and larvae of zebrafish, *Brachydanio rerio*. *Arch Environ Contam Toxicol*. 21:126-134.

Dell'Omo G (2000) *Behavioural Ecotoxicology*. Dell'Omo G (Eds.) John Wiley & Sons, Inc., Hoboken, New Jersey, USA.

Eagles-Smith CA, Suchanek TH, Colwell AE, Anderson NL (2008) Mercury trophic transfer in a eutrophic lake: the importance of habitat-specific foraging. *Ecol Appl.* 18:196-212.

Eisler R (1987) Mercury hazards to fish, wildlife, and invertebrates: a synoptic review. U.S. Fish and Wildlife Service Biological Report 85 (1.10).

Engenheiro EL, Hankard PK, Sousa JP, Lemos MF, Weeks JM, Soares AMVM (2005) Influence of dimethoate on acetylcholinesterase activity and locomotor function in terrestrial isopods. *Environ Toxicol Chem*. 24:603-609.

EPA - United States Environmental Protection Agency (1997) Fate and transport of mercury in the environment. In: Mercury study report to congress (Vol. III). EPA- 452/R-97-005.

Evans MS, Muir D, Lockhart WL, Stern G, Ryan M, Roach P (2005) Persistent organic pollutants and metals in the freshwater biota of the Canadian Subarctic and Arctic: an overview. *Sci Total Environ*. 351-352:94-147.

Faria MS, Re A, Malcato J, Silva PCLD, Pestana J, Agra AR, Nogueira AJA, Soares AMVM (2006) Biological and functional responses of in situ bioassays with *Chironomus riparius* larvae to assess river water quality and contamination. *Sci Total Environ*. 371:125-137.

Gerhardt A, Svensson E, Clostermann M, Fridlund B (1994) Monitoring of behavioral patterns of aquatic organisms with an impedance conversion technique. *Environ Int*. 20:209-219.

Gerhardt A, Janssens de Bisthoven L (1995) Behavioural, developmental and morphological responses of *Chironomus* gr. *thummi* larvae (Diptera, Nematocera) to aquatic pollution. *J Aquat Ecosyst Health*. 4:205-214.

Gerhardt A, Carlsson A, Ressemann C, Stich KP (1998) New Online Biomonitoring System for *Gammarus pulex* (L.) (Crustacea): In Situ Test Below a Copper Effluent in South Sweden. *Environ Sci Technol*. 32:150-156.

Gerhardt A (2000) Recent trends in biomonitoring for water quality control. In: *Biomonitoring of Polluted Water.* Gerhardt A (Ed.). Trans Tech Publications Ltd., Zürich, 95–118.

Gerhardt A, Janssens de Bisthoven L, Soares AMVM (2005) Effects of acid mine drainage and acidity on the activity of *Choroterpes picteti* (Ephemeroptera: Leptophlebiidae). *Arch Environ Contam Toxicol*. 48:450-458.

Gerhardt A, Ingram MK, Kang IJ, Ulitzur S (2006) In situ on-line toxicity biomonitoring in water: recent developments. *Environ Toxicol Chem*. 25:2263-2271

Gilbert SG, Grant-Webster KS (1995) Neurobehavioral Effects of Developmental Methylmercury Exposure. *Environ Health Perspect Supplem*. 103:135-142.

Gillis PL, Diener LC, Reynoldson TB, Dixon DG (2002) Cadmium-induced production of a metallothioneinlike protein in *Tubifex tubifex* (Oligochaeta) and *Chironomus riparius* (Diptera): correlation with reproduction and growth. *Environ Toxicol Chem*. 21:1836-1844.

Goodyear KL, McNeill S, (1999) Bioaccumulation of heavy metals by aquatic macro-invertebrates of different feeding guilds: a review. *Sci Total Environ*. 229:1-19.

Hall G, Pelchat P (1997) Evaluation of a direct solid sampling atomic absorption spectrometry for the trace determination of mercury in geological samples. *Analyst* 122:921–924.

Hammerschmidt CR, Fitzgerald WF (2005) Methylmercury in Mosquitoes Related to Atmospheric Mercury Deposition and Contamination. *Environ Sci Technol*. 39:3034-3039.

Ingersoll CG (1995) Sediment Tests. In: *Fundamentals of Aquatic Toxicology (2nd ed.)*, Rand GM (Ed.), Taylor and Francis, Washington, DC, 231-255.

Janssens de Bisthoven L, Gerhardt A, Soares AMVM (2004) Effects of acid mine drainage on larval *Chironomus* (Diptera, Chironomidae) measured with the Multispecies Freshwater Biomonitor™. *Environ Toxicol Chem*. 23:1123-1128.

Jensen PD, Sorensen MA, Walton WE, Trumble JT (2007) Lethal and sublethal responses of an aquatic insect *Culex quinquefasciatus* (Diptera: Culicidae) challenged with individual and joint exposure to dissolved sodium selenate and methylmercury chloride. *Environ Toxicol*. 22:287-294.

Loureiro S, Soares AMVM, Nogueira AJA (2005= Terrestrial avoidance behaviour tests as screening tool to assess soil contamination. *Environ Pollut*. 138:121-131.

Macedo-Sousa JA, Pestana JLT, Gerhardt A, Nogueira AJA, Soares AMVM (2007) Behavioural and feeding responses of *Echinogammarus meridionalis* (Crustacea, Amphipoda) to acid mine drainage. *Chemosphere*. 67:1663-1670.

Martinez EA, Moore BC, Schaumloffel J, Dasgupta N (2004) Effects of exposure to a combination of zinc- and lead-spiked sediments on mouthpart development and growth in *Chironomus tentans*. *Environ Toxicol Chem*. 23:662-667.

Mason AZ, Jenkins KD (1995) Metal detoxification in aquatic organisms. Tessier A, Turner DR (Eds). *Metal Speciation and Bioavailability in Aquatic Systems*. John Wiley, West Sussex, UK. 479–609.

Mason RP, Reinfelder JR, Morel FMM (1995) Bioaccumulation of mercury and methylmercury. *Water Air Soil Pollut*. 80:915-921.

Milani D, Reynoldson TB, Borgmann U, Kolasa J (2003) The relative sensitivity of four benthic invertebrates to metals in spiked-sediment exposures and application to contaminated field sediment. *Environ Toxicol Chem*. 22:845-854.

Minitab I (2006) Minitab Statistical Software 14.20.

Morel FMM, Kraepiel AML, Amyot M (1998) The chemical cycle and bioaccumulation of mercury. *Annu Rev Ecol Syst*. 29:543-566.

Natal-da-Luz T, Amorim MJM, Römbke J, Sousa JP (2008) Avoidance tests with earthworms and springtails: Defining the minimum exposure time to observe a significant response. *Ecotoxicol Environ Saf*. 71:545–551.

OECDa (2004) Guideline 219 - Sediment-water chironomid toxicity test using spiked water. 21.

OECDb (2004) Guideline 218 - Sediment-water chironomid toxicity test using spiked sediment. 21.

Pereira M, Lillebø A, Pato P, Válega M, Coelho J, Lopes C, Rodrigues S, Cachada A, Otero M, Pardal M, Duarte A (2008) Mercury pollution in Ria de Aveiro (Portugal): a review of the system assessment. *Environ Monit Assess.* 155:39-49.

Péry ARR, Mons R, Flammarion P, Lagadic L, Garric J (2002) A modeling approach to link food availability, growth, emergence, and reproduction for the midge *Chironomus riparius*. *Environ Toxicol Chem*. 21:2507-2513.

Pestana JLT, Loureiro S, Baird DJ, Soares, AMVM (2009) Fear and loathing in the benthos: Responses of aquatic insect larvae to the pesticide imidacloprid in the presence of chemical signals of predation risk. *Aquat Toxicol*. 93:138-149.

Postma JF, Kyed M, Admiraal W (1995) Site specific differentiation in metal tolerance in the midge *Chironomus riparius* (Diptera, Chironomidae). *Hydrobiologia*. 315:159-165.

Qureshi SA, Saxena AB, Singh VP (1980) Acute toxicity of four heavy metals to benthic fish food organisms from the River Khan, Ujjain. *Internat J Environ Studies.* 15:59-61.

Rasmussen PE (1994) Current Methods of Estimating Atmospheric Mercury Fluxes in Remote Areas. *Environ Sci Technol*. 28:2233-2241.

Rieradevall M, García-Berthou E, Prat N (1995) Chironomids in the diet of fish in Lake Banyoles (Catalonia, Spain). In: Cranston P (Ed.). *Chironomids: from genes to ecosystems*. Melbourne, Australia: CSIRO Australia.

Ristola T (2000) Assessing of sediment toxicity using the midge *Chironomus riparius* (Diptera: Chironomidae). PhD Thesis. University of Joensuu, Joensuu, Finland.

Rossaro B, Gagging GF, Marchetti R (1986) Accumulation of mercury in larvae and adults, Chironomus riparius (Meigen). *Bull Environl Contam Toxicol*. 37:402-406.

Samson JC, Shenker J (2000) The teratogenic effects of methylmercury on early development of the zebrafish, *Danio rerio*. *Aquat Toxicol*. 48:343-354.

Schroeder WH, Munthe J, Lindqvist O (1989) Cycling of mercury between water, air, and soil compartments of the environment. *Water Air Soil Pollut*. 48:337-347.

Servia M, Péry A, Heydorff M, Garric J, Lagadic L (2006) Effects of copper on energy metabolism and larval development in the midge *Chironomus riparius*. *Ecotoxicology*. 15:229-240.

Sibley PK, Benoit DA, Ankley GT (1997) The significance of growth in *Chironomus tentans* sediment toxicity tests: relationship to reproduction and demographic endpoints. *Environ Toxicol Chem*. 16:336-345.

Sibley PK, Ankley GT, Benoit DA (2001) Factors affecting reproduction and the importance of adult size on reproductive output of the midge C*hironomus tentans*. *Environ Toxicol Chem*. 20:1296-1303.

Sildanchandra W, Crane M (2000) Influence of sexual dimorphism in *Chironomus riparius* Meigen on toxic effects of cadmium. *Environ Toxicol Chem*. 19:2309-2313.

Suchanek TH, Richerson PJ, Holts LJ, Lamphere BA, Woodmansee CE, Slotton DG, Harner EJ, Woodward LA (1995) Impacts of mercury on benthic invertebrate populations and communities within the aquatic ecosystem of Clear Lake, California. *Water Air Soil Pollut*. 80:951-960.

Timmermans KR, Peeters W, Tonkes M (1992) Cadmium, zinc, lead and copper in *Chironomus riparius* (Meigen) larvae (Diptera, Chironomidae): uptake and effects. *Hydrobiologia*. 241:119-134.

ToxRat (2003) Software for the statistical analysis of biotests. Copyright: ToxRat Solutions GmbH, Alsdorf, Germany

Tremblay A, Cloutier L, Lucotte M (1998) Total mercury and methylmercury fluxes via emerging insects in recently flooded hydroelectric reservoirs and a natural lake. *Sci Total Environ*. 219:209-221.

Vázquez-Núñez R, Méndez N, Green-Ruíz C (2007) Bioaccumulation and Elimination of Hg in the Fireworm Eurythoe complanata (Annelida: Polychaeta) from Mazatlan, Mexico. *Arch Environ Contam Toxicol*. 52:541-548.

Vermeulen AC, Liberloo G, Dumont P, Ollevier F, Goddeeris B (2000) Exposure of *Chironomus riparius* larvae (diptera) to lead, mercury and β-sitosterol: effects on mouthpart deformation and moulting. *Chemosphere*. 41:1581-1591.

Vidal DE, Horne AJ (2003) Mercury Toxicity in the Aquatic Oligochaete Sparganophilus pearsei: I. Variation in Resistance Among Populations. *Arch Environ Contam Toxicol*. 45:184-189.

Walker CH, Hopkin SP, Sibly RM, Peakall DB (2006) *Principles of Ecotoxicology (3rd ed)*. Walker CH, Hopkin SP, Sibly RM, Peakall DB (Eds.) CRC Press Taylor & Francis Group, Boca Raton, FL, 315.

Wallschläger D, Kock HH, Schroeder WH, Lindberg SE, Ebinghaus R, Wilken RD (2000) Mechanism and significance of mercury volatilization from contaminated floodplains of the German river Elbe. *Atmos Environ*. 34:3745-3755.

Wiener JG, Krabbenhoft DP, Heinz GH, Scheuhammer AM (2003) Ecotoxicology of mercury. In: *Handbook of Ecotoxicology (2nd ed).* Hoffman DJ, Rattner BA, Burton GA, Cairns J (Eds.) CRC Press, Boca Raton, Florida, 409–463.

Wolfe MF, Schwarzbach S, Sulaiman RA (1998) Effects of mercury on wildlife: a comprehensive review. *Environ Toxicol Chem*. 17:146-160.

Wong AHK, McQueen DJ, Williams, DD, Demers E (1997) Transfer of mercury from benthic invertebrates to fishes in lakes with contrasting fish community structures. *Can J Fish Aquat Sci*. 54:1320-1330.

Zar JH (1996) *Biostatistical Analysis.* Prentice-Hall International, Inc., New Jersey.

Žižek S, Horvat M, Gibičar D, Fajon V, Toman MJ (2007) Bioaccumulation of mercury in benthic communities of a river ecosystem affected by mercury mining *Sci Total Environ*. 377:407-415.

Chapter 5

Bioaccumulation and elimination of mercury in the midge larvae *Chironomus riparius* Meigen (Diptera: Chironomidae): a link to behaviour

5. BIOACCUMULATION AND ELIMINATION OF MERCURY IN THE MIDGE LARVAE *CHIRONOMUS RIPARIUS* MEIGEN (DIPTERA: CHIRONOMIDAE): A LINK TO BEHAVIOUR.

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Abstract

The benthonic midges *Chironomus riparius* (Meigen) lives in close contact with water and as a result it should not be neglected as an exposure pathway. In this study we assessed mercury kinetics and behavioural effects in larvae of under a water-only exposure. Both uptake and elimination of waterborne mercury were effectively described by using a one-compartment kinetic model. After the exposure period, test solutions were replaced by clean ASTM hard water. Results show that the midges were able to readily accumulate the heavy metal, presenting a very fast uptake, while its elimination was very slow, with only ~39% of the total mercury in the larvae being depurated after 48 hours in clean ASTM hard water. Behaviour did not present differences upon exposure or elimination, but a trend to increase ventilation was noticed during the exposure period.

Keywords Toxicokinetics; *Chironomus riparius*; Bioaccumulation; Behaviour; Mercury

5.1. Introduction

The Minamata (Japan) incident in the 1950's (Ekino et al., 2007) triggered the global concerns regarding mercury contamination. This metal is very common in the environment and can have natural sources – such as volcanism – or anthropogenic origins such as mine tailings or industrial effluents (Wolfe et al., 1998; Wiener et al., 2003). Due to its high volatility, it can also be dispersed via atmospheric transportation and deposited in other regions (Morel et al., 1998; Boening, 2000) mainly as Hg(II) (EPA, 1997), thus being available to biota even in regions far away from any point source. In spite imposed legislation have reduced/minimize and intend to eliminate mercury discharges to the environment in the last decades, mercury legacy in sediments and soils continue to be a matter of great concern at global scale, because mercury is a persistent contaminant that bioaccumulates even at low concentrations and exhibits high toxicity towards aquatic organisms (Suchanek et al., 1995; Tremblay and Lucotte, 1997; Azevedo-Pereira and Soares, 2010 – Chapter 4). In fact, mercury bioaccumulation can present an increased ecological risk because it can be biomagnified through the trophic chain (Morel et al., 1998). In aquatic environments, benthic fauna plays an important role in the food web (Armitage et al., 1995), representing a link with higher trophic levels. This way, metal contamination affecting these ecological communities can affect the distribution and abundance of benthic fauna and any deleterious effects of metals on these organisms can be consequently reflected in the whole ecosystem (Hare, 1992; Fleeger et al., 2003).

One of the most ubiquitous benthic groups is Chironomus spp.. Its a cosmopolitan aquatic diptera that can be found in both lotic and lentic environments (Armitage et al., 1995) which play an important role as preys for fish or aquatic birds (Garcia-Berthou, 1999). These larvae also have a high potential to play an important role as sentinel organisms in environmental monitoring (Choi et al., 1998) and are consequently used to assess sediment toxicity in situ and in the laboratory (OECD, 2004; Faria et al., 2007) but can also be used in water only toxicity tests (Lydy et al., 2000; Stuijfzand et al., 2000).

Previous tests regarding the effects of mercury on chironomids have focused on bioaccumulation (Rossaro et al., 1986) or morpho-physiological and behavioural parameters (Vermeulen et al., 2000; Azevedo-Pereira and Soares, 2010 – Chapter 4). Because accumulation of mercury by aquatic organisms is rapid, its depuration is slow and has a high potential for biomagnification (Eisler, 1987), assessing bioaccumulation and relate it with other parameters is important to understand how toxic agents and their concentration in the tissues can affect the organism physiological responses.

Any contaminant affecting an organism implies an uptake of the toxicant and is associated to the capability that the organism has to accumulate and eliminate the substance. According to Nuutinen et al. (2003; after McCarty and Mackay, 1993), toxicological processes may comprise three general phases: the first one includes the period of time upon which an organism is exposed to a certain chemical and the relative bioavailability of that chemical during the exposure (exposure phase); the second includes the uptake, distribution, metabolism, and elimination of the bioavailable portion of the toxicant (toxicokinetics phase); and the third involves the biological response resulting from the chemical arriving at the site(s) of toxic action in the organism and acting to produce its toxic effect(s) in a time dependant manner (toxicodynamics phase). Since behaviour is considered to be one of the most sensitive indicators of chemical stress and the first line of defence to environmental stimuli (Beitinger, 1990), integrating the organism physiological processes and mechanisms with the environmental stimuli that cause them (Dell'Omo, 2002), by understanding when an environmental perturbation (e.g. metal accumulation) induces a behavioural response of the organism can help to reach a comprehensive perception of what happens in contaminated ecosystems. These behavioural alterations can be measured by using biomonitors, giving us a quick, measurable and discriminated sub-lethal response. In the past decade, several biomonitors have been used in multiple ecotoxicological tests (Engenheiro et al., 2005; Macedo-Sousa et al., 2007) also including tests with chironomids (Janssens de Bisthoven et al., 2004).

The study aimed to evaluate the kinetic performance (bioaccumulation and elimination) of waterborne mercuric choride in the benthic invertebrate

Chironomus riparius Meigen and to establish a relationship between toxicokinetics and organisms' behaviour.

5.2. Material and Methods

5.2.1. Test organism

Chironomus riparius Meigen (Diptera, Chironomidae) larvae were obtained from a laboratory culture established for more than four years in standard conditions at 20ºC and in a 16-8h light-dark period. Midges were kept in various small 4L aquaria and separated according to their life stage, placed inside a large acrylic container to allow adult swarming. All aquaria had a layer of acid-washed inorganic fine sediment as substrate and ASTM hard water (ASTM, 1980), provided with aeration. Sediment was bought as commercial sand, being subjected to a 24h acid wash (10% HNO3) in order to remove any heavy metal ions, after which it was washed thoroughly with distilled water and the organic matter was removed by loss-on-ignition combustion for 8h at 450ºC. Water and sediment were renewed on a weekly basis. Organisms were fed twice a week ad libitum with macerated fish flakes, Tetramin® (Tetrawerke, Melle, Germany). Prior to the test, two egg ropes were removed from the culture and transferred into a crystallizing dish with hard water ASTM medium and placed at 20ºC. When the larvae ecloded we re-transferred them into a beaker with acid-washed inorganic fine sediment as substrate and ASTM hard water until they reached the size needed for the tests.

5.2.2. Test chemical

Mercuric (II) Chloride (HgCl2; Merck KGaA, Darmstadt, Germany) was used to prepare the appropriate stock solutions of mercury with ultra-pure water, and the concentration determined by a Mercury Analyser from LECO – AMA254 (St. Joseph, Michigan, USA). Stock solution concentration was 9,113 mg Hg L^{-1} and it was stored at room temperature, protected from light and periodically analysed. Tests solutions were prepared by diluting the stock solution in ASTM hard water, in order to reach the pre-established concentration (38 µg Hg L^{-1}).

5.2.3. Uptake experiment

This bioassay was performed in static conditions, at 20 ± 1 ºC with a 16h:8h light:dark photoperiod, as a water only toxicity test. No substrate was used in the uptake experiments to avoid possible sorption to sediment. Forty 350-ml plastic beakers containing 150 ml of test solution were prepared. One control and one concentration (38 µg L^{-1} Hg) were used, separated in 20 beakers per medium. The concentration was chosen based on the EC_{20} for growth, obtained in previous tests with the same species (Azevedo-Pereira & Soares, 2010 – Chapter 4): EC_{20} 95%-CL = 31.77 µg L^{-1} (7.50 – 50.30). Medium and plastic beakers were allowed to stabilize for 4h, upon which five 3rd instar larvae were carefully transferred into each test vessel with a plastic pipette. The larvae were previously unfed for 24 hours in order to clear the guts of any organic particles, obtained from our laboratory culture. The larvae remained unfed until the end of the test. All beakers were covered with perforated Parafilm™ in order to slow evaporation. Tests began when 50% of the tests organisms were placed in the beakers.

Replicates were analysed at 1.30, 3, 6, 12 and 24 hours after the beginning of the exposure period. These sample times were chosen from preliminary tests. Three replicates per concentration were removed and water and animals were analysed. Animals were removed and placed in a crystallizing dish with clean ASTM water

for 5 minutes, in order to remove any possible Hg particles they might have adsorbed to their surface. Afterwards, the animals were quickly passed through filter paper to remove any superficial water and weighed (fresh weight) on a Kern ALS220-4N analytical balance before being analysed as a composite sample of 5 animals per replicate. Forty ml of the test solutions were acidified (pH<2.00, HNO3 65% Fluka, Switzerland) and preserved for a maximum period of 96h. Forty µl of each test solution was then analyzed as a measure of waterborne Hg.

Simultaneously, a behavioural study using the Multispecies Freshwater Biomonitor (MFB) was conducted: this equipment records online the behaviour of aquatic species quantitatively, by measuring the changes in impedance caused by organisms that move freely inside test chambers (acrylic tubes with 7cm/2cm length/diameter) with electrodes that generate an alternating current and detect the changes in this electrical field (Gerhardt et al., 1994). As a result of these changes in the electrical field, different chironomid behaviours can be assigned to different frequencies, such as locomotion (low frequency movements like crawling, on the range 0.5-2.5 Hz) or ventilation (high frequency movements which involve undulation of the body in a regular pattern, on the range 3.0-8 Hz). These frequencies are obtained by previous tests comprising visual observation and simultaneous recording with the equipment. The data generated is created from the percentage of time that the organism spends on each activity.

In this behavioural study, the test chambers were placed in 3L plastic beakers with the respective concentration (one beaker for control – ASTM hard water – and another for the 38 µg L^{-1} Hg concentration). Seven animals for control and ten animals for the 38 µg L^{-1} Hg concentration were exposed individually in the chambers. For each beaker, three additional empty chambers were added as blank replicates. The MFB recorded automatically for 4 minutes in every 10 minutes (equivalent to 6 recordings per hour) during a 24 hour period. After that period, 3 larvae were removed and analysed for mercury, following the procedures stated above. Water samples from the beakers containing the chambers were also acidified for posterior analysis.

Bioconcentration factor (BCF) was also estimated, at 24h, using the following equation:

$$BCF = \frac{C_a}{C_w} \qquad (1)$$

Where Ca = concentration of mercuric chloride in the animal (µg g^{-1}) and Cw = concentration of mercuric chloride in the water (µg g^{-1}).

5.2.4. Elimination experiment

After 24h of exposure, larvae were gently removed from each test beaker, placed in a crystallizing dish with clean ASTM water and transferred to glass vials with clean hard water ASTM, following the procedure adopted in the previous contaminated replicates. The larvae remained unfed. A time frame was established to analyse elimination of Hg in these specimens. Three replicates were removed per treatment and water and biological samples were analysed at 3, 6, 12, 24, 48 and 72 hours after being placed in clean medium. Animals were removed from the beakers and transferred to a crystallizing dish with clean ASTM water for 5 minutes. Afterwards, the animals were blotted dry on filter paper to remove any superficial water and weighed (fresh weight) before being analysed as a pooled sample of 5 animals per replicate. 40 ml of the test solutions were acidified (pH < 2, HNO3 65% Fluka) and preserved for a maximum period of 96h. 40 µl of each test solution were then analysed for waterborne Hg.

Regarding the behavioural patterns, the animals that were inside the MFB test chambers during the exposure experiments were removed, rinsed through ASTM hard water and then transferred individually into new test chambers that were placed inside 3L plastic beakers containing clean ASTM hard water, using the same procedure adopted in the previous contaminated test chambers. During this procedure, the MFB was turned off for 45 minutes, in order to fulfil all the procedures. The MFB maintained the same recording patterns as described above, until the end of the post-48h exposure period. Physical-chemical parameters were measured at the beginning and every 48h until the end of test.

5.2.5. Mercury analysis

The test solutions that were used to contaminate the medium were analysed before being added to the test vessels. All samples, including biota and preserved acidified water samples, were analysed directly by atomic absorption spectrometry (AAS), using an Advanced Mercury Analyser (AMA254 – Mercury Analyser from LECO, St. Joseph, Michigan, USA). This procedure involves a thermal decomposition of the sample and collection of the mercury vapour on a gold amalgamator, as described by Hall and Pelchat (1997). Our procedure, however, involved different time periods (drying time 60 seconds, decomposition time of 150 seconds and waiting time of 45 seconds), in accordance with the volume and weight of the material we used here. The accuracy of the data was assessed using the reference materials: DORM-3 (fish protein certified reference material for trace metals) from the National Research Council Canada.

5.2.6. Kinetics

In these experiments, the uptake and elimination kinetics of mercuric chloride in the organisms was described using a one-compartment model, allowing a simultaneous estimation of the assimilation (a) and elimination (k) rates. We used the constant exposure model (Sousa et al., after Van Brummelen & Van Straalen, 2000), applying the following equations:

For

$$Q_t = \frac{a}{k}\left(1 - e^{-kt}\right) \qquad (2)$$

And for

$$Q_t = \frac{a}{k}\left(1 - e^{-kt_c}\right)e^{-k(t-t_c)} \qquad (3)$$

Where Qt = concentration in the organisms at time t (µg Hg g^{-1} animal); a = assimilation rate (µg Hg g^{-1} animal d^{-1}); k = elimination rate constant (d^{-1}); t = time (d); and tc = time at which the animals were transferred to uncontaminated medium (d).

5.2.7. Statistical analysis

The parameters used in the toxicokinetics model were estimated using the nonlinear estimation module of STATISTICA© 7.0 statistical package (StatSoft, Inc., 2004) with the Quasi-Newton method for calculating least squares. Data from behavioural experiments were arcsin square root transformed to stabilise variances across treatments (Zar, 1996) and two-way ANOVA's were performed using the Minitab 14.20 statistical package (Minitab, 2006), with mercuric chloride concentrations and hours as treatments. Where applicable, results are presented as mean ± SE. For all statistical tests the significance level was set at $p \leq 0.05$.

5.3. Results

In the uptake and elimination experiments the physical parameters pH, dissolved oxygen and conductivity averaged 7.86 ± 0.11, 6.67 mg L^{-1} ± 0.3, 500 µS cm^{-1} ± 2.9, respectively, during the whole test duration. Upon stabilization, concentrations in the contaminated beakers were maintained relatively constant throughout the uptake experimental period (31 ± 3 µg L^{-1}). Mortality was measured as immobilization and was observed in exposed beakers only at 48 hours test period, with 13% mortality for controls at the end of the test. Cannibalism among chironomids was registered at 96 hours, hence observations made at that time were not considered because results could be flawed.

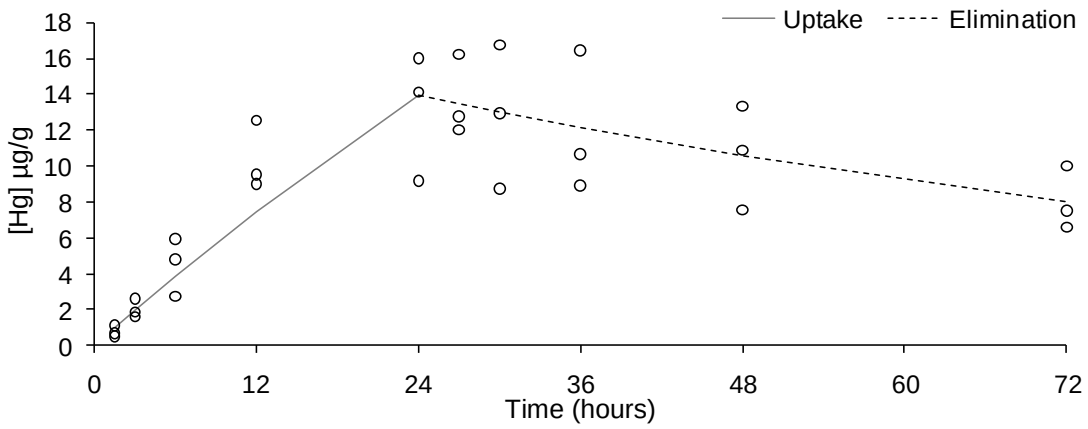

Figure 5.1 - Kinetic behaviour of mercuric chloride in *Chironomus riparius* during uptake and elimination phases. Organisms were exposed to contaminated ASTM hard water during the first 24h. Data was fitted by nonlinear regression (see text for further explanation).

The uptake of mercuric chloride by the test organisms was rapid, being detected at the first sampling period just after initial exposure (Fig. 5.1), reaching an average value of 13.1 µg Hg g^{-1} animal at the end of this period. During the elimination phase, mercury concentration in the organisms started to decrease mildly just after they were transferred to clean ASTM hard water.

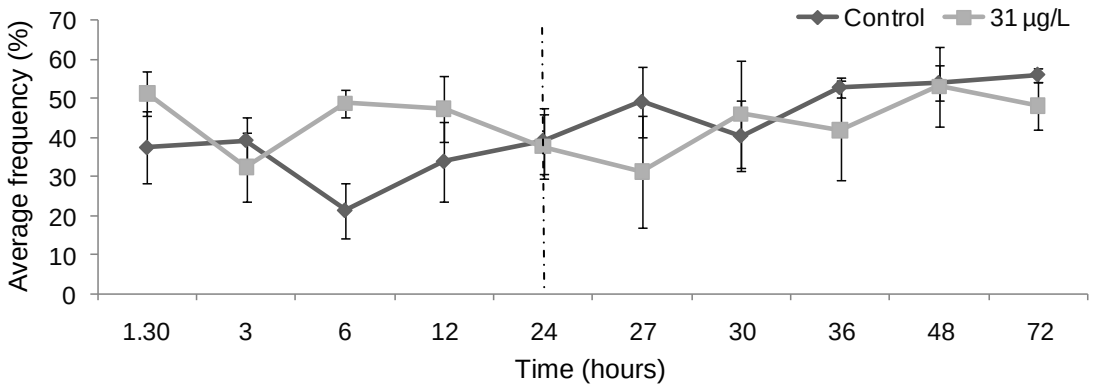

Figure 5.2 - Average activity frequencies of locomotion of *Chironomus riparius* when exposed to a concentration of 31 µg L^{-1} [Hg] for a period of 72h. Dashed line represents the end of the uptake phase and the beginning of the elimination phase.

Within the 48h time frame upon which the elimination period occurred, the organisms were not able to effectively eliminate the mercury, reaching an average value of 8.03 µg Hg g^{-1} animal at the end of this period (only ~39% of the total mercury was eliminated). The estimated kinetic parameters were 0.663 µg Hg g^{-1}

animal day^{-1} for the assimilation rate (a) and 0.012 day^{-1} for the elimination rate constant (k). The BCF value found for mercury was 450.

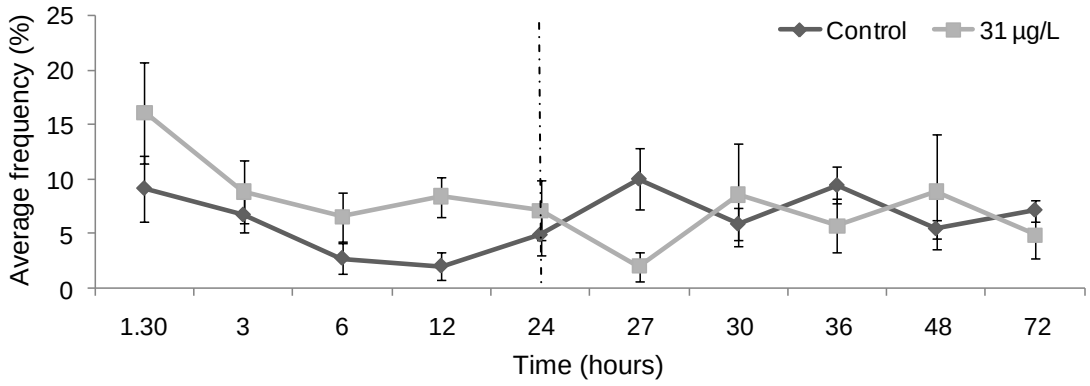

Figure 5.3 - Average activity frequencies of ventilation of *Chironomus riparius* when exposed to a concentration of 31 µg L^{-1} [Hg] for a period of 72h. Dashed line represents the end of the uptake phase and the beginning of the elimination phase.

The behavioural pattern responses of the midges were not affected by the presence of the stress agent. No statistically significant differences were found for locomotion or ventilation, neither among the experiment time frame (two-way ANOVA, $p>0.05$), nor between exposed and control organisms (two-way ANOVA, $p>0.05$). In fact, a very similar pattern was found between the control and exposed midges in locomotion activities, in both uptake and elimination phases (Fig. 5.2).
During the uptake phase, organisms exposed to the stress agent presented a higher ventilation activity frequency than the ones that were in clean test medium, albeit showing no significant differences with the control; when placed in clean ASTM hard water during the elimination phase, ventilation activities of both control and pre-exposed midges stabilized (Fig. 5.3).

5.4. Discussion

Our preliminary tests indicated that a 24h exposure period was sufficiently long to ensure adequate accumulation of mercury in the midges and to reach an

accumulation plateau, thus the experiments were designed to last for 24 hours. This short term bioassay was planned to understand the toxicokinetics of waterborne mercury, avoiding the integration of possible confounding factors such as metal adsorption by sediment or inorganic contaminated particles ingestion, as well as food intake by the organisms. Other short term kinetic bioassays with chironomids, with no sediment or food addition, have been successful in determining toxicokinetic parameters and developing kinetic models that describe uptake and elimination of other chemicals such as DDE and 2-chlorobiphenyl (Lydy et al., 2000).

The use of a one compartment model allowed the simultaneous estimation of the uptake and elimination rates in ASTM hard water. The rate of Hg uptake in *C. riparius* larvae was a quite rapid process and comparable with the cadmium (Cd; Group B metal, like Hg) bioaccumulation in the same species (Timmermans et al., 1992) and Hg bioaccumulation rate in other invertebrates, like marine macrobenthonic species [*Scrobicularia plana* and *Hediste diversicolor* (Cardoso et al., 2009)]. The elimination rate (0.012 day^{-1}) was however substantially lower than the one found for the same species in Cd toxicokinetic studies [0.20 day^{-1} for larvae from non-adapted populations (Postma et al., 1996)]. In our experiments, only ~39% of the total mercury was eliminated. Tsui and Wang (2007) also predicted a slow elimination of Hg for *Daphnia magna* and Vázquez-Núñez et al. (2007) reported a similar pattern for the marine fireworm *Eurythoe complanata*. The BCF found for mercury in our experiment was 450, much lower than the BCF found for Cd (6850) by Timmermans et al. (1992) and the BCF found for Hg (1657; unpublished data). Both experiments comprised feeding and the presence of substrate (paper fibers for the first and sediment for the latter). The low BCF in this experiment might be due not only to the fact that we established a small elimination period, but also because this metal was relatively less bioavailable, since the midges were not fed nor had sediment in the beakers.

Any possible adsorption of Hg to the exoskeleton that could influence the results of the AAS analysis (and consequently have an impact on the elimination phase results) was not measured, but other authors also reported that metal adsorption to the exoskeleton was low compared with the uptake in internal tissues, like Cd

uptake in the same species, where only an insignificant portion was adsorbed to the exoskeleton (Timmermans et al., 1992) or where 90% of the metal was found in the guts (Postma et al., 1996); and Hg uptake in crustaceans (Wright et al., 1991; Ruelas-Inzunza et al., 2004), where the exoskeleton was one of the structures that accumulated less Hg. Even rinsing larvae in ASTM hard water in spite of acidified water did not prove to have significant differences among concentrations of metal obtained from exposed larvae (Timmermans et al., 1992, for Cd).

Faster elimination rates of metals in studies with feeding might be related with desorption and repartioning of the metal between the gut and clean sediment particles passing through it or even with higher excretion rates (since food intake can lead to higher metabolic rates). In fact, Tsui and Wang (2007) refer that for *Daphnia magna* assimilation efficiency of Hg is somewhat dependent on food density. Without feeding, one can find a high risk of false positives (Ankley et al., 1994), thus in this work any confounding factors that could affect waterborne bioaccumulation were avoided (as stated above), since fine sediments particles could adsorb Hg and therefore affect water concentration, as well as could be ingested by the larvae (Pinder, 1986) influencing the excretion of non tissue adsorbed metals.

In this research, no statistical significant differences where found for either behavioural parameters among treatments (e.g. exposed and control midges) and among the experimental period. This is probably due to the fact that 24 hours toxicant exposure was not enough to produce significant effects on either locomotion or ventilation of the midges. Azevedo-Pereira and Soares (2010, Chapter 4) also did not found statistical significant differences for *C. riparius* locomotion at a similar concentration (40.88 µg Hg L^{-1}) after 4 days exposure to the same toxicant, reporting effects only after 10 days exposure. During the first 24 hours, ventilation activities of the exposed midges were higher than the ones in clean medium, and this is usually linked with attempts to escape from contaminated areas (Janssens de Bisthoven et al., 2004). After being transferred to clean medium, pre-exposed larvae rapidly normalised their behavioural activities when compared with non-exposed larvae, thus suggesting that a quick

contamination period is not enough to produce different behavioural effects on exposed midges.

This type of experiments does not always represent ecologically realistic scenarios, but are important to know the evolutionary pattern of a chemical in the environment and in the organisms with time, evidencing the importance of the exposure route. The model developed for this study underestimates the feeding exposure route, since in water environments the conjugation with particles dominates the movement and fate of mercury (Schoellhamer after Jones, 1996), but highlights the toxic effects of waterborne Hg, as well as gives us an understanding of how *C. riparius* slowly eliminates Hg. This rapid bioaccumulation and slow elimination can promote serious consequences at higher trophic levels.

References

Ankley GT, Benoit DA, Balogh JC, Reynoldson TB, Day KE, Hoke RA (1994). Evaluation of potential confounding factors in sediment toxicity tests with three freshwater benthic invertebrates. Environmental Toxicology and Chemistry. 13: 627-635.

ASTM (1980). Standard practise for conducting acute toxicity tests with fishes, macroinvertebrates and amphibians.E-729-80, ASTM Philadelphia, P.A.

Armitage PD, Cranston PS, Pinder LCV (1995). The Chironomidae: The biology and ecology of non-biting midges. Armitage PD, Cranston PS, Pinder LCV (Eds.) Chapman & Hall, London, UK.

Azevedo-Pereira HMVS, Soares AMVM (2010). Effects of Mercury on Growth, Emergence, and Behavior of *Chironomus riparius* Meigen (Diptera: Chironomidae). Archives of Environmental Contamination and Toxicology. 59: 216-224.

Beitinger TL (1990). Behavioral reactions for the assessment of stress in fishes. Journal of Great Lakes Research. 16: 495–528.

Boening DW (2000). Ecological effects, transport, and fate of mercury: a general review Chemosphere. 40: 1335-1351.

Cardoso PG, Lillebø AI, Pereira E, Duarte AC, Pardal MA (2009) Different mercury bioaccumulation kinetics by two macrobenthic species: the bivalve *Scrobicularia plana* and the polychaete *Hediste diversicolor*. Marine Environmental Research. 68: 12-18.

Choi J, Rivoal F, Roche H, Caquet T (1998). Identification de biomarqueurs d'ecotoxicite chez deux organismes sentinelles potentials, le chironome (*Chironomus riparius* (Mg.)) et la lymnee (*Lymnaea palustris* (Muller)). Ichtyophysiologica Acta 21: 89-106.

Dell'Omo G (2002). Behavioural Ecotoxicology. Dell'Omo G (Eds.) John Wiley & Sons, Inc., Hoboken, New Jersey, USA.

Eisler R (1987). Mercury hazards to fish, wildlife, and invertebrates: a synoptic review. U.S. Fish and Wildlife Service Biological Report 85 (1.10).

Ekino S, Susa M, Ninomiya T, Imamura K, Kitamura T (2007). Minamata disease revisited: An update on acute and chronic manifestations of mercury poisoning. Journal of the Neurological Sciences. 262: 131-144.

Engenheiro EL, Hankard PK, Sousa JP, Lemos MF, Weeks JM, Soares AMVM (2005). Influence of dimethoate on acetylcholinesterase activity and locomotor function in terrestrial isopods. Environmental Toxicology and Chemistry. 24: 603-609.

EPA (1997). Mercury Study Report to Congress, Volume III: Fate and Transport of Mercury in the Environment EPA-452/R-97-005.

Faria M, Lopes RJ, Nogueira AJA, Soares AMVM (2007). In situ and laboratory bioassays with *Chironomus riparius* larvae to assess toxicity of metal contamination in rivers: the relative toxic effect of sediment versus water contamination. Environmental Toxicology and Chemistry. 26: 1968–1977.

Fleeger JW, Carman KR, Nisbet RM (2003) Indirect effects of contaminants in aquatic ecosystems. Science of the Total Environment. 317: 207–233.

Garcia-Berthou E (1999) Food of introduced mosquito fish: ontogenetic diet shift and prey selection. Journal of Fish Biology. 55: 135-147.

Gerhardt A, Svensson E, Clostermann M, Fridlund B (1994). Monitoring of behavioural patterns of aquatic organisms with an impedance conversion technique. Environment International. 20: 209–219.

Hall G, Pelchat P (1997). Evaluation of a direct solid sampling atomic absorption spectrometry for the trace determination of mercury in geological samples. Analyst. 122: 921–924.

Hare L (1992). Aquatic Insects and Trace Metals: Bioavailability, Bioaccumulation, and Toxicity. Critical Reviews in Toxicology. 22: 327 - 369.

Janssens de Bisthoven L, Gerhardt A, Soares AMVM (2004). Effects of acid mine drainage on larval *Chironomus* (Diptera, Chironomidae) measured with the Multispecies Freshwater Biomonitor™. Environmental Toxicology and Chemistry. 23: 1123-1128.

Jones, AB, Slotton DG (1996). Mercury Effects, Sources and Control Measures, San Francisco Estuary Regional Monitoring Program. San Francisco Estuary Institute.

Lydy MJ, Lasater JL, Landrum, PF (2000). Toxicokinetics of DDE and 2-Chlorobiphenyl in *Chironomus tentans*. Archives of Environmental Contamination and Toxicology. 38: 163–168.

Macedo-Sousa JA, Pestana JLT, Gerhardt A, Nogueira AJA, Soares AMVM. (2007) Behavioural and feeding responses of *Echinogammarus meridionalis* (Crustacea, Amphipoda) to acid mine drainage. Chemosphere. 67: 1663-1670.

Morel FMM, Kraepiel AML, Amyot M (1998). The chemical cycle and bioaccumulation of mercury. Annual Review of Ecology and Systematics. 29: 543-566.

Minitab (2006). Minitab Statistical Software 14.20, Minitab Inc., State College.

Nuutinen S, Landrum PF, Schuler LJ, Kukkonen JVK, Lydy MJ (2003). Toxicokinetics of Organic Contaminants in *Hyalella azteca*. Archives of Environmental Contamination and Toxicology. 44: 467-475.

OECD (2004). Guideline 218: sediment-water chironomid toxicity test using spiked sediment.

Pinder LCV (1986). Biology of Freshwater Chironomidae. Annual Review of Entomology. 31: 1-23.

Postma JF, VanNugteren P, Buckert-De Jong, MB (1996). Increased cadmium excretion in metal-adapted populations of the midge *Chironomus riparius* (diptera). Environmental Toxicology and Chemistry. 15: 332-339.

Rossaro B, Gaggino GF, Marchetti R (1986). Accumulation of mercury in larvae and adults, *Chironomus riparius* (Meigen). Bulletin of Environmental Contamination and Toxicology. 37: 402-6.

Ruelas-Inzunza J, García-Rosales SB, Páez-Osuna F (2004). Distribution of mercury in adult penaeid shrimps from Altata-Ensenada del Pabellón lagoon (SE Gulf of California). Chemosphere. 57: 1657–1661.

Sousa JP, Loureiro S, Pieper S, Frost M, Kratz W, Nogueira AJA, Soares AMVM (2000). Soil and plant diet exposure routes and toxicokinetics of lindane in a terrestrial isopod Environmental Toxicology and Chemistry. 19: 2557-2563.

StatSoft Inc. (2004). STATISTICA for Windows [version 7.0]. Tulsa, USA.

Stuijfzand SC, Helms M, Kraak MHS, Admiraal W (2000). Interacting effects of toxicants and organic matter on the midge *Chironomus riparius* in polluted river water. Ecotoxicology and Environmental Safety. 46: 351–356.

Suchanek TH, Richerson PJ, Holts LJ, Lamphere BA, Woodmansee CE, Slotton DG, Harner EJ, Woodward LA (1995). Impacts of mercury on benthic invertebrate populations and communities within the aquatic ecosystem of Clear Lake, California. Water, Air, & Soil Pollution. 80: 951-960.

Timmermans KR, Peeters W, Tonkes M (1992). Cadmium, zinc, lead and copper in *Chironomus riparius* (Meigen) larvae (Diptera, Chironomidae): uptake and effects. Hydrobiologia. 241: 119-134.

Tremblay A, Lucotte M (1997). Accumulation of total mercury and methyl mercury in insect larvae of hydroelectric reservoirs. Canadian Journal of Fisheries and Aquatic Sciences. 54: 832-841.

Tsui MT-K, Wang W-X (2007). Biokinetics and tolerance development of toxic metals in *Daphnia magna*. Environmental Toxicology and Chemistry. 26: 1023-1032.

Vázquez-Núñez R, Méndez N, Green-Ruíz C (2007). Bioaccumulation and Elimination of Hg in the Fireworm *Eurythoe complanata* (Annelida: Polychaeta) from Mazatlan, Mexico. Archives of Environmental Contamination and Toxicology. 52: 541-548.

Vermeulen AC, Liberloo G, Dumont P, Ollevier F, Goddeeris B (2000). Exposure of *Chironomus riparius* larvae (diptera) to lead, mercury and β-sitosterol: effects on mouthpart deformation and moulting. Chemosphere. 41: 1581-1591.

Wiener JG, Krabbenhoft DP, Heinz GH, Scheuhammer AM (2003). Ecotoxicology of mercury. In: Hoffman DJ, Rattner BA, Burton Jr. GA & Cairns Jr. J (eds), Handbook of Ecotoxicology, Lewis publishers, London, 409-463.

Wolfe MF, Schwarzbach S, Sulaiman RA (1998). Effects of mercury on wildlife: a comprehensive review. Environmental Toxicology and Chemistry. 17: 146-160.

Wright DA, Welbourn PM, Martin AVM (1991) Inorganic and organic mercury uptake and loss by the crayfish *Orconectes propinquus*. Water, Air and Soil Pollution. 56: 697-707.

Zar JH (1996) Biostatistical Analysis. Prentice-Hall International, Inc., New Jersey.

Chapter 6

General conclusions and final remarks

6. GENERAL CONCLUSIONS AND FINAL REMARKS

The focus of the present thesis was to investigate behavioural responses and other standard endpoints of the benthic invertebrate *Chironomus riparius* towards mercury and imidacloprid exposure. In the previous chapters, it has been noticed that the behavioural responses are an important tool to rapidly and non-destructively assess toxicity towards the chosen model species. A causal link between altered behaviour and environmental equilibrium is also addressed here.

In ecotoxicology, the use of standard guideline tests using know and reliable endpoints under controlled laboratory conditions has proven to be an efficient process to assess the toxicity of chemicals towards model species. In this thesis, a new behavioural approach was developed and added as an additional endpoint to link the underlying physiological and ecological consequences of potential environmental contamination. With the use of online biomonitors we can get fast and sensitive results (registering behaviour as an early warning parameter), and can obtain results at ecological relevant concentrations (e.g. concentrations often found in the field). This is particularly important since ecotoxicologists have strived to use bioassays that can quickly give valuable information of contaminant effects on organisms. By improving our understanding of behavioural responses and by obtaining a quantifiable measure of behaviour we can acquire these answers.

Being the first line of defence towards environmental stimuli (Beitinger, 1990), animal behaviour is an excellent indicator of environmental changes or inflow of toxicants, as well as by being a representation of physiological alterations in the organism. Behaviour can be assessed in the laboratory by using the Multispecies Freshwater Biomonitor (MFB), developed by Gerhardt et al., (1994), in short term studies (Janssens de Bisthoven et al., 2004), in long term studies with constant recordings (Macedo de Sousa et al., 2007) or in long term tests with phased recordings as shown in this thesis.

Regarding the model species, benthic invertebrates like *C. riparius* combine the features of living in the sediment, can be often found in the water column, are a major food source for other species, and are distributed worldwide, thus making

them strong candidates for sentinel species for these types of pollutants in aquatic environments. In this thesis, evidence was provided that sub-lethal concentrations of the selected contaminants can promote significant harmful consequences to this species.

Our results support that exposure to imidacloprid (IMI) will affect the growth and behaviour of the midge larvae and that organisms can in fact recover from a short exposure to the insecticide. But one also needs to take into consideration that IMI has negative effects on aquatic insects, especially in the case of high concentrations or even repeated pulses of contamination (Pestana et al. 2009). Thus, carefully planned pesticide application intervals in agricultural fields should be considered, because if runoff occurs it will give aquatic organisms the possibility to recover from these pulses, contrary to continuous applications that might have more severe population implications. The results also show that when subjected to IMI, *C. riparius* ventilation behaviour is a more sensitive endpoint than locomotion and biochemical responses.

Sub-lethal concentrations of mercury on *C. riparius* promoted a trend of impairment in behavioural patterns with increasing concentrations of the toxicant. Growth was also impaired and delayed emergence rates / development time were also noticed. This was probably due to reductions in behavioural activities that even at low concentrations can decrease the amount of time spent foraging, producing effects at the morpho-physiological levels, and thus severely affecting the chironomid performance in the environment. Besides, these larvae can quickly bioaccumulate mercury in unfed conditions and present a slow depuration of the heavy metal, which can elicit serious consequences to the ecosystem balance.

Due to the fact that chironomids are one of the most widespread and abundant macroinvertebrates in freshwater ecosystems, being important preys for higher trophic levels (e.g. fish and aquatic birds), any factor that can affect the population (behavioural impairment → decreased foraging → growth reduction + delay in development time) may promote a negative impact on the freshwater ecosystem. Since the effects of sub-lethal levels of contaminants at the population levels are only detected, in many cases, after several generations, the development and validation of early warning systems like these behavioural assessments –

behaviour, as an adaptive response to a particular level of stimuli, can be obliterated or produce an aberrant response when the animal is exposed to a higher (or different) level of stimulation (Beitinger, 1990) – can help to predict possible alterations at the field population levels even faster than conventional standard ecotoxicological tests (survival, growth or emergence).

Integrating behaviour with regularly used endpoints in ecotoxicology evidences the sensitivity and non lethality of this endpoint, allowing (in the case of online biomonitors) not only an evaluation of toxicity at the end of the test, but also a continuous, quantifiable and real time measurement of how the animals are reacting during the experiments. Despite the non-use of sediment in the test chambers, the selected species presented excellent and reliable behavioural responses towards the toxicity at which they were subjected, not only because it was a short term quantifiable measurement of behaviour in select periods throughout the experiment, but also because these larvae also exhibit good swimming and locomotor activities in water. In fact, these results highlighted the understanding of the chironomids' behaviour responses as an early warning parameter.

The use of these behavioural endpoints and online biomonitoring as a sub-lethal ecotoxicogical relevant parameter in toxicology will therefore increase the versatility of the tests, allowing a measurable and quantitative behavioural response at the whole-organism level, giving a representation of the organism physiological response to environmental factors by offering an ecologically relevant, integrative, sensitive, fast and non-destructive assessment, thus certifying that this approach can be used in further assays.

Further studies should bear in mind more ecologically relevant behavioural studies using online biomonitors like MFB: when assessing toxicity on long term contamination of sediment organisms, the behavioural approach revealed in this study can produce reliable results, but if there is the possibility to use more replicates, one could assess sediment toxicity by applying a two compartment chamber (water and sediment, as referred by Gerhardt and Schmidt, 2002), measuring percentages of time that the animals stays in each compartment

(somewhat like measuring avoidance for 24 hours during 7 days); and when assessing water toxicity, this equipment reveals ideal conditions to do long term monitoring of water column model species (e.g. *Daphnia magna*; *Danio rerio*), using flow-through devices and the possibility of establishing pollution pulses intercalated with clean periods.

References

Beitinger TL (1990). Behavioural reactions for the assessment of stress in fishes. Journal of Great Lakes Research. 16: 495–528.

Gerhardt A, Svensson E, Clostermann M, Fridlund B (1994). Monitoring of behavioral patterns of aquatic organisms with an impedance conversion technique. Environment international. 20: 209-219.

Gerhardt A, Schmidt S (2002). The Multispecies Freshwater Biomonitor as tool for sediment biotests and biomonitoring. Journal of Soils and Sediments, 2: 67-70.

Janssens de Bisthoven L, Gerhardt A, Soares AMVM (2004). Effects of acid mine drainage on larval *Chironomus* (Diptera, Chironomidae) measured with the Multispecies Freshwater BiomonitorTM. Environmental Toxicology and Chemistry. 23: 1123-1128.

Macedo-Sousa JA, Pestana JLT, Gerhardt A, Nogueira AJA, Soares AMVM (2007). Behavioural and feeding responses of *Echinogammarus meridionalis* (Crustacea, Amphipoda) to acid mine drainage. Chemosphere. 67: 1663-1670.