Universidade de Aveiro Departamento de Biologia

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Fátima Cristina Paulino Simão

Avaliação ecotoxicológica de PAHs utilizando a planária de água doce, *Girardia tigrina*

Ecotoxicological assessment of PAHs using the freshwater planarian *Girardia tigrina*

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o júri

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palavras-chave

Planárias de água doce; ecotoxicologia; hidrocarbonetos aromáticos policíclicos; regeneração; comportamento; biomarcadores bioquímicos; reprodução sexuada; bioacumulação diferencial; transferência trófica.

resumo

As planárias de água doce são animais que possuem uma variedade de características que as tornou fundamentais para várias áreas do conhecimento, tais como a investigação em células estaminais, a neurofarmacologia ou a investigação sobre envelhecimento. O interesse por estes animais deve-se a atributos únicos como a existência de células estaminais distribuídas pelos seus corpos, a presença de um cérebro simples, mas com semelhanças ao sistema nervoso dos vertebrados, ou a sua aparente ausência de envelhecimento. O interesse pelas planárias como animais experimentais difundiu-se à área da investigação ecotoxicológica, não só devido à sua fácil manutenção em contexto laboratorial e sensibilidade a contaminantes ambientais, mas também à vasta gama de respostas que podem ser usadas para avaliar a teratogénese, a carcinogénese, a toxicidade comportamental e reprodutiva ou a neurotoxicidade. Para além disto, as planárias podem ser usadas em testes com múltiplas espécies como invertebrados epibentónicos predadores. Neste contexto, o presente trabalho pretendeu explorar os parâmetros ecotoxicológicos das planárias de água doce em resposta a contaminantes ambientais, através da utilização de hidrocarbonetos aromáticos policíclicos (PAHs) como compostos modelo. Os PAHs são contaminantes ubíquos no ambiente, devido à variedade de fontes emissoras, tanto naturais, como antropogénicas, e à sua capacidade de serem transportados através do ar por longas distâncias. Contudo, a avaliação de risco ambiental dos PAHs tem-se mostrado difícil, devido à variabilidade das capacidades de metabolização destes compostos nos invertebrados e à grande variedade de efeitos que os PAHs podem provocar nos organismos. Para alguns grupos de invertebrados, como é o caso das planárias, os efeitos destes compostos são (quase) desconhecidos. No sentido de explorar a potencial utilização das planárias de água doce no contexto da ecotoxicologia, este trabalho utilizou a espécie *Girardia tigrina* como organismo modelo e três PAHs com diferentes números de anéis aromáticos, o fenantreno, o pireno e o benzo[a]pireno (B[a]P), de 3, 4 e 5 anéis aromáticos, respetivamente. Foram delineados vários objetivos

específicos: o desenvolvimento de ensaios para a avaliação dos parâmetros ecotoxicológicos nas planárias de água doce, a avaliação da toxicidade dos PAHs e a potencial acumulação dos PAHs em diferentes cenários de exposição. Ao longo de 6 capítulos experimentais, os protocolos para a avaliação dos parâmetros ecotoxicológicos foram refinados e, mais especificamente, foi desenvolvido um protocolo para avaliar a inibição alimentar de acordo com as características específicas da planária *G. tigrina*. Também se verificou que esta espécie de planária é sensível aos PAHs, tendo sido observada mortalidade, desintegração de tecidos, atrasos na regeneração, alterações comportamentais, alterações bioquímicas, diminuição da taxa reprodutiva e efeitos em planárias recém-nascidas. A severidade dos efeitos observados aumentou de acordo com as concentrações internas de PAHs nos tecidos das planárias. No entanto, apesar das semelhanças químicas entre os compostos, cada um dos PAHs provocou diferentes efeitos nas planárias. A avaliação da taxa de alimentação e locomoção mostrou ser sensível em resposta aos contaminantes em exposições de curta duração, e evidenciou o potencial destes parâmetros para a avaliação ecotoxicológica. Em períodos de exposição mais longos, a fecundidade mostrou ser um parâmetro sensível. Para além disso, através da observação dos recém-nascidos resultantes de progenitores expostos ao B[a]P, foi verificado que estes apresentavam anomalias comportamentais, evidenciando, assim, a importância da avaliação da condição dos recém-nascidos como um parâmetro reprodutivo e em exposições multigeracionais. Constatou-se ainda que, nos tecidos das planárias, os PAHs se acumularam em maior quantidade na zona cefálica, potencialmente indicando que estes compostos poderão ser neurotóxicos para as planárias, como sugerem também as alterações comportamentais. Para além disto, a bioacumulação de PAHs em planárias expostas a sedimentos contaminados ou que consumiram presas contaminadas com B[a]P, demonstrou o potencial destes animais para testes de avaliação ecotoxicológica de sedimentos e na avaliação da transferência trófica de contaminantes. Os resultados obtidos no presente trabalho indicam que as planárias são sensíveis a contaminantes, permitem a avaliação de uma grande variedade de parâmetros e podem também ser usadas como predadores em testes com múltiplas espécies. Assim, a utilização das planárias de água doce para a investigação ecotoxicológica é fortemente recomendada.

keywords

Freshwater planarians; ecotoxicology; Polycyclic aromatic hydrocarbons; regeneration; behaviour; biochemical biomarkers; sexual reproduction; differential accumulation; trophic transfer.

abstract

Freshwater planarians are animals that possess a set of features that has made them pivotal for research areas such as stem cell research, neuropharmacology, or ageing research. The appeal of these animals has stemmed from features such as the existence of adult stem cells distributed over their bodies, the presence of a simple brain sharing similarities with the vertebrate nervous system, or their apparent lack of ageing. The interest in freshwater planarians as experimental animals has spread to ecotoxicity testing, given features such as easy maintenance in laboratory setting, their sensitivity to environmental contaminants, and also the range of effects that can be evaluated in response to contaminants, such as teratogenicity, carcinogenicity, reproductive and behavioural toxicity or neurotoxicity. Their predatory nature offers yet another interesting opportunity to include them in multi-species tests as epibenthic invertebrate predators. In the context of exploring the sensitivity of freshwater planarian endpoints to environmental contaminants, the present work used polycyclic aromatic hydrocarbons (PAHs) as model compounds. PAHs are ubiquitous contaminants in the environment, given the multitude of natural and anthropogenic sources and their ability to be transported over long distances through the air. As a major contaminant class in the environment, the risk assessment of PAHs has been challenging, given the array of metabolizing abilities among invertebrates, as well as the multitude of effects these compounds can elicit. For some invertebrate groups, such as freshwater planarians, there is virtually no (eco)toxicological information. With the ultimate goal of exploring freshwater planarians in the context of ecotoxicological research, this work used the freshwater *Girardia tigrina* as experimental animal and three PAHs with distinct number of aromatic rings, the 3-, 4- and 5-ringed phenanthrene, pyrene and benzo[a]pyrene, respectively. Several specific objectives were addressed: development of novel planarian bioassays, evaluation of PAH toxicity potential accumulation in *G. tigrina* under different exposure scenarios. Along 6 experimental chapters, experimental protocols were refined and, more specifically, a feeding protocol was developed in accordance with the specific features of *G. tigrina*. This freshwater planarian was also shown to be sensitive to PAHs, with mortality, disintegration of tissues, regeneration delays, behavioural im-

pairments, biochemical changes, decreases in reproductive output and decreased newborn fitness being observed: The severity of effects was well related with the concentration-dependent increase of PAH-type compounds in planarian tissues. However, despite chemical similarities, each PAH elicited different effects in planarians. The evaluation of feeding and locomotion in response to chemical stress was evidenced to be sensitive at short exposure periods, showcasing the potentialities of planarian behavioural endpoints for ecotoxicity testing. With longer exposure periods, fecundity was shown to be a sensitive endpoint, while increased behavioural anomalies in unexposed newborns resulting from B[a]P-exposed parents, evidenced the importance of newborn condition as a reproduction-related endpoint in planarians and for transgenerational studies. The evaluation of internal concentrations of PAH-type compounds in the head and tail portions of exposed planarians revealed that these compounds accumulated more in the heads of planarians, providing a hint on the potential neurotoxicity of these compounds, as suggested by the observed behavioural effects. Moreover, the accumulation of PAH-type compounds in *G. tigrina* exposed to B[a]P-contaminated sediments or to contaminated prey, evidenced their potential for ecotoxicological experiments with contaminated sediments and to evaluate trophic transfer of contaminants. The results obtained in the present work indicate that planarians are sensitive to chemical stress, with a multitude of available endpoints, while showing potential for inclusion as invertebrate predators in multispecies studies as invertebrate predators. Therefore, their usage for ecotoxicological research is strongly recommended.

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Chapter I – Introduction, objectives and thesis structure

1 Freshwater planarians, a brief introduction

Planarians are free living flatworms belonging to the Platyhelminthes (platy – flat; helminth – worm) phylum (Riutort et al. 2012; Noreña et al. 2014). This is the 4th largest animal phylum, with around 20000 described species, nearly 1300 being from freshwater habitats (Riutort et al. 2012; Noreña et al. 2014). This phylum encompasses both free living and parasitic worms living in marine, terrestrial and freshwater environments (Schockaert et al. 2007). Among the free-living flatworms, the Order Tricladida is the most studied and well-known, with 426 freshwater species (Noreña et al. 2014) and biogeographical data indicate that they have been around for at least ~ 300 million years (Vila-Farré and Rink 2018). This Order will be the main focus of the following sections and the term "planarian" will be used throughout this thesis to refer members of the Order Tricladida, as commonly practiced throughout the literature.

1.1 General description

Planarians are soft-bodied and dorso-ventrally flattened animals, without exoskeleton or any hardened cuticle (Rink 2013; Ramm 2017). Their size ranges from a few to around 40 mm in some forms (Tachet et al. 2006) .External appearance (Figure I-1) is simple, they are bilaterally symmetrical with a triangular-shaped head and an elongated tail. On the dorsal side, photoreceptors and auricles are visible, on the head region (Newmark and Sánchez Alvarado 2002; Roberts-Galbraith and Newmark 2015). On the ventral side, they possess the pharyngeal opening. In sexually mature planarians, the gonopore is also visible, posteriorly to the pharynx (Noreña et al. 2014; Vila-Farré and Rink 2018). Planarians usually move by gliding, using muscles to direct movement and ciliary beating (over a mucus layer) (Rompolas and Patel-King 2009a; Cochet-Escartin et al. 2015; Cebrià 2016). The freshwater forms usually have dull colours, mainly grey to light brown, probably to aid in camouflaging (Lindsay-Mosher and Pearson 2018).

1.2 Habitat, distribution and ecosystem interactions

Freshwater planarians occur all over the world, with the exception of some islands (Schockaert et al. 2007; Vila-Farré and Rink 2018). The most widespread family is the Dugesiidae, occurring in 5 continents (Vila-Farré and Rink 2018). Most of the described species are from the Palearctic, reflecting the taxonomic efforts on this region (Schockaert et al. 2007; Vila-Farré and Rink 2018). Freshwater planarians can inhabit a great variety of environments, including ponds, lakes, streams, rivers and temporary water bodies (Tachet et al. 2006; Noreña et al. 2014; Vila-Farré and Rink 2018). They are ectothermic animals that generally tolerate a wide range of temperatures, although temperature will influence development rates and reproduction cycles (Tachet et al. 2006; Noreña et al. 2014; Vila-Farré and Rink 2018). Most planarian species seem to be able to survive in waters

with pH ranging from 4 to 9, while water hardness does not seem to greatly influence their survival or distribution, although some exceptions exist (Rivera and Perich 1994; Vila-Farré and Rink 2018). High salinity (NaCl) levels may preclude the survival of many species, but some may inhabit brackish waters (Rivera and Perich 1994; Noreña et al. 2014; Vila-Farré and Rink 2018). Dissolved oxygen is the most limiting factor dictating planarian distribution, with these animals not tolerating low oxygen levels (Rivera and Perich 1994; Vila-Farré and Rink 2018).

Planarians are epibenthic animals, living in contact with sediment and below leaves or attached to the underside of stones (Tachet et al. 2006; Schockaert et al. 2007; Kotpal 2012; Noreña et al. 2014). Planarians are photonegative and are mainly active during the night, remaining hidden from light during the day (Lombardo et al. 2011a; Noreña et al. 2014). However, chemical cues from prey items can attract them to sunlit places in order to feed (Lombardo et al. 2011a). Freshwater planarians are predators and hunt live prey or consume recently dead animals (Vila-Farré and Rink 2018). Some are very active hunters, while others mostly prey on injured or less active animals (Vila-Farré and Rink 2018). They can feed on a great variety of food items, such as gastropods and their eggs, insect larvae and adults, small crustaceans, oligochaetes and even amphibian eggs (Pickavance 1971; Lombardo et al. 2011b; Vila-Farré and Rink 2018). To hunt, they make use of their powerful muscles and wrap themselves around prey. Mucus secretion aids in trapping and capturing prey, preventing their escape (Noreña et al. 2014; Vila-Farré and Rink 2018). Some spe-

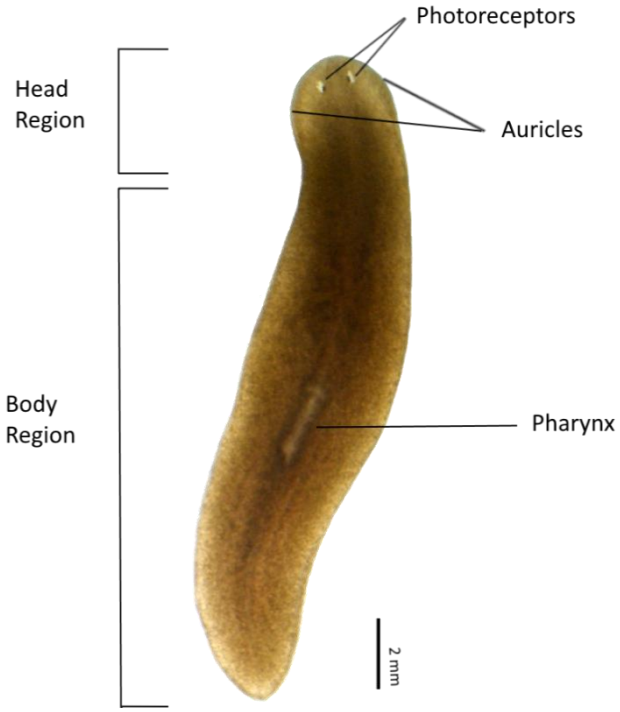


Figure I-1 – External morphology of a freshwater planarian, *Schmidtea polychroa* (From Brandl et al. 2016). Modified under the Creative Commons licence: https://creativecommons.org/licenses/by/4.0/deed.en

cies are gregarious, living and hunting in groups, which allows for the capture of bigger prey items (Pickavance 1971; Kotpal 2012). Some animals are known to prey upon planarians: invertebrates such as dragonfly nymphs, caddisfly larvae or adult beetles, as well as vertebrates such as newts and fish (Reynoldson 1983; Noreña et al. 2014; Vila-Farré and Rink 2018). In some environments, planarians can be top predators (Vila-Farré and Rink 2018).

Planarian populations seem to be mainly regulated through inter and intraspecific competition for food, since predation seems to play a minor role (Reynoldson 1983; Vila-Farré and Rink 2018). The ability to degrow (for further details see 1.3.6 - Digestive system – Feeding, digestion and energy reserves) and stand long periods of starvation contributes to this phenomenon (Vila-Farré and Rink 2018). The dispersal abilities of planarians are limited, given their sensitivity to desiccation, and absence of mechanisms and structures to survive the lack of water. The long-range dispersion through cocoons is also unlikely, since these are sessile structures that remain attached to the substrate (Vila-Farré and Rink 2018).

1.3 Morphology and physiology

Planarians are acoelomate, triploblastic animals, possessing distinct tissues and organs (Newmark and Sánchez Alvarado 2002). They have a simple body plan, with an outermost layer of epithelial cells, followed by layers of muscles and parenchyma tissue filling the spaces between organs and gut (Noreña et al. 2014). Planarians possess nervous, digestive, excretory, muscular, epidermal and reproductive systems, along with sensorial organs (Roberts-Galbraith and Newmark 2015).

1.3.1 Epidermis, glands and secretions

The body wall of planarians is composed by multiciliated epithelial cells attached to a basement membrane, without any cuticle or hardened elements (Tyler and Hooge 2004; Noreña et al. 2014; Roberts-Galbraith and Newmark 2015). Cilia are mostly found on the ventral side, allowing synchronized beating and gliding movement over a layer of mucus (Newmark and Sánchez Alvarado 2001; Rompolas and Patel-King 2009a). The surface of the body is covered by a mucus layer, the glycocalyx, a carbohydrate complex, that protects the body surface (Tyler and Hooge 2004; Noreña et al. 2014). Other mucoid secretions are produced by glands and reach the body surface through thin channels between epidermal cells (Tyler and Hooge 2004; Noreña et al. 2014). A feature of Platyhelminthes is the presence of rhabdites, rod-shaped structures of proteinous nature, that are present in epidermal cells or below the epidermis, especially on the dorsal side of the body (Pedersen 1959; Tyler and Hooge 2004; Noreña et al. 2014). The functions of these structures are not fully elucidated yet, but it is believed that upon contact with water, these structures lead to formation of mucus (Kotpal 2012; Noreña et al. 2014; Hayes 2017). Planarian mucous secretions play roles in locomotion, prey capture, protection from physical injury and microorganisms, adhesion to surfaces and possibly in repulsion from predators and cocoon formation (Jennings 1957; Newmark

and Sánchez Alvarado 2002; Noreña et al. 2014; Hayes 2017). Since planarians do not possess a respiratory system, oxygen is obtained by diffusion through the tegument (Newmark and Sánchez Alvarado 2002).

1.3.2 Muscular system

Planarians possess a net of muscle fibres arranged in layers, that constitute the body wall musculature. These three layers are arranged in different orientations: an outermost circular layer, an innermost longitudinal layer and a diagonal layer, between the others (Newmark and Sánchez Alvarado 2001; Noreña et al. 2014; Cebrià 2016). Some species, such as *Schmidtea mediterranea* and *Girardia tigrina* possess a 4th layer of longitudinal fibres between the circular and diagonal layers (Noreña et al. 2014; Cebrià 2016). Although the muscular system has a role in the locomotion of planarians, it is mainly used to direct movement during cilia-driven locomotion and to maintain the shape and structure of the animal (Noreña et al. 2014; Cebrià 2016). Besides the body wall musculature, muscle fibres are associated with the reproductive and digestive organs (Cebrià 2016). The pharynx is also a highly muscular and tubular organ, possessing a circular and a longitudinal muscle layers (Cebrià et al. 1999; Cebrià 2016).

1.3.3 Parenchyma

Filling the space between epidermis, gut and organs is the parenchyma, a tissue of mesenchymal origin (Newmark and Sánchez Alvarado 2002). It serves as a frame for the other tissues and is constituted by an extracellular matrix (composed of collagenous fibres) and several cell types: pigment cells, gland cells, embedded cells from other organs and tissues, fixed parenchyma cells and neoblasts (González-Estévez and Saló 2010; Noreña et al. 2014). Pigment cells contain pigment granules; two different pigment types have been described: ommochrome and porphyrin (Lindsay-Mosher and Pearson 2018). At least for some species, such as *Schmidtea mediterranea* both pigment types have been found (Lindsay-Mosher and Pearson 2018). These pigments are thought to act as camouflage or to protect from UV light (Birkholz and Beane 2017; Lindsay-Mosher and Pearson 2018). Fixed parenchyma cells connect the other cell types, play a role in nutrient storage and distribution and are also believed to act in the balancing of ionic concentrations (González-Estévez and Saló 2010; Noreña et al. 2014). Neoblasts are the planarians stem cells, that are found throughout the parenchyma (Rink 2013).

1.3.3.1 Neoblasts and regeneration in planarians

One of the most striking features of planarians is their ability to regenerate a complete organism from tiny pieces (Newmark and Sánchez Alvarado 2002). This regeneration potential is provided by the stem cells, the neoblasts, that exist scattered throughout the planarian body. Neoblasts are

small roundish cells, of 5 – 10 µm, that are found throughout the parenchyma (Rink 2013). Neoblasts generally comprise 25% - 30% of the cellular population in a planarian (Baguñà and Romero 1981) and are the only cells that will undergo division, being the cellular source for tissue turnover and regeneration (Newmark and Sánchez Alvarado 2001; Newmark and Sánchez Alvarado 2002). The mitotic rate of neoblasts appears to be very high, leading to a fast replacement of tissues, with complete turnover achieved in a matter of weeks (Rink 2013). The regions in front of the photoreceptors and the pharynx do not possess neoblasts and are, therefore, incapable of regeneration if excised (Rink 2013). Recently, studies have suggested that, even though neoblasts appear to be a homogenous cell population, this may not be the case. Some neoblasts appear to really be totipotent (cNeoblasts) (Wagner et al. 2011), but others seem to be committed to certain cellular lineages (Zhu and Pearson 2016; Brown and Pearson 2017).

When animals are injured, neoblasts migrate to the wound site and the division rate is increased, giving rise to a regeneration blastema, an unpigmented structure of undifferentiated cells from which the lost tissues will be regrown (Newmark and Sánchez Alvarado 2001; Rink 2013; Cebrià et al. 2015). The regeneration process can be divided into several steps; 1st is the contraction of muscles at the cut surface and closure of the wound; 2nd is the cellular proliferation of neoblasts and formation of a blastema; 3rd is the differentiation of neoblasts and morphogenesis (Cebrià et al. 2015). The regeneration process also involves the remodelling of pre-existing tissues, so that a completely proportional body will be obtained (Cebrià et al. 2015).

The high regeneration potential is closely linked and is generally higher in species that reproduce asexually by fission (Egger et al. 2007) (for further details, see section 1.3.8 - Reproduction and life cycle). Still, many species with strictly sexual populations, have a high regeneration potential and others, such as *Bdelloura candida*, have limited regeneration abilities and cannot regenerate a new head upon decapitation (Egger et al. 2007). Interestingly, the limited regenerative abilities appear to be related to the inability to direct the formation of structures, such as the brain, rather than to a decreased regeneration potential (Egger et al. 2007).

1.3.4 Nervous system

The planarian central nervous system is composed of bi-lobed cerebral ganglia in the anterior part of the body, connected by nervous commissures, and two longitudinal nerve cords in the ventral part of the body, that run the length of the animal (Newmark and Sánchez Alvarado 2002; Gustafsson et al. 2002; Cebrià 2007). The longitudinal nerve cords are connected by numerous transverse commissures, giving it a ladder-like appearance (Gustafsson et al. 2002). Subepidermal and submuscular nerve plexus, as well as nerve plexus associated with the gut and reproductive organs constitute the peripheral nervous system (Gustafsson et al. 2002; Cebrià 2007; Ross et al. 2017). From the brain to the margins of the body, sensory nerves are projected, connecting to sensorial structures (Gustafsson et al. 2002; Cebrià 2007). The planarian nervous system is constitut-

ed by unipolar, bipolar and multipolar neurons (Cebrià 2007). The presence of several neurotransmitters and neuroactive molecules has also been shown in planarians. These include serotonin (5-hydroxytryptamine), dopamine, octopamine, gamma-aminobutyric acid (GABA), acetylcholine, glutamate and aspartate among other neuroactive substances (Cebrià 2007; Buttarelli et al. 2008; Cebrià et al. 2015; Ross et al. 2017). Moreover, at least 140 neuropeptide-encoding genes have been described for *S. mediterranea* (Ross et al. 2017). Electrical synapses are also present in the form of gap junctions (Peiris and Oviedo 2013).

The planarian nervous system is able to process signals from the environment, with animals responding to stimuli such as light, temperature, chemical gradients, touch, water currents, or electric and magnetic fields (Nicolas et al. 2008; Inoue et al. 2015; Ross et al. 2017). The planarian brain is also able to decide the appropriate behaviour when presented with multiple stimuli, prioritizing some behaviours over others, in accordance with the intensity of the stimuli (Inoue et al. 2015). There is also evidence that planarians have the ability of long-term memory and learning (Wisenden and Millard 2001; Prados et al. 2013; Shomrat and Levin 2013). Another function that is believed to be played by the planarian nervous system is that of an endocrine system. Since they do not possess coelom, circulatory system, nor endocrine glands, transmission of neuroactive, hormone-like compounds is believed to be achieved directly from nervous cells or through intercellular spaces to their targets, regulating processes such as growth, development and reproduction (Fairweather and Skuce 1995; Reuter and Kreshchenko 2004; Cebrià 2007; Cebrià et al. 2015).

Planarians are regarded as the first animals evolving a true brain (Sarnat and Netsky 2002). Additionally, the planarian brain shares more features with the vertebrate nervous system than with the nervous system of other invertebrates (Buttarelli et al. 2008). Common features with the vertebrate brain include predominance of chemical over electrical synapses, shape of neurons, common neurotransmitters and low spontaneous electrical activity (Sarnat and Netsky 2002).

1.3.5 Senses and sense organs

Planarians can detect a variety of stimuli from the environment and behave accordingly. They possess photosensitive structures termed photoreceptors, located on the dorsal side of the body in the head portion, and are generally two (Roberts-Galbraith and Newmark 2015). The planarian light-sensing structures are composed of several photoreceptor neurons with projections nested in a pigment cup. The black pigment in optic cup cells is composed of eumelanin (Hase et al. 2006; Roberts-Galbraith and Newmark 2015). The *Polycelis* genus constitute an exception to this design; they possess up to hundreds of photoreceptors along the margins of the body with single pigment cells associated to a photoreceptor cell (Aikawa and Shimozawa 1991; Roberts-Galbraith and Newmark 2015). The planarian photoreceptors can detect light intensity and direction, but are unable to form images (Cebrià et al. 2015), so these are mainly used for phototactic behaviour. Additionally, planarians have been shown to discriminate light of differing wavelengths and show

stronger avoidance towards shorter wavelengths, such as UV light (Paskin et al. 2014). Some experimental evidences also suggest that planarians are able to sense light through some other mechanism, since planarians without photoreceptors still avoid light (Birkholz and Beane 2017). Porphyrin-type pigments occurring in the bodies of certain planarian species have been proposed as being related to this phenomenon (Lindsay-Mosher and Pearson 2018).

Other than photoreceptors, planarians have numerous sensorial receptors distributed all over the body, on the head margins and auricles (Skaer 1961; Noreña et al. 2014). These receptors allow detection of several environmental stimuli, such as chemical cues (chemotaxis), water currents (rheotaxis), thermal gradients (thermotaxis) and touch (thigmotaxis) (Inoue et al. 2015; Ross et al. 2017).

1.3.6 Digestive system – Feeding, digestion and energy reserves

Planarians have a highly branched digestive system. The name Tricladida stems from the existence of three main branches, 1 anterior and 2 posterior (Newmark and Sánchez Alvarado 2002; Roberts-Galbraith and Newmark 2015; Felix et al. 2018). Since planarians do not possess a circulatory system, it is believed that the highly branched nature of the digestive system is a mean of distributing nutrients to all body parts (Forsthoefel et al. 2011). The digestive system possesses only one opening, the mouth, located on the ventral side, slightly above the middle part of the body that connects to the branched intestine (Noreña et al. 2014; Roberts-Galbraith and Newmark 2015; Felix et al. 2018). Two cell types form the intestine: glandular cells that secrete digestive enzymes, and phagocytic cells that engulf food particles (Forsthoefel et al. 2011; Noreña et al. 2014; Roberts-Galbraith and Newmark 2015). An eversible highly muscular and mobile tube, the pharynx, is used in the feeding process and waste disposal (Noreña et al. 2014; Roberts-Galbraith and Newmark 2015; Felix et al. 2018). This structure generally rests inside the pharyngeal chamber and is protruded through the mouth by elongation of the muscles (Jennings 1962). When planarians capture a prey, the pharynx is inserted inside the prey's body (Noreña et al. 2014). Proteolytic enzymes are released, with tissue and fluids being sucked in by the pharynx (Jennings 1957; Jennings 1962). Digestion progresses in the gut lumen, where more enzymes are discharged, and continues inside vacuoles in the gastrodermis cells (Jennings 1957; Jennings 1962). After a few days, the cells are completely clear of food particles; any undigested particles in the gut are released through the pharynx (Jennings 1957; Jennings 1962). After feeding events, the cellular division rate increases sharply, leading to growth of the animals (Rink 2013).

Planarians possess large reserves of proteins and fat; the former are located on cells of the gastrodermis, while the latter are located, in the form of globules, in parenchyma cells (Jennings 1957). They also possess small hydrocarbon reserves in the form of glycogen, dispersed throughout the parenchyma and in the gastrodermis cells (Jennings 1957). The energy reserves are able to sustain starving planarians for several months (Jennings 1957). In such periods without food, planari-

ans possess the ability to decrease in size, a process termed degrowth. This ability is so powerful, that a fully grown planarian can decrease to a size inferior to that of a newborn (Newmark and Sánchez Alvarado 2002). Despite the decreased size, their physiological functions are maintained; only reproduction is hindered and the sexual organs (if present) resorbed (González-Estévez et al. 2012; Rink 2013; Felix et al. 2018). Degrowth is achieved by reducing the number of cells, with body and cell-type proportions remaining roughly the same (Newmark and Sánchez Alvarado 2002; Takeda et al. 2009). Even during starvation, neoblast division continues at the same rate to achieve tissue turnover, with energy being derived from autophagic processes (González-Estévez and Saló 2010; Rink 2013; Felix et al. 2018). This process is completely reversible, and when fed again, planarians will grow, with the sexual strains forming sexual structures *de novo* (Rink 2013; Felix et al. 2018). It is even hypothesized by some authors that cycles of growth and degrowth might have a rejuvenating effect on individuals (Egger et al. 2007).

1.3.7 Excretory system

In planarians, excretion and osmoregulation are achieved by the protonephridial system, by the body wall and lining of the gut (Hertel 1993). Freshwater planarians are hyperosmotic regulators (Hart et al. 1991). The protonephridial system consists of a network of branched tubules arranged in units termed protonephridia (Noreña et al. 2014; Roberts-Galbraith and Newmark 2015). On one end, flame cells filter the excretion products from the parenchyma and use cilia to drive the flow of the resulting fluid. These are linked to tubules that end in a pore on the epidermis, from which fluid exchange occurs (Noreña et al. 2014; Roberts-Galbraith and Newmark 2015).

1.3.8 Reproduction and life cycle

Reproduction in planarians is a complex subject, with great diversity of reproductive strategies. Sexual and / or asexual reproductive strategies can be employed. Some species alternate between reproductive modes depending on the season and according to environmental conditions (Stocchino and Manconi 2013; Ramm 2017). Other species possess strict sexual and asexual strains, such as *S. mediterranea*, with “strains” that are distinguishable by a chromosomal translocation (Newmark and Sánchez Alvarado 2002; Ramm 2017). Others exist as sexual or parthenogenetic individuals, that may coexist and cross (Pongratz et al. 2003). Typically, asexual and sexual strains differ in their ploidies, with sexual animals generally being diploid and asexual ones polyploid, but asexual mixoploids also occur (Beukeboom et al. 1998; Lázaro et al. 2009). Up to this date, many questions remain about the origin of the asexual populations and their relationship with the sexual ones (Lázaro et al. 2009; Álvarez-Presas and Riutort 2014).

The most typical form of asexual reproduction in planarians is transverse fission in the post-pharyngeal region. Usually, a portion of the caudal region is separated from the rest of the body by

fixing the tail portion and stretching until 2 fragments are formed, with each fragment regenerating a complete individual (Newmark and Sánchez Alvarado 2002; Thorp and Covich 2010; Malinowski et al. 2017). This process is termed architomy, meaning that the animal will divide without prior formation of new organs, with these being formed afterwards (Reuter and Kreshchenko 2004). Some species, such as *S. mediterranea* can also divide into small fragments, each regenerating a new individual, in a process called fragmentation (Kenk 1967; Quinodoz et al. 2011). A rare form of asexual reproduction in planarians is paratomy, a process in which the structures of the new individual are formed prior to fission (Egger et al. 2007; Vila-Farré and Rink 2018). Animals that employ these reproductive strategies lack sexual organs (Stocchino and Manconi 2013). Regulation of fission is dependent on several factors such as temperature, population density, size of the animal and light-dark cycles (Newmark and Sánchez Alvarado 2002). Some studies suggest that melatonin is synthesized (from serotonin) in the planarian head region, according to photoperiod in a circadian manner, and plays a role in the regulation of fission (Morita and Best 1993; Asano et al. 1998; Itoh et al. 1999).

In planarians, the sexual organs are formed when animals reach a large size (Newmark and Sánchez Alvarado 2002). No germ cells are specified during embryogenesis and are only formed when the sexual structures develop (Newmark and Sánchez Alvarado 2002; Newmark et al. 2008). The sexual structures of planarians are characterized by high developmental plasticity and can be resorbed when animals degrow, and formed again when animals resume growth (Newmark and Sánchez Alvarado 2002; Newmark et al. 2008). Planarians are hermaphrodites and possess a set of female and male organs and accessory glands (Newmark and Sánchez Alvarado 2002; Roberts-Galbraith and Newmark 2015). The female organs are comprised of a pair of ovaries (located ventrally, behind the cephalic ganglia), connected to ciliated oviducts that run the length of the animal and link to the copulatory apparatus and gonopore (Newmark and Sánchez Alvarado 2002; Newmark et al. 2008). The male organs consist of a great number of testis, dorso-laterally located, linked to the seminal vesicles and to the copulatory apparatus (Newmark and Sánchez Alvarado 2002; Newmark et al. 2008). In planarians, sexual reproduction requires cross fertilization and animals cannot self-fertilize (Hughes 1989; Kobayashi et al. 2012), with rare exceptions (Anderson 1952). Mating occurs mainly during the night; generally, animals face away from each other, lift the tails and simultaneously introduce their penises into the other's gonopore. They stay attached for some time, several minutes to several hours, exchanging sperm (Peters et al. 1996; Vreys et al. 1997). The exchanged sperm may be used to fertilize eggs, stored for later use or digested (Pongratz and Michiels 2003; Vila-Farré and Rink 2018). Stored sperm can be used to fertilize eggs up to several months after exchange (Pongratz and Michiels 2003). There are also some planarians that employ a parthenogenic reproductive mode. These are morphologically similar to sexual planarians, but mating and sperm of donors only serves to trigger the parthenogenic development of the egg (Vreys et al. 2002; Ramm 2017). In natural environments, parthenogenetic animals coexist with sexual forms; they can cross and, occasionally, parthenogenetic animals may

use the donor's sperm to fertilize eggs, leading to occasional sex (Pongratz et al. 2003; D'Souza et al. 2006; D'Souza and Michiels 2010). Factors that are known to influence sexual reproduction include diet composition, crowding, dissolved oxygen and salinity (Kostelecky et al. 1989; Knakievicz et al. 2006). Little is known about the mechanisms that control and regulate sexual reproduction in planarians, although a few studies have uncovered some genes expressed and required for this function, evidencing that reproductive development might be controlled by neuroendocrine substances released by the nervous system (Collins et al. 2010; Rouhana et al. 2017). Moreover, Miyashita et al. (2011) have suggested the presence of a steroid hormone system with possible roles in the formation of reproductive organs in planarians.

After fertilization, several eggs, along with numerous yolk cells, become enclosed in a proteinous shell, a cocoon, that is deposited through the gonopore (Corso et al. 2006; Harrath et al. 2013; Vila-Farré and Rink 2018). Cocoons are ovoid in shape, with a white to yellow coloration immediately after deposition, becoming progressively darker as they harden (Tachet et al. 2006; Rouhana et al. 2017). Planarian development usually takes some weeks but depends on species and ambient temperature. Development is direct, with no larval stages, with newly hatched planarians only differing from adults on size and absence of sexual organs (Newmark and Sánchez Alvarado 2001; Vila-Farré and Rink 2018).

The lifecycles of planarians are complex. Typically, fissiparous animals are considered immortal and seem to lack metabolic ageing (Sahu et al. 2017). Sexual forms are believed to breed either one season and die (semelparous), or along several seasons (iteroparous) (Stocchino and Manconi 2013; Vila-Farré and Rink 2018). Some species can also alternate between sexual and asexual reproductive modes, depending on season (Stocchino and Manconi 2013). Nevertheless, some evidences suggest that non-fissiparous planarians may also be long-lived, with negligible ageing (Mouton et al. 2011).

2 Planarians as experimental animals

Planarians have been the focus of in-depth studies for over a century, since their fascinating features raised the attention of numerous researchers (for hystorical overview see Rieger 1998; Newmark and Sánchez Alvarado 2002). This interest has spread to a plethora of research fields and, nowadays, many have recognised planarians as useful experimental animals. Particularly, the feature of regeneration provided by neoblasts has led to their inclusion or proposition as models for developmental biology (Newmark and Sánchez Alvarado 2002), regeneration research (Ivankovic et al. 2019; Sánchez Alvarado 2006), epigenetic regulation in stem cells (Dattani et al. 2019) or *in vivo* model for screening of carcinogenic compounds (Stevens et al. 2017). The interesting features of planarian stem cells have also led to attempts of establishing neoblast cell cultures (Schurmann and Peter 2001). Moreover, common features with the vertebrate nervous system and the behavioural consequences of disrupting planarian neurochemical pathways, have made them interesting

for neuropharmacological research (Buttarelli et al. 2008), and their apparent immortality (at least of the asexual strains) has been the focus of ageing research (Sahu et al. 2017). Interestingly, planarian mitochondria share a very rare regulation feature with human heart and skeletal muscle mitochondria, namely a strong control of oxidative phosphorylation exerted by the phosphorylation system, not known in any of the other currently used models (including mice and rats), which shows promise for the study of the role of mitochondrial metabolism in human diseases (Lemieux and Warren 2012). Other areas have also recognized the potential of planarians as experimental animals, like ciliary research (Rompolas and Patel-King 2009b), phylogeography (Álvarez-Presas and Riutort 2014), or for the study of the biology of human parasites, as surrogates (Collins and Newmark 2013), and the number of research fields that use planarians as experimental animals seems to be increasing.

The unique features of planarians and their potential have also been recognized for ecotoxicological research. Their usage has been increasing in the past decades, with previous works pointing out some of their advantages as experimental animals for the screening of environmental contaminants (Best and Morita 1991; Knakievicz 2014; Hagstrom et al. 2015; Rodrigues et al. 2016; Ofoegbu et al. 2016; Hagstrom et al. 2016; Wu and Li 2018; Ofoegbu et al. 2019b; Ofoegbu et al. 2019a). The following section summarizes the main aspects of planarians as relevant experimental animals focusing on their possible ecotoxicological endpoints.

2.1 **Advantages and potential for ecotoxicological studies**

Planarians possess several desired features as experimental animals for ecotoxicological studies. First, they are easy to maintain in laboratory cultures and have low maintenance needs, with feeding being performed once a week, and media renewal and cleaning of containers performed twice a week (Oviedo et al. 2008). They are easily obtained in large numbers, by taking advantage of their regeneration potential to get numerous fragments that will regenerate new individuals (Oviedo et al. 2008). Alternatively, cultures can be left to reproduce on their own, which will naturally occur if animals are in good condition and well fed (personal observation). Field collection is also easily performed, given the frequency and abundance of planarians in many freshwater habitats (Vila-Farré and Rink 2018). Their predatory nature further facilitates this, being possible to deploy simple traps with “bait” (e.g. liver) in freshwaters and collect these animals with little effort (Noreña et al. 2014). Alternatively, planarians can be purchased from specialized suppliers (e.g. https://www.carolina.com/platyhelminthes/brown-planaria-living/132954.pr). Most planarian species used for toxicity testing belong to the Dugesiidae Family, namely *G. dorotocephala*, *G. tigrina*, *Dugesia japonica* and *S. mediterranea*. Other members of this Family have been used to a lesser extent. From the Planariidae Family, *Polycelis felina* has been the most used. Some species appear to have been chosen based on *in situ* occurrence, availability from commercial suppliers or popularity for other research areas.

The abundance, low dispersal abilities, commonness, wide geographical distribution and tolerance to a wide range of environmental factors of freshwater planarians (Schockaert et al. 2007; Vila-Farré and Rink 2018) make their use relevant in the evaluation of contaminant effects across distinct biogeographical regions. As epibenthic animals (Vila-Farré and Rink 2018), planarians have the potential to be used in ecotoxicity testing of contaminated water or sediments. Given the small size of planarians, ecotoxicity testing may be performed without generating a large amount of wastes. Moreover, they could be used in multispecies tests and trophic transfer studies as invertebrate predators (e.g. Majdi et al. 2016). The shared features of their nervous system with that of the vertebrates' (see section 1.3.4 - Nervous system) makes them suitable for the screening of neuroactive compounds, while offering the advantages of not being subjected to the same ethical restrictions as vertebrates (Hagstrom et al. 2016). Another interesting possibility is the potential to obtain clonal cultures, even if the planarians belong to a sexual strain, providing genetically uniform organisms for testing, thus removing genetic variability as an influential factor. Moreover, these clonal organisms can, for instance, provide the opportunity to evaluate differences in the effects of toxicants on the nervous system of regenerated versus intact planarians (Hagstrom et al. 2015).

The other research areas currently focused on freshwater planarians present an advantage for ecotoxicological research, in the sense that they can provide relevant information and tools. Finally, using planarians, a plethora of endpoints can be evaluated in response to contaminant stress, offering the opportunity to investigate teratogenicity, carcinogenicity, behavioural effects, reproductive toxicity, or neurotoxicity in the same animal model. The following sections will further detail the effects of contaminant stress in freshwater planarians.

2.1.1 Toxicity assessment using planarians

2.1.1.1 Effects on survival and tissue integrity

Just as with other organisms, mortality has been the most evaluated effect in (eco)toxicological studies using planarians. Unlike other animals, when planarians die, their tissues usually disintegrate (Brøndsted 1955). Therefore, mortality is easily evaluated in planarians, as dead animals completely disintegrate. However, this disintegration prevents further examination of the tissues (Hagstrom et al. 2016). Interestingly, for many contaminants, the disintegration starts in the cephalic region and progresses caudally, for instance, in response to pesticides (Kouyoumjian and Uglow 1974; Villar et al. 1994; Horvat et al. 2005) or metals (Best et al. 1981b; Calevro et al. 1998; Franjević et al. 2000b). In the more extreme cases, this disintegration leads to death, but planarians can lose a large part of the anterior portion and still be able to regenerate after exposure ceases (Horvat et al. 2005) or even during exposure (Best et al. 1981b). Some authors have used head resorption / loss to further characterize the acute effects of contaminants (Best et al. 1981a; Best et al. 1981b), since this phenomenon occurs at lower concentrations than mortality, but is already a

sign of extreme stress. Thus, head tissue integrity has the potential to be used to characterize contaminant stress as an easily observable acute effect, although not all contaminants elicit this response. Other observable effects can be elicited in planarians after short exposures to contaminants, such as alterations on pigmentation, enhanced mucus production (Grebe and Schaeffer 1991b; Calevro et al. 1998; Kovačević et al. 2009; Ofoegbu et al. 2016).

2.1.1.2 Effects on Regeneration

The ability to regenerate after injury has been used to evaluate contaminant effects in planarians. Since regeneration of missing body parts is complex, involves stem cell proliferation and differentiation, as well as remodelling of tissues, entailing many of the processes that occur in embryonic development, it is expected that it could be affected by a variety of chemicals (Best and Morita 1991; Reddien and Alvarado 2004). By evaluating regeneration, the teratogenicity of contaminants can also be assessed by examining the presence of malformations (Best and Morita 1991). Cephalic regeneration can also be used to study the effects of contaminants towards the developing brain, by observing the reappearance of normal behaviours and hence, if the contaminants affect the process of nervous system development and cause developmental toxicity (Best and Morita 1991; Hagstrom et al. 2015). By following the reappearance of phototaxis, chemotaxis, thermotaxis or other behavioural responses to environmental stimuli, it might be possible not only to infer on contaminant neurotoxicity, but also help to pinpoint the affected neuronal populations (Best and Morita 1991; Hagstrom et al. 2015). Typically, cephalic regeneration, after surgical decapitation, is used to evaluate contaminant effects, since the regeneration of cephalic structures follows a sequence of events culminating in the reappearance of easily identifiable structures (Calevro et al. 1998). Hence, in a period of days (depending on the species), it is possible to visually score the reappearance (or not) of photoreceptors and auricles, as well as any malformations. Additionally, the size of the blastema can be measured, providing an additional metric on the course of the developmental / regeneration process. Some observed effects in regenerating animals include: delays on the reappearance of photoreceptors and / or auricles, abnormal shape of the blastema, changes in blastema size, abnormal wound cicatrisation, malformations (e.g. variable number of photoreceptors) or complete inhibition of head regeneration followed by death (Best et al. 1981b; Villar et al. 1993; Villar et al. 1994; Calevro et al. 1998; Knakievicz and Ferreira 2008; Kustov et al. 2014; Ribeiro and Umbuzeiro 2014; Ofoegbu et al. 2016; Córdova López et al. 2019; Ofoegbu et al. 2019a). Moreover, some studies observed changes in behavioural responses of planarians regenerated in the presence of contaminants, indicating potential developmental or behavioural teratogenicity (Best and Morita 1991; Hagstrom et al. 2015). These studies indicate that alterations to the normal regeneration process are important effects to evaluate in ecotoxicological studies.

2.1.1.3 Effects on Behaviour

The most studied behavioural alterations in ecotoxicological studies with planarians have been locomotion / motility effects. Following a neuropharmacology work by Raffa et al (2001), in which authors showed a simple but sensitive method to evaluate planarian locomotor velocity (pLMV), many have used this assay, or a variation of it, to evaluate the effects of contaminants on the locomotion of planarians (e. g. Knakievicz and Ferreira 2008; Zhang et al. 2010; Plusquin et al. 2012; Zhang et al. 2012; Alonso and Camargo 2015; Rodrigues et al. 2016; Ofoegbu et al. 2016). This simple method consists of using a millimetric sheet with lines every 0.5 cm and counting the number of gridlines crossed by individual planarians over several minutes. Since similarly sized planarians (1.0 – 2.6 cm) show a stable locomotor behaviour over the evaluated period, the locomotor velocity can be calculated for individual planarians (Raffa et al. 2001). More recently, some works have used automated video-tracking systems to evaluate locomotor behaviour in planarians (Rodrigues et al. 2016; Saraiva et al. 2018; Zhang et al. 2018b), thus eliminating some drawbacks over manual scoring, such as time-consumption or observer bias. Locomotion can also be evaluated in several time-points throughout the exposure period on the same animals, providing the possibility to track the evolution of locomotor responses and potential recovery. Most studies have reported decreases in locomotor velocity of planarians exposed to environmental contaminants, such as organotins, pesticides or metals (Knakievicz and Ferreira 2008; Rodrigues et al. 2016; Ofoegbu et al. 2016), but some psychiatric drugs were shown to increase planarian locomotor velocity (Ofoegbu et al. 2019b). Evaluating planarian locomotion in response to contaminants may be a very sensitive endpoint when compared to mortality or regeneration and provides relevant information on the health status, as well as on potential adverse enduring effects of contaminants.

Some other behavioural assays have been used to a lesser extent in planarian studies. Righting / flip-over response is an example. It is a simple assay that evaluates the time needed for a planarian to return to the “normal” position, after being placed with its ventral side up by the experimenter (Kouyoumjian and Uglow 1974; Best and Morita 1991). Contaminant-exposed planarians usually take more time to flip-over than control animals, indicating a potential neurotoxic effect (Kouyoumjian and Uglow 1974). Feeding activity / feeding inhibition / prey capture has also been previously used to assess contaminant effects, evaluating the ability of planarians to capture and ingest live prey such as adult Daphnids over a 30 min period (Best and Morita 1991) or *Chironomus riparius* larvae over 12 or 24-h periods (Rodrigues et al. 2016; Ofoegbu et al. 2016; Córdova López et al. 2019). These studies have observed decreases in the feeding activity / feeding rate of planarians exposed to contaminants.

Additionally, there is potential to develop and refine simple protocols that evaluate planarian behaviour in response to contaminants, similarly as performed in the study by Inoue et al. (2015) to evaluate chemotaxis, thigmotaxis, thermotaxis and phototaxis. In recent years, some authors have been proposing the usage of high-throughput screening platforms to evaluate contaminant effects on several planarian features, including behaviour. Among behavioural parameters, these plat-

forms evaluate gliding speed and activity level, phototaxis, thermotaxis and / or scrunching behaviour (Hagstrom et al. 2015; Zhang et al. 2018b).

Planarians can display stereotypical behaviours such as “bridge-like position”, “walnut position”, “snake-like” and “screw-like” movements, which have been described in neuropharmacological studies as a result of stimulation / blockade of specific receptors in the planarian nervous system (see review by Buttarelli et al. 2008). Some works dealing on the effects of environmental contaminants in planarians have described such stereotypical behaviours (Villar et al. 1993; Horvat et al. 2005; Ofoegbu et al. 2016), which might indicate effects on their nervous system. Moreover, a scoring system was proposed by Grebe and Schaeffer (1991b) to characterize behavioural responses elicited by contaminants, encompassing 18 possible response types categorized in 5 response groups: locomotive, morphological, neurological, morbidity and protective. This scoring system has since been used in several planarian studies (Grebe and Schaeffer 1991a; Kapu and Schaeffer 1991; Villar et al. 1993), and some have applied it with modifications (Wu et al. 2014).

There is a growing interest in evaluating planarian behaviours for the screening of environmental contaminants, since they can be easily evaluated, can be more sensitive than mortality by orders of magnitude and might provide clues on the underlying mechanisms of action of contaminants. Moreover, the non-invasiveness of the assays allows the further usage of animals in different time periods, thus reducing the number of animals for testing.

2.1.1.4 Effects on Reproduction

As planarians possess both sexual and asexual reproductive modes, it is possible to evaluate the effects of contaminants on both fission and sexual reproduction, depending on the chosen species / populations. Reproduction is a key aspect that should be evaluated in response to chronic environmental contaminant stress, since it is fundamental for the survival of populations (Breitholtz et al. 2006). Most toxicological studies that evaluate effects on planarian reproduction, have focused on fission. Effects on the fission process can be simply evaluated as the percentage of individuals that have undergone fission (Best et al. 1981b; Best and Morita 1991), or the number of fission fragments produced over a given time period (Ofoegbu et al. 2019b; Ofoegbu et al. 2019a). Hence, planarians of appropriate size and condition should be chosen, i.e. animals large enough to undergo fission and with “tails tapering to a point” as an indication of not recently having undergone fission (Best et al. 1981a; Best and Morita 1991). Moreover, since animals selected for these experiments are “fission ready”, the time frame needed can be relatively short, with exposure periods ranging from 9 (Ofoegbu et al. 2019b; Ofoegbu et al. 2019a), 10 (Best et al. 1981a; Best and Morita 1991), 12 (Best et al. 1981b) to 24 days (Kouyoumjian and Uglow 1974). Still, longer exposure periods can also be employed (7 weeks; Nelson et al. 1994), depending on the specific objectives of the work. Moreover, as fission rate also depends on population density (Newmark and Sánchez Alvarado 2002), it is possible to manipulate this test parameter in order to evaluate if fis-

sion-control mechanisms might be altered by contaminants (see Best et al. 1981b). The above-mentioned studies have observed no effects, delays or even complete inhibition of fission behaviour in response to contaminants. Moreover, a study found that chlordane produced a biphasic response, increasing fission at low and inhibiting it at high concentrations (Best et al. 1981a). As the occurrence of fission depends on several aspects of planarian physiology, such as the ability of neoblasts to successfully divide and differentiate, or the production of neuroactive substances by the nervous system and successful transmission to the correct cellular targets (Reuter and Kreshchenko 2004), it is not surprising that many contaminants can impair it. The sensitivity of fission when compared to mortality (Kouyoumjian and Uglow 1974), and its potential disruption by neuroactive compounds such as pharmaceuticals (Ofoegbu et al. 2019b; Ofoegbu et al. 2019a), makes this an important and relevant parameter when evaluating effects of environmental contaminants, even more considering that disruption of fission might have implications for the maintenance of natural populations.

A few studies have evaluated contaminant effects on sexual reproduction of planarians and evidenced that it can be sensitive to contaminant stress (Indeherberg et al. 1999a; Indeherberg et al. 1999b; Knakievicz and Ferreira 2008; Ribeiro and Umbuzeiro 2014; Córdova López et al. 2019). Sexual reproduction can be evaluated by using sexually mature specimens with cocoon-laying abilities and exposing them to contaminants in groups (Knakievicz and Ferreira 2008; Ribeiro and Umbuzeiro 2014; Córdova López et al. 2019) or as isolated individuals (Indeherberg et al. 1999b) for several weeks. Isolated individuals can be evaluated in this way, since sperm can be stored from previous mating events and used to continue cocoon production (Pongratz and Michiels 2003). Parameters such as fecundity (number of cocoons) and fertility (number of newborns) have been shown to be partially or even totally inhibited by metals, dyes and herbicides (Indeherberg et al. 1999a; Indeherberg et al. 1999b; Knakievicz and Ferreira 2008; Ribeiro and Umbuzeiro 2014; Córdova López et al. 2019). Regarding cocoons, there is little information on their specific sensitivity towards contaminant exposure, although Best & Morita (1991) have reported lower hatching rate and smaller progeny from cocoons incubated in ethanol-containing media (as high as 0.1%). It is unclear if cocoons can be useful to evaluate contaminant stress, with this subject meriting further research, given the implications for natural planarian populations. Moreover, other parameters can also be important for ecotoxicity testing, including size of newborns, number of days until cocoon hatching, or newborn condition / malformations. The evaluation of sexual organ formation and sexual reproduction in planarians could also provide further information on reproductive effects of contaminants in freshwater invertebrates. Still, as remarked by Wu and Li (2018), there is a need for more information regarding the regulation of sexual reproduction in planarians to improve interpretation of results.

2.1.1.5 Effects on Biochemical markers

Biochemical biomarkers have been successfully applied to uncover aspects of contaminant effects on planarians. The homogenization of planarian tissues for biomarker assessment is easily performed, since they do not possess hardened structures. Most planarian works studying biochemical effects of contaminants have evaluated neurotoxicity, oxidative stress or, in the case of metals, metallothionein concentration (Villar et al. 1994; Wu et al. 2011; Plusquin et al. 2012; Wu et al. 2012; Zhang et al. 2014). Biochemical studies using planarians may provide important information on the sub-organismal effects of environmental contaminants, providing links to higher levels of biological organization. Additionally, there is already available information on planarian biochemistry, as several enzymes of phase I and II of xenobiotic metabolism have been detected and measured (Li 2016) and several enzymes usually employed in biochemical biomarker studies have been characterized (e.g. Phenoloxidase - Pang et al. 2010; Cholinesterase - Hagstrom et al. 2017; Catalase - Zhang et al. 2018a).

2.1.1.6 Genotoxic and tumorigenic effects

The genotoxicity of chemicals, using analysis of chromosome aberrations, as well as the micronucleus and comet assays, have also been successfully applied in planarians, detecting deleterious effects of environmental contaminants. Evaluated contaminants include mercuric chloride (Kalafatić et al. 2004b), norflurazon (Horvat et al. 2005), copper (Knakievicz and Ferreira 2008), cadmium and zinc (García-Medina et al. 2013), tributyltin (Ofoegbu et al. 2016), carbamazepine and fluoxetine (Ofoegbu et al. 2019b) as well as environmental water samples (Prá et al. 2005). Moreover, some studies have proposed planarians as potential organisms to evaluate the tumorigenic potential of contaminants. This was based on observations that exposure to known carcinogenic chemicals led to the formation of abnormal growths (neoplasias), with features resembling mammalian tumours (Foster 1963). More recently, planarians have been proposed as *in vivo models* to discriminate between genotoxic and non-genotoxic carcinogenic compounds, with the authors indicating its advantages as “non-vertebrate, fast, sensitive and low-cost procedure” (Stevens et al. 2017).

2.1.1.7 Effects on Degrowth, cellular dynamics and histology

Degrowth in planarians can also be used to evaluate the effects of contaminant exposure. This is simply based on the principle that a planarian will degrow when starved (Felix et al. 2018), but the degrowth will be more pronounced if contaminant stress is added (see Indeherberg et al. 1999b). Even if fed, planarians might degrow under chemical stress (see Plusquin et al. 2012). Some works have used body size / area changes to evaluate metal stress (Indeherberg et al. 1999b; Plusquin et al. 2012), although, as pointed by Indeherberg et al. (1999b), contraction and immobilization of animals as a consequence of chemical stress, might preclude the accurate measument of animals. Still, the evaluation of degrowth can be simple and useful, especially in response to contaminants

that do not induce behavioral anomalies. Experimental setups to evaluate degrowth have the advantage of assessing contaminant effects over longer periods without some confounding effects, such as adsorption of contaminants to food particles, and offer a possible way to disentangle effects of contaminants on metabolic costs (i.e.,increased metabolism and consequent reduction of enery reserves and / or reduced food intake).

Histological preparations allow for the detection of cellular damage, changes in size of cells, changes in structure of cellular layers, or deposition of contaminants in tissues (Franjević et al. 2000a; Franjević et al. 2000b; Horvat et al. 2005; Kovačević et al. 2009; Plusquin et al. 2012). Moreover, as the only dividing cells in a planarian, neoblasts can be monitored and mitotic rate can be evaluated (Kalafatić et al. 2004a; Plusquin et al. 2012). This approach can help explain some aspects of contaminant effects and standard protocols can be easily found for the preparation of planarian samples (e.g. Stevenson and Beane 2010; Brubacher et al. 2014).

2.1.2 Planarian sensitivity to contaminants

Most studies have focused on the effects of metals and pesticides in planarians and, more recently, on effects of several emerging contaminants. Some of the toxicological studies using planarians are detailed in Table I-1. Other studies have summarized the lethal concentration ranges for these compounds in the available planarian studies (see Knakievicz 2014; Wu and Li 2018). The study by Wu and Li (2018) performed species sensitivity distribution (SSD) models using data from the literature to compare the sensitivities of planarians towards environmental pollutants with other freshwater invertebrates, including model organisms such as *Daphnia*. They concluded that, generally, planarian sensitivity towards environmental pollutants fall within the range of sensitivities when compared to the most commonly used freshwater invertebrates in ecotoxicology. Moreover, their relative sensitivity varies with the contaminant. For instance, it was observed that *D. japonica* seems to be quite tolerant to metals (cadmium, copper and zinc) when compared to many freshwater invertebrates (including the planarian *G. tigrina*), which seemed to be related to low uptake rates of these compounds (Wu and Li 2017). On the other hand, the sensitivity of *S. mediterranea* to tributyltin (TBT) (96 h LC_{50} of 1.31 µg L^{-1}) (Ofoegbu et al. 2016) seems to be in the same range as for the most sensitive freshwater species (96 h LC_{50} of 1.14 µg L^{-1} for *Hydra oligactis;* EPA 2003). Moreover, given planarian's nervous system similarities with the vertebrate brain, pharmaceuticals may elicit more effects upon freshwater planarians than to other commonly tested invertebrate species, as hinted by a study evidencing a high sensitivity of *S. mediterranea* to carbamazepine and fluoxetine (Ofoegbu et al. 2019b).

Regarding metal exposure, there seems to be some degree of variation in the sensitivity towards the different metals, with planarians seeming especially sensitive to mercury or tin (Best et al. 1981b; Ofoegbu et al. 2016). Moreover, a variety of responses have been observed in freshwater planarians exposed to metals, ranging from genotoxicity, changes in biochemical biomarker levels

(oxidative stress or increased metallothionein levels), behavioural defects (such as stereotypical behaviours), changes in morphology, depigmentation, regeneration delays or failure, locomotion / mobility changes, decreased fission, decreased fecundity and fertility, and mortality. As expected, mortality was the least sensitive effect detected in planarians, with regeneration (e.g. Best et al. 1981b; Ofoegbu et al. 2016), locomotion (e.g. Plusquin et al. 2012; Zhang et al. 2015; Ofoegbu et al. 2016) or biochemical biomarkers (e.g. Plusquin et al. 2012; Wu et al. 2012) being responsive at much lower levels of contamination (some at concentrations ≈150 times lower; see Table I-1).

Pesticide effects on planarians range from genotoxicity, neurotoxicity (measured as decreases in acetylcholinesterase activity), behavioural defects (such as stereotypical behaviours), disintegration of tissues and acephaly, depigmentation, regeneration delays or failure, impaired locomotion, increased righting time, feeding inhibition, decreased fission rate and mortality (Kouyoumjian and Uglow 1974; Best et al. 1981a; Hill et al. 1992; Villar et al. 1993; Villar et al. 1994; Horvat et al. 2005; Rodrigues et al. 2016; Saraiva et al. 2018; Córdova López et al. 2019). Regarding toxicity differences between pesticide classes, planarians seem to have a greater sensitivity towards insecticides (see Wu and Li 2018), with several organophosphorus insecticides causing severe inhibition of acetylcholinesterase (AChE) activity in exposed planarians (Villar et al. 1994). Moreover, a few works evidence the sensitivity of sublethal effects when evaluating toxicity of pesticides, with regeneration, behaviour, reproduction, or DNA damage (Horvat et al. 2005; Saraiva et al. 2018; Córdova López et al. 2019) detecting deleterious effects at much lower concentrations than lethality (some at concentrations ≈100 times lower; see Table I-1).

Other freshwater contaminants, such as UV filters, surfactants, paraben preservatives or natural and synthetic hormones have been tested in planarians (Li 2012a; Li 2012b; Li 2013). The works dealing with benzophenone-type UV filters plus paraben preservatives, or natural and synthetic sex hormones, focused on generating lethality values for *D. japonica* (Li 2012a; Li 2013), while for natural and synthetic surfactants lethality, mobility and cholinesterase and ATPase activities were also evaluated (Li 2012b). Moreover, other chemicals, such as solvents, like dimethyl sulfoxide (DMSO) and phenol have been shown to cause lethal and / or sublethal effects on planarians (Grebe and Schaeffer 1991b; Pagán et al. 2006). The latter study recommends that DMSO should not be used above 0.1% in planarian studies. The synthetic dye disperse red 1 was found to cause delays in regeneration, behavioural anomalies, increased mucus production and fecundity decreases (Ribeiro and Umbuzeiro 2014). Recently, studies have reported the effects of low concentrations of psychiatric drugs on planarians, indicating that carbamazepine increased planarian locomotor activity, while fluoxetine increased locomotor activity, decreased feeding activity, induced DNA damage and inhibited fission rate (Ofoegbu et al. 2019b). Moreover, the combined effects of NaCl and fluoxetine exposure revealed that increased salinity levels might enhance deleterious effects of these pharmaceuticals on freshwater planarians (Ofoegbu et al. 2019a).

Table I-1 – Some toxicological studies with planarians, evidencing 96 h lethal concentrations (LC_{50}). Exposure time and lowest observed effect concentrations (LOECs) for sub-lethal endpoints are also detailed.

Chemical	Mortality 96 h LC_{50}	Other effects	Sub-lethal endpoints - LOEC	Exposure time	Species	Reference
Metals						
Aluminium	(5d) 1100 mg L^{-1}	Abnormal behaviours, morphological alterations	≥ 200 mg L^{-1}	5 d	*Polycelis felina*	(Kovačević et al. 2009)
Cadmium	1.17 ± 0.13 mg L^{-1}			4 d	*Dugesia japonica* newborns	(Zhang et al. 2010)
Cadmium	1.45 ± 0.14 mg L^{-1}	Locomotion	1.37 ± 0.08 mg L^{-1}	4 d	*D. japonica* adults	(Zhang et al. 2010)
Cadmium	(3d) 8.45 mg L^{-1}	Accumulation; body size; neoblast proliferation; gene expression	281.03 µg L^{-1}	3 w	*Schmidtea mediterranea*	(Plusquin et al. 2012)
Cadmium	(2d) 1.25 mg L^{-1}	Accumulation in the head, body and intact animals; biochemical biomarkers	0.625 mg L^{-1}	7 d	*Dugesia japonica*	(Wu et al. 2011)
Copper	1.23 mg L^{-1}	Gene expression; biochemical biomarkers	40 µg L^{-1}	1 d	*Girardia schubarti*	(Guecheva et al. 2003)
Copper	0.12 ± 0.02 mg L^{-1}	Micronucleus assay; Locomotion	0.10 mg L^{-1}	4 d	*Girardia tigrina* newborn	(Knakievicz and Ferreira 2008)

Copper	0.42 ± 0.08 mg L^{-1}	Accumulation; micronucleus assay; Loco-motion; sexual reproduction.	0.05 mg L^{-1}	Up to 5w	*Girardia tigrina* adult	(Knakievicz and Ferreira 2008)
Copper	0.48 ± 0.13 mg L^{-1}	Regeneration	0.11 mg L^{-1}	1 - 5w	*Girardia tigrina* Regenerat-ing adults	(Knakievicz and Ferreira 2008)
Potassium dichromate	12.9 ± 2.8 mg L^{-1}				*Girardia tigrina* newborns	(Preza and Smith 2001)
Copper	4.23 mg L^{-1}	Biochemical biomarkers	50 µg L^{-1}	1 - 15 days	*Dugesia japonica*	(Zhang et al. 2014)
Mercury	(5d) 0.16 – 0.40 mg L^{-1}	Head disinte-gration; Re-generation; Fission	79.89 µg L^{-1}	Up to 12 d	*Girardia dorotoceph-ala*	(Best et al. 1981b)
Lead	13.80 mg L^{-1}	Locomotion; Feeding	0.5 mg L^{-1}	4 d	*Dugesia japonica*	(Zhang et al. 2015)
Pesticides						
Chlorantranil iprole		Locomotion; Feeding	26.2 µg L^{-1}	4 d	*Dugesia subtentacu-lata*	(Rodrigues et al. 2016)
Chlordane	(5d) 5 - 10 mg L^{-1}	Fission; Head Dissolution	0.2 - 1 mg L^{-1}	13 d	*Girardia dorotoceph-ala*	(Best et al. 1981a)
Chlorpyrifos	(7d) 4.3 mg L^{-1}	Regeneration; Abnormal behaviours;		7 d	*Girardia dorotoceph-ala*	(Villar et al. 1993)
Cyprocona-zole	(2d) 47.38 mg L^{-1}	Regeneration; Locomotion; Feeding	0.45 mg L^{-1}	8 d	*Girardia tigrina*	(Saraiva et al 2018)

Diazinon	0.63 ± 0.2 mg L^{-1}	Regeneration; Fission; Biochemical biomarkers		15 d	*Girardia tigrina*	(Villar et al. 1994)
Fenitrothion	2.9 ± 0.1 mg L^{-1}	Regeneration; Fission; Biochemical biomarkers		15 d	*Girardia tigrina*	(Villar et al. 1994)
Malathion	(7d) 13.1 mg L^{-1}	Regeneration; Abnormal behaviours;		7 d	*Girardia dorotocephala*	(Villar et al. 1993)
Malathion	4.4 ± 0.8 mg L^{-1}	Regeneration; Fission; Biochemical biomarkers		15 d	*Girardia tigrina*	(Villar et al. 1994)
Methyl parathion	(7d) 4.2 mg L^{-1}	Regeneration; Abnormal behaviours;		7 d	*Girardia dorotocephala*	(Villar et al. 1993)
Methyl parathion	4.1 ± 0.2 mg L^{-1}	Regeneration; Fission; Biochemical biomarkers		15 d	*Girardia tigrina*	(Villar et al. 1994)
p-Nitrophenol	(7d) 10.2 mg L^{-1}	Regeneration; Abnormal behaviours;		7 d	*Girardia dorotocephala*	(Villar et al. 1993)
p-Nitrophenol	12.1 ± 2.1 mg L^{-1}	Regeneration; Fission; Biochemical biomarkers		15 d	*Girardia tigrina*	(Villar et al. 1994)
Roundup	(2d) 35.94 mg L^{-1}	Regeneration; Locomotion; Feeding; Reproduction	1.87 mg L^{-1}	Up to5 w	*Girardia tigrina*	(Córdova López et al. 2019)

TBT	1.31 µg L^{-1}	Regeneration; DNA damage; Locomotion; Feeding	8 ng L^{-1}	4 d	*Schmidtea mediterra-nea*	(Ofoegbu et al. 2016)
Other Chemicals						
Carbamaze-pine	> 10 mg L^{-1}	Regeneration; DNA damage; Locomotion; Feeding	0.1 µg L^{-1}	Up to 9 d	*Schmidtea mediterra-nea*	(Ofoegbu et al. 2019)
Disperse red 1	75 mg L^{-1}				*Girardia tigrina* newborns	(Ribeiro and Umbuzeiro 2014)
Disperse red 1	152 mg L^{-1}	Regeneration; Sexual repro-duction	1 mg L^{-1}	Up to 5 w	*Girardia tigrina adults*	(Ribeiro and Umbuzeiro 2014)
Fluoxetine	160.01 µg L^{-1}	Regeneration; DNA damage; Locomotion; Feeding	0.1 µg L^{-1}	Up to 9 d	*Schmidtea mediterra-nea*	(Ofoegbu et al. 2019)

Environmental stress can also affect freshwater planarians (see review by Cao et al. 2020). Elevated concentrations of inorganic nitrogen compounds such as ammonia and nitrite, have been shown to cause mortality and / or locomotion impairments in freshwater planarians (Alonso and Camargo 2008; Alonso and Camargo 2011). The effects of altering water quality parameters, such as hardness, dissolved oxygen, temperature, salinity and pH have also been studied in planarians (Rivera and Perich 1994; Knakievicz et al. 2006). Species-specific differences exist on the tolerance to varying levels of water hardness, temperature or salinity, however dissolved oxygen levels seem to be a critical factor for the survival of these animals (Rivera and Perich 1994). Other factors, such as magnetic fields seem to influence the planarian regeneration process (Jenrow et al. 1996).

2.1.3 *Girardia tigrina* (*Dugesia tigrina*) as a model organism in ecotoxicology

Girardia tigrina (Girard, 1850) is a freshwater planarian belonging to the Dugesiidae Family. It is a common species, native of the American continent, that was introduced in Europe around 1920

(Tachet et al. 2006). Since then, it has been reported in several European countries, Japan, Australia and South America (Vila-Farré et al. 2011). External appearance is characterized by a triangular head and brown coloration. Two main colour patterns can be distinguished, spotted or with a light-coloured stripe along the midline (Kenk 1944). Natural populations of *G. tigrina* can reproduce either through fission, by sexual reproduction, or can alternate reproductive modes according to season (Kenk 1944; Knakievicz et al. 2006; Vila-Farré et al. 2011).

G. tigrina is a relatively well studied planarian that has been used in (eco)toxicological studies. This species has been shown to be sensitive to metals and pesticides and it seems that *G. tigrina* has similar or slightly higher sensitivity to some contaminants (Knakievicz and Ferreira 2008; Zhang et al. 2014; Van Huizen et al. 2017) when compared to other commonly used planarian species. Moreover, newborns of this species have been proposed for toxicity testing, given the higher sensitivity to environmental contaminants in comparison with adults (Preza and Smith 2001). It is also one of the two planarian species for which chemical tumorigenicity has been demonstrated (Wu and Li 2018). Its wide geographical distribution and tolerance to changes in water quality parameters (such as pH, temperature or hardness) (Rivera and Perich 1994; Vila-Farré et al. 2011), makes this a suitable species for testing in a variety of environmental conditions.

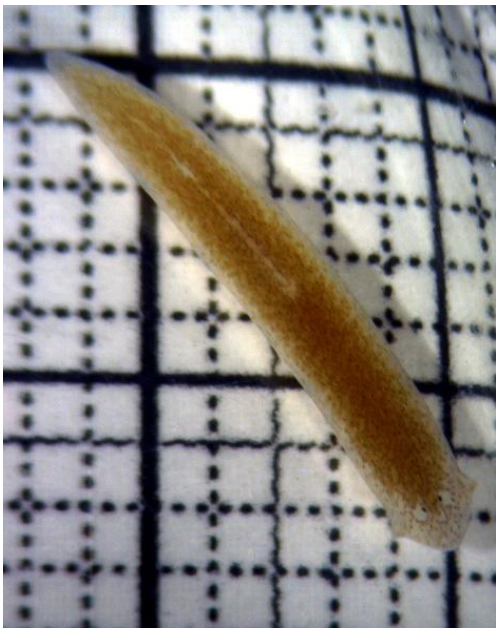


Figure I-2 – *Girardia tigrina*, a freshwater planarian.

3 PAHs

Polycyclic aromatic hydrocarbons (PAHs) have long been identified as chemicals of relevance and disease-causing agents in humans and other organisms (EPA 1987). Some are considered persistent organic pollutants (POPs), because they can remain in the environment and organism's tissues for long periods of time, are toxic, and can be transported over long distances (Nadal et al.

2015). Because PAHs are continually produced by a variety of natural and anthropogenic sources and can be transported over long distances, they are virtually ubiquitous. This is extremely concerning, given the difficulties in limiting emissions for compounds that are produced and released to the environment by such a variety of sources (Burgess et al. 2003; Meador 2008). Despite the similarities in physicochemical properties, the differences between individual PAHs influence their distribution in the environment, and the effects they have upon organisms (Logan 2007). Added to this, these compounds occur as complex mixtures and may be released in combination with non-aromatic hydrocarbons or nonhydrocarbons (Albers 2003). These issues make risk assessment of PAHs extremely difficult (Logan 2007). Given the toxicity of PAHs, they have been identified as priority substances by U.S.A., Canadian, Australian and European Union environmental agencies (CEPA 1999; EPA 1987; NPI 2011; European Commission 2015). The 3, 4 and 5-ringed PAHs, phenanthrene, pyrene and benzo[a]pyrene, respectively, were chosen as model compounds for the present work, given their frequency in freshwater sediments in PAH-contaminated areas. Moreover, despite the studies evaluating PAH effects upon aquatic invertebrates, there is almost no information on their effects in animals of the Platyhelminthes phylum. The following sections will comprise an overview of PAH features, with special emphasis on aquatic environments.

3.1 **Physicochemical properties**

Polycyclic aromatic hydrocarbons (PAHs) or polyarenes are chemicals composed of carbon and hydrogen atoms, having 2 or more fused benzene rings (Harvey 1998; Baird et al. 2005; Haritash and Kaushik 2009). PAHs can further be classified as alternants, constituted only by fused aromatic rings, and non-alternants, compounds with non-aromatic rings attached to benzene rings (for instance fluoranthene) (Harvey 1998). Furthermore, compounds with nitrogen, oxygen or sulphur in the aromatic ring are termed heterocyclic (as opposed to homocyclic) and are included in the broader definition of polycyclic aromatic compounds (PACs) that includes all the afore-mentioned chemicals. Thousands of compounds are included in the PAC category, including alkylated forms (Harvey 1998; Burgess et al. 2003; Albers 2003; Meador 2008). Compounds that are unsubstituted and without alkyl groups are termed parent PAH (Meador 2008). Depending on the number of aromatic rings, PAHs can be also categorized as low molecular weight (LMW; 2 - 3) and high molecular weight (HMW; ≥ 4) PAHs (Alegbeleye et al. 2017).

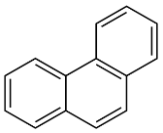
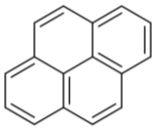
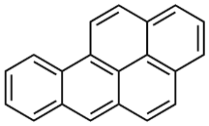
Common features of PAHs include low vapor pressure, low water solubility and high melting and boiling points, being white, yellow or colourless solids at room temperature. Water solubility usually deceases with increasing mass (Douben 2003; Haritash and Kaushik 2009). Due to their aromatic structures, PAHs can absorb light in the UV-visible region and emit light of longer wavelength. This fluorescence is characteristic for each PAH structure and can be used to identify compounds (Yu 2002; Beyer et al. 2010). Some of the chemical properties of phenanthrene, pyrene and benzo[a]pyrene are listed in Table I-2.

3.2 **PAHs synthesis and sources**

PAHs can be grouped in several categories depending on how they were formed: pyrogenic, petrogenic, diagenetic and biological (Burgess et al. 2003; Albers 2003; Abdel-Shafy and Mansour 2016). Pyrogenic PAHs are formed through incomplete combustion of organic matter at high temperatures, leading to thermal decomposition (pyrolysis) of molecules, followed by the thermal assemblage of the formed radicals (pyrosinthesis) (Hoffman et al. 2002; Burgess et al. 2003; Albers 2003; Haritash and Kaushik 2009). Petrogenic PAHs are formed when organic matter in sediments is subjected to relatively low temperatures (100 – 300 °C) over geological time scales, being part of the complex chemical mixtures that we know as fossil fuels (Burgess et al. 2003; Albers 2003). Diagenetic PAHs are formed from biogenic precursors, after deposition in sediments over relatively short-time periods, as compared to petrogenic PAHs (Meyers and Ishiwatari 1993). Biogenic PAHs are formed by organisms, such as plants, fungi or bacteria (Burgess et al. 2003; Albers 2003). Generally, the processes that give rise to PAHs, do not produce them as individual compounds, but as complex mixtures of possibly thousands of different substances, as in the case of petroleum, containing PACs and other compound classes (Burgess et al. 2003). Depending on the processes by which they were formed, PAH mixtures possess distinct composition, being possible to identify the source by its PAH “fingerprint” (Hylland 2006; Tobiszewski and Namieśnik 2012; Stogiannidis et al. 2015). While petroleum sources are mainly enriched in 2 – 3 ringed PAHs with a high degree of alkylated forms, pyrogenic sources produce many 4 – 6 ringed and unsubstituted PAHs (Lee and Anderson 2005; Anderson and Lee 2006; Pampanin and Sydnes 2013).

Sources of PAHs include both naturally occurring and anthropogenic processes (Albers 2003; Haritash and Kaushik 2009). Natural sources of PAHs include forest fires, volcanoes, oil seeps and production by living organisms (plants, fungi or bacteria), while anthropogenic sources include burning of oil and wood, production of coke and charcoal, internal combustion engines, petroleum spills, municipal waste burning or asphalt (Albers 2003; Haritash and Kaushik 2009; Ball and Truskewycz 2013; Abdel-Shafy and Mansour 2016). Some industrial processes use PAHs for the manufacturing of products, such as pigments, dyes, plastics, or agrochemicals (Liu et al. 2008; Abdel-Shafy and Mansour 2016). Despite the many possible origins, the majority of PAH releases to the environment are derived from combustion processes, although major spills of fossil fuels may release great amounts of these compounds in a short timeframe (Albers 2003; Abdel-Shafy and Mansour 2016).

Table I-2 – Chemical properties of the three selected PAHs, phenanthrene, pyrene and benzo[a]pyrene (From Latimer and Zheng 2003).

PAH	Molecular formula	Molecular weight (g mol^{-1})	Melting point (°C)	Boiling point (°C)	Solubility (mg L^{-1})	Log K_{ow*}	Structure
Phenanthrene	$C_{14}H_{10}$	178.23	101	339	1.1	4.55	
Pyrene	$C_{16}H_{10}$	202.28	156	360	0.132	4.88	
Benzo[a]pyrene	$C_{20}H_{12}$	252.3	175	495	0.0038	6.13	

***K_{ow}: octanol/water partition coefficient**

3.3 **Occurrence and distribution of PAHs in the environment**

PAHs are ubiquitous in the environment, given the multitude of anthropogenic and natural sources added to constant emissions (see section 3.2 - PAHs synthesis and sources) (Burgess et al. 2003). PAHs can enter terrestrial, marine or freshwater environments, by direct contamination from PAH sources, by atmospheric deposition or by runoff (Latimer and Zheng 2003; Albers 2003; Ball and Truskewycz 2013; Alegbeleye et al. 2017). Contamination of locations far away from PAH sources can occur, since PAHs can be transported over long distances through the air, as volatiles or associated with particles (Arey and Atkinson 2003; Nadal et al. 2015). Still, a large portion of PAHs will remain near the areas of emission, meaning that they will tend to be more concentrated in more populated and industrialized areas, or near locations that have frequent forest fires or volcanic activity (Eisler 2000). Although they occur in every environmental compartment, soils and sediments will be the sinks of PAHs, given the affinity of these compounds to bind to organic matter (Haritash and Kaushik 2009). PAHs can persist for long periods of time bound to soil or sediment particles, with studies observing half-lives of more than 100 days or even years in surface sediments, depending on the prevailing physical conditions and PAH solubility (Wilcock et al. 1996; Nikolaou et al. 2009). PAHs persist for long periods of time, because they do not degrade easily, with degradation rates decreasing and persistence increasing with number of aromatic rings (Haritash and Kaushik 2009). Among degradation mechanisms we can include photooxidation, photolysis, chemical and microbial degradation, with the latter being the major degradation pathway and performed by fungi, algae or bacteria (Hoffman et al. 2002; Hylland 2006; Haritash and Kaushik 2009).

Within the context of climate change, the distribution of PAHs has the potential to be affected, due to shifts in the environmental factors that affect and govern their distribution (Nadal et al. 2015). For instance, temperature increases can lead to enhanced volatilization of semi-volatile compounds, or even to shifts in the phase that compounds are usually found (e.g. pyrene that partitions between volatile and solid) (Macdonald et al. 2005; Nadal et al. 2015). Moreover, the higher temperatures coupled with drier conditions predicted for some regions, will enhance the probability of wildfires, potentially leading to increased PAH emissions and risk for nearby waterbodies (Macdonald et al. 2005; Woodward et al. 2016). Additionally, severe storm events, might enhance the runoff of contaminants from land to waterbodies, and can potentially lead to resuspension of contaminants from sediments (Schiedek et al. 2007; Nadal et al. 2015).

3.3.1 PAHs in the aquatic environment

PAHs can end up in the aquatic environment through atmospheric deposition, oil spills, wastewater effluents, urban runoff or ash runoff in recently burned areas (Hoffman et al. 2002; Meador 2008; Ball and Truskewycz 2013; Alegbeleye et al. 2017). Despite being ubiquitous substances, high environmental levels are generally related to the proximity of PAH sources (Alegbeleye et al. 2017). For instance, oil spills can lead to high environmental concentrations in surface waters, as observed after the Gulf War, along the Kuwait coast, where total PAHs were estimated as 21.14 to 320.5 µg/L on surface waters (Bu-Olayan et al. 1998). A recent review has summarized the levels of POPs on China's surface waters, and found that the PAH levels in the dissolved phase ranged from 0.5 to 474000 ng/L, with most analysed rivers having maximum PAHs levels over 10000 ng/L. The high levels found in many of these water courses were mainly found near highly populated and industrialized areas, leading to the high pollution levels (Han and Currell 2017). Concentration of individual PAHs in surface waters with low pollution levels, generally do not exceed 50 ng/L, with concentrations above this indicating nearby PAH-pollution sources (WHO 2003). Nevertheless, concentrations in surface waters can be quite variable, since PAHs in the water column have the tendency to bind to particulate matter and settle onto sediments (Latimer and Zheng 2003; Alegbeleye et al. 2017). It is in sediments that the highest PAH concentrations can be found, with lakes around the world containing from 0.159 to 33090 ng g^{-1} of total PAHs (Viguri et al. 2002; Barakat et al. 2011; Du and Jing 2018).

3.3.1.1 PAHs effects in aquatic organisms

Once in the aquatic environment, PAHs will accumulate in organisms or adsorb to the organic matter in sediments, with a small portion remaining in the water column (Alegbeleye et al. 2017). Sediments will act as a sink for PAHs, but can also act as a source for overlying water and organisms (Beasley and Kneale 2002). The fraction that will be available for organismal uptake will depend on factors such as hydrophobicity / solubility of PAHs, grain size and amount of organic matter on

sediments, and partitioning among different phases (Altenburger et al. 2003; Thorsen et al. 2004; Meador 2008; Alegbeleye et al. 2017). Some variability also exists regarding bioavailability of pyrogenic versus petrogenic PAHs, since the former are frequently strongly associated with particles and therefore, may not be so readily bioavailable (Hylland 2006; Menzie and Coleman 2007). Other features influencing the bioavailability of PAHs include environmental transformation (e.g. microorganism degradation) the life-style and functional feeding mode of the organism, i.e. benthic detritivores and predators will be more prone to PAHs accumulation (Kaag et al. 1997; Beasley and Kneale 2002; Altenburger et al. 2003; Meador 2008; Alegbeleye et al. 2017). The exposure of aquatic animals to PAHs will thus mainly be through contact with contaminated sediment, body surfaces, such as skin or respiratory structures and ingestion of contaminated food particles (Hoffman et al. 2002; Meador 2008).

After uptake from the surrounding environment, the fate of PAHs in organisms will depend on the partitioning between tissues, metabolism and elimination (Altenburger et al. 2003; Meador 2008). In vertebrates, PAH molecules are known to enhance the activity of xenobiotic metabolism by binding to aril hydrocarbon receptors (AHR), which leads to the transcription of genes encoding cytochrome P-450 enzymes (van der Oost et al. 2003; Shimada 2006; Ball and Truskewycz 2013). These cytochrome P-450 enzymes, among others, are known to metabolize PAHs (van der Oost et al. 2003; Shimada 2006; Ball and Truskewycz 2013; Moorthy et al. 2015). The metabolization of PAHs results in more hydrophilic and reactive compounds, that may have a greater toxicity than the parent compounds (Miller and Ramos 2001; Meador 2008; Ball and Truskewycz 2013). In fact, the well-studied carcinogen benzo[a]pyrene needs to undergo metabolic activation in order for its reactive metabolites to form DNA adducts that can lead to cancer and genetic mutations (Miller and Ramos 2001; Ball and Truskewycz 2013; Moorthy et al. 2015). PAH metabolites can also form adducts with proteins and lead to the formation of reactive oxygen species (ROS) that can damage cellular components (Miller and Ramos 2001; Ball and Truskewycz 2013; Moorthy et al. 2015). Nevertheless, the hydrophilic nature of metabolites makes them also more easily and usually rapidly excreted by vertebrates (Meador 2008; Ball and Truskewycz 2013). The major PAH biotransformation organ in vertebrates is the liver, although other organs may also show metabolic activity towards PAHs (Altenburger et al. 2003; Ball and Truskewycz 2013). On the other hand, invertebrates may greatly vary on their PAH-metabolizing abilities, with some being able to extensively do so (like crustaceans), while others (like molluscs) having low metabolic activity toward PAHs (Meador 2008). However, even closely related species may have very different metabolizing abilities concerning PAHs (McElroy et al. 2000; Verrengia Guerrero et al. 2002; Rust et al. 2004; Meador 2008). It is also noteworthy that the cytochrome P-450 isoenzymes responsible for PAH metabolization in many invertebrates, seem to be different from those of vertebrates, potentially leading to formation of distinct PAH metabolites (Altenburger et al. 2003; Jørgensen et al. 2008). Moreover, many invertebrates seem to possess AHRs that do not have the same affinity for PAHs

as vertebrate AHRs, thus not being able to induce xenobiotic metabolism in the same way (Jørgensen et al. 2008; Ball and Truskewycz 2013).

The extent to which organisms are able to metabolize PAHs is related to accumulation, meaning that organisms that have low or no metabolic activity towards PAHs may accumulate them in tissues, and organisms that extensively metabolize PAHs will have lower body residue levels, due to elimination (Eisler 2000; Rust et al. 2004; Meador 2008). Since PAHs are fast and efficiently metabolized in vertebrates, accumulation generally decreases with increasing octanol / water partition coefficient (K_{ow}), contrary to what would be expected without biotransformation and elimination (Altenburger et al. 2003; van der Oost et al. 2003; Logan 2007; Ball and Truskewycz 2013). The lipid content of organisms and of individual organs also seems to play a role in the accumulation of PAHs, with lipid-rich tissues, such as brain and gametes, potentially accumulating more (Meador 2008; Xu et al. 2011; Ball and Truskewycz 2013). Nevertheless, the extent to which PAH-metabolizing organisms accumulate PAHs or their metabolites is unknown (Meador 2008). Due to the extensive metabolization, PAHs are generally regarded as not being biomagnified. In vertebrate-dominated assemblages this seems to be the case (see Wan et al. 2007). However, it is unknown if the same is also verified in communities where invertebrates are the top predators (Meador 2008). Added to this, the ability and extent to which PAH metabolites can be transferred through feeding on PAH-exposed prey is also unknown (see Carrasco Navarro et al. 2013), since very few studies have dealt with this subject. Nevertheless, even a low transfer of PAHs to top predators does not necessarily mean an absence of effects (see Rocha et al. 2012).

It is, however, worth mentioning that although different PAHs may possess similar chemical structures, their metabolic induction and biochemical interactions may be quite variable, both within and between species, leading to the variability of observable effects (Altenburger et al. 2003; Incardona et al. 2006). PAHs can cause a great variety of effects upon aquatic organisms. Mortality caused by PAHs is generally low and related to non-specific mechanisms, such as narcosis, due to accumulation in cell membranes and disruption of their functioning and integrity (Escher and Hermens 2002; Altenburger et al. 2003; Meador 2008). Mortality is mostly observed under exposure to low molecular weight and relatively soluble PAHs (Arfsten et al. 1996; Billiard et al. 2008; Meador 2008). However, acute effects may be more pronounced in organisms with low biotransformation abilities, given PAH accumulation in tissues (Meador 2008). Moreover, photoactivation of PAHs (by UV or visible light) to reactive compounds has been demonstrated (Arfsten et al. 1996; Meador 2008; Fu et al. 2012). This phenomenon can greatly increase the toxicity of (some) PAHs (Bellas et al. 2008; Meador 2008; Fu et al. 2012). However, in the aquatic environment this phenomenon might not be so relevant, except for organisms that inhabit close to the water surface (Logan 2007; Meador 2008; Fu et al. 2012). Mortality may also be induced by a compromised immune system, since PAHs have been shown to affect vertebrate and invertebrate immune systems (Reynaud and Deschaux 2006; Meador 2008; Ball and Truskewycz 2013). In wild fish exposed to PAHs, lesions

on fins, gills or skin elicited by opportunistic pathogens have been indicated as possibly resulting from compromised immune systems (Reynaud and Deschaux 2006; Logan 2007).

Some PAHs are procarcinogenic, meaning that their metabolites can induce DNA damage and form adducts with macromolecules that can lead to cancer, as observed for humans and other vertebrates (Ball and Truskewycz 2013; Moorthy et al. 2015). In fact, benzo[a]pyrene is the most well-studied PAH, given its known (pro)carcinogenic properties to humans (Miller and Ramos 2001). Development can also be affected by PAHs, with studies showing skeletal malformations, oedemas or cardiac dysfunction in fish and / or fish embryos exposed to PAHs (Logan 2007; Billiard et al. 2008; Meador 2008; Ball and Truskewycz 2013). Added to this, PAHs have also been evidenced to cause endocrine disruption, and cause reproductive toxicity in both vertebrates and invertebrates (Logan 2007; Manzetti 2012; Zhang et al. 2016; Bolden et al. 2017). Interestingly, although some evidence suggests that exposure to some PAHs, most notably benzo[a]pyrene, can lead to neurotoxic effects in humans, few studies have investigated this phenomenon (Tang et al. 2003; Wormley et al. 2004; Schroeder 2011). PAHs have also been shown to reduce growth, both in vertebrates and invertebrates, and this may be related with reduced energy budget (Payne et al. 2003; Meador 2008; Ball and Truskewycz 2013).

The behaviour of organisms can be impaired by exposure to sub-lethal concentrations of PAHs. Avoidance of contaminated sediments, changes in ventilation, altered swimming behaviour or feeding reductions have been observed in aquatic animals exposed to PAHs, among other behavioural changes (Lotufo 1997; De Lange et al. 2006; Gravato and Guilhermino 2009; Gauthier et al. 2016). Behavioural alterations may have implications in escaping from predators, energy intake or relocation to uncontaminated areas, which may compromise the survival of organisms on the long term (Payne et al. 2003; Hellou 2011).

Table I-3 – Effects of the selected PAHs, phenanthrene, pyrene and benzo[a]pyrene in aquatic organisms.

Species	PAH	Effect	Test duration	Effect concentration (µg L^{-1})	Reference
Freshwater Invertebrates					
Daphnia magna[1]	Phenanthrene	Mortality	48 h	LC_{50} - 730.67	(Verrhiest et al. 2001)
Daphnia magna[1]	Phenanthrene	Mortality	48 h	LC_{50} - 949.96 (853.7 - 1076.5)	(Xie et al. 2006)
Daphnia magna[1]	Phenanthrene	Mortality	48 h	LC_{50} – 570.3 (516.9 - 623.8)	(Feldmannová et al. 2006)
Daphnia magna[1]	Phenanthrene	Mortality	48 h	LC_{50} – 0.275 (0.199 – 0.351)	(Wu et al. 2015)
Daphnia magna[1]	Phenanthrene	Reproduction – nº of offspring	21 d	EC_{10} - 60	(Wu et al. 2015)
Daphnia pulex[1]	Phenanthrene	Reproduction – nº of offspring	16 d	LOEC: 60	(Savino and Tanabe 1989)
Daphnia magna[1]	Pyrene	Mortality	48 h	LC_{50} - 135.8	(Brausch and Smith 2009)
Daphnia magna[1]	Pyrene	Immobilization	48 h	EC_{50} - 67.9 ± 7.90	(Clément et al. 2005)
Daphnia magna[1]	Benzo[a]pyrene	Mortality	48 h	LC_{50} – 250 ± 40	(Atienzar et al. 1999)
Daphnia magna[1]	Benzo[a]pyrene	Immobilization	24 h	EC_{50} - 29.3 (13.32–242.2)	(Ha and Choi 2009)
Daphnia magna[1]	Benzo[a]pyrene	Reproduction – nº of offspring	14 d	LOEC: 25.0	(Atienzar et al. 1999)
Daphnia magna[1]	Benzo[a]pyrene	Reproduction- nº of offspring	21 d	LOEC: 0.02	(Ha and Choi 2009)
Chironomus sancticaroli[2]	Phenanthrene	Mortality	96 h	LC_{50} - 1210	(Richardi et al. 2018)
Chironomus	Phenanthrene	Mortality	96 h	LC_{50} - 1600	(Morais et al.

sancticaroli[2]				(1510 – 1655)	2014)
Chironomus plumosus	Phenanthrene	Mortality	96 h	LC_{50} - 462 (390 – 559)	(Wu et al. 2015)
Chironomus riparius[3]	Phenanthrene	Mortality	96 h	LC_{50} - 162.2 (146.2 - 181.8)	(Bleeker et al. 2002)
Chironomus riparius[3]	Pyrene	Mortality	96 h	LC_{50} - 38.43 (22.25 - 64.72)	(Bleeker et al. 2002)
Chironomus riparius[2]	Benzo[a]pyrene	Mortality	24 h	LC_{50} - 9873 (7426–14610)	(Ha and Choi 2008)
Fish					
Danio rerio[4]	Phenanthrene	Mortality	120 h	LC_{50} - 310	(Vergauwen et al. 2015)
Danio rerio[4]	Phenanthrene	Mortality	120 h	LC_{50} – 486 ± 40	(Gündel et al. 2012)
Danio rerio[4]	Phenanthrene	Reproduction – Egg production	120 d	LOEC: 5.0	(Peng et al. 2019)
Pomatoschistus microps[5]	Pyrene	Mortality	96 h	LC_{50} - 0.871	(Oliveira et al. 2012)
Danio rerio[4]	Benzo[a]pyrene	Mortality	3 dpf	LC_{50} - 1286.781	(Weigt et al. 2011)
Danio rerio[6]	Benzo[a]pyrene	Reproduction – Egg production	49 d	LOEC: 1.63	(Hoffmann and Oris 2006)
Algae					
Scenedesmus subspicatus	Phenanthrene	Growth rate	7 d	EC_{50} - 5024 (3488 – 7250)	(Djomo et al. 2004)
Scenedesmus subspicatus	Pyrene	Growth rate	7 d	EC_{50} - 18.72 (16.98–20.70)	(Djomo et al. 2004)
Scenedesmus subspicatus	Benzo[a]pyrene	Growth rate	7 d	EC_{50} - 1.48 (1.28–1.72)	(Djomo et al. 2004)

1 – Tests performed in neonates; 2 – Tests performed with 4th instar larvae; 3 – Tests performed with 1st instar larvae; 4 – Tests performed in embryos; 5 – Tests performed in juveniles; 6 – Tests performed in 2-3 months old animals.

3.3.1.2 Monitoring of PAHs effects using aquatic organisms

The monitoring of PAH effects on aquatic environments has much relied on fish species, probably given their economic and recreational relevance, as well as extensive knowledge on general aspects of their metabolism towards PAHs (Logan 2007). Nevertheless, birds, mammals and invertebrates have also been used for this purpose, although to a lesser extent. As vertebrates are known to extensively metabolize and excrete PAHs, the levels of PAH metabolites can be indicative of recent exposure (up to 1 week). Since PAH metabolites are fluorescent, techniques such as fixed fluorescence wavelength (FF) or synchronous fluorescence spectrometry (SFS), among others, can be used to detect the presence of this compounds in organisms (van der Oost et al. 2003; Logan 2007; Beyer et al. 2010). The detection of PAH metabolites in fish bile has been extensively used in many monitoring studies, with both laboratory and field studies evidencing it as a good indicator of fish exposure to PAHs from both pyrogenic and petrogenic sources (Altenburger et al. 2003; van der Oost et al. 2003; Lee and Anderson 2005). Interestingly, the measurement of PAH metabolites in crab urine can also be indicative of PAH presence and bioavailability in aquatic environments, since crustaceans are known to metabolize PAHs using cytochrome P450 enzymes (Galloway 2006; Dissanayake and Bamber 2010). On the other hand, PAH body residues can be indicative of pollution levels in species that have low metabolic activity towards these compounds, such as bivalves (Pampanin and Sydnes 2013; Kasiotis and Emmanouil 2015).

The expression of cytochrome P4501A enzymes is induced by PAHs, which in turn are involved in PAH metabolism in vertebrates. Field and laboratory studies have evaluated hepatic cytochrome P4501A protein or mRNA levels, or alternatively, their catalytic activity, by the aryl hydrocarbon hydroxylase (AHH) and ethoxyresorufin O-deethylase (EROD) assays (Sarasquete and Segner 2000; van der Oost et al. 2003). Some field studies have shown that, induction of hepatic cytochrome P4501A levels in fish occurs in areas contaminated with PAHs, (Van Veld et al. 1990; Sundt et al. 2012), although not all studies show evidence of induction (Payne et al. 1984; Gorbi and Regoli 2004). As cytochrome P4501A levels in fish might be influenced by several physiological factors, by the PAH composition (2-3 ring PAHs are not strong inducers) and by other contaminants, caution is needed when interpreting results in field studies (Altenburger et al. 2003; Lee and Anderson 2005). Moreover, although cytochrome P450 enzymes have been detected in invertebrates and both EROD and AHH activities (characteristics of cytochrome P4501A in vertebrates) have been measured in crustaceans, further studies are needed to characterize these enzymes in invertebrates, since cytochrome P4501A genes have not been identified in invertebrates (Lee and Anderson 2005; Rodrigues and Pardal 2014). The presence and levels of PAH-DNA adducts in fish liver or in mussel tissues can be indicative of the extent of PAH exposure (van der Oost et al. 2003; Skarphéðinsdóttir et al. 2007). PAH-DNA adducts can result from cumulative exposure to PAHs, being an indicator of genotoxicity that can be evaluated months to more than a year after exposure occurs (van der Oost et al. 2003; Pampanin and Sydnes 2013). Evidence suggests that PAH-DNA adducts can be a good predictor of the occurrence of neoplasia in fish and correlates well with PAH

levels in bivalve tissues (Altenburger et al. 2003; Skarphéðinsdóttir et al. 2007). Moreover, some studies use multi-biomarker approaches, in order to better evaluate and characterize the effects of environmental contaminant mixtures, which often contain PAHs (Galloway et al. 2004; Guimarães et al. 2012). Some of these include the measurement of enzymes and cofactors related with antioxidant defences, indexes of oxidative damage, neurotoxicity biomarkers, energy related biomarkers and energy reserves (Galloway et al. 2004; Anderson and Lee 2006; Guimarães et al. 2012; Rodrigues et al. 2013). Neoplasms and lesions have also been observed in animals (mainly fish and bivalves) in locations with high PAH concentrations, being found in combination with higher PAH metabolites or residues in tissues (Anderson and Lee 2006; Logan 2007). However, it may be hard to draw relations between these types of lesions to specific contaminants, especially in natural contexts (van der Oost et al. 2003).

Aquatic environments contaminated with PAHs have been documented to possess altered communities when compared to low contamination areas. Some of the observed changes include lower diversity, changed dominance patterns, loss of sensitive species or altered trophic structure (Jackson et al. 1989; Venturini and Tommasi 2004; Scoggins et al. 2007; Hallare et al. 2011). It is important to mention that attributing community-level effects to a contaminant or a contaminant class is a hard challenge in natural environments, given the plethora of variables and the presence of other xenobiotics. Still, PAHs have been identified as effect-causing toxicants in sediment samples containing several other contaminants (Brack et al. 2007), indicating that these can be responsible for toxic effects in natural environments. Ultimately, PAH contamination in freshwater systems may lead to toxic effects on aquatic organisms and potentially alter community patterns and ecosystem functioning (Albers 2003; Mehler et al. 2010).

4 Thesis objectives and outline

The present work explores the potential of freshwater planarians in the context of ecotoxicological research, using PAHs as model contaminants. The unique features of planarians (outlined in section 1) make them interesting alternative models for toxicity testing, especially considering the variety of endpoints that can be evaluated in response to contaminant stress (outlined in section 2). Furthermore, considering their epibenthic nature, planarians will likely be exposed to PAHs in natural environments, given the persistence and ubiquity of these compounds in sediments. This approach will not only be an important step for the risk assessment of PAH effects for a group of organisms for which (almost) no information exists, but will also allow for the testing of a great variety of planarian endpoints. The choice of several PAHs differing in the number of aromatic rings will further provide insights into the potential differences between each compound's effects upon a single species. With this in mind, 3 PAHs were selected as test compounds, phenanthrene, pyrene and benzo[a]pyrene, and a widespread freshwater planarian, *Girardia tigrina*, was selected as test

species. In this work, and besides this general introduction, several specific research objectives were addressed and entail the different sections / chapters of this thesis:

Development and adaptation of assays for the testing of planarian endpoints

The potential utilization of planarians for ecotoxicological research depends on the development of protocols that can effectively evaluate the deleterious effects of contaminants on several aspects of these organisms' biology. With this in mind, the planarian endpoints utilized throughout this thesis were chosen based on several reasons: common usage in ecotoxicological studies (mortality and reproduction), unique planarian features (cephalic regeneration), simple but sensitive to contaminant stress (behavioural endpoints), and ability to provide insights on potential mechanisms of action behind other observed effects (biochemical biomarkers). Along the chapters of this thesis, the protocols to evaluate the chosen ecotoxicological endpoints were adapted to *G. tigrina*. This was based on the existing planarian literature and using standardized testing conditions of ecotoxicological studies of temperate freshwaters when possible (e.g. the use of ASTM hard water medium, maintenance temperature of 20°C, etc.). More specifically, Chapter II deals with the development / adaptation of two behavioural assays using *G. tigrina*: a simple protocol to evaluate feeding rate in response to contaminant stress and a protocol to evaluate locomotion using video tracking system. To ensure that these behavioural endpoints can detect effects under differently acting stressors, their sensitivity was evaluated in response to 2 common stressors in freshwaters, inorganic mercury ($HgCl_2$) and salinity (using NaCl as surrogate).

Evaluation of PAH effects on freshwater planarians

To evaluate the responsiveness and sensitivity of several planarian endpoints, the effects elicited by the chosen PAHs were investigated. First, we assessed whole-organism level effects of phenanthrene, pyrene and benzo[a]pyrene based on mortality, disintegration of tissues, cephalic regeneration, locomotion, feeding rate and presence of PAH residues in tissues (Chapter III). To further investigate and obtain some mechanistic understanding on the effects of phenanthrene, pyrene and benzo[a]pyrene in freshwater planarians, some effects at the biochemical level were evaluated, including biomarkers of neurotoxicity, oxidative stress and damage, as well as energy reserves and consumption (Chapter IV). The PAHs with most potential to cause chronic effects, pyrene and benzo[a]pyrene, were further investigated concerning their potential long-term effects upon the planarian *G. tigrina*, while also exploring the potential of planarian reproduction-related endpoints for ecotoxicological research (Chapter V).

Potential PAH accumulation in different scenarios

Some important but frequently overlooked issues in previous planarian works concern the uptake of chemical compounds. Some evidence suggests that PAHs can accumulate in lipid-rich tissues and target organs such as the brain. Considering that accumulation of contaminants in the cephalic region has been previously observed in planarians, the possibility of pyrene and benzo[a]pyrene preferential accumulation in the head portion of planarians was investigated (Chapter VI). Moreo-

ver, almost no work has used contaminated sediments to evaluate toxicant effects upon freshwater planarians, and it is unclear if planarians may be useful for sediment ecotoxicity studies. It is also unclear if the specific feeding mode of planarians may preclude the trophic transfer of contaminants from contaminated prey. Hence, the potential transfer of B[a]P (a PAH that is usually tightly bound to organic matter) was investigated by performing planarian toxicity tests with spiked sediments and by investigating the trophic transfer of B[a]P from bottom-dwelling insect larvae to planarians, thus evaluating the potential of *G. tigrina* to be used in such types of assays (Chapter VII).

General discussion and future work

This final chapter consists on the general discussion of the obtained results and final considerations regarding the use of *G tigrina* in ecotoxicological research while some avenues for future research are also mentioned (Chapter VIII).

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Chapter II – Planarian behavioural endpoints in ecotoxicology: a case study evaluating mercury and salinity effects in *Girardia tigrina*

Planarian behavioural endpoints in ecotoxicology: a case study evaluating mercury and salinity effects in *Girardia tigrina*

Abstract

The evaluation of behavioural endpoints in response to contaminant stress has been gaining relevance for ecotoxicological research. This is because behavioural endpoints such as feeding rate or locomotion may provide important information on the health status of organisms and can potentially establish links between biochemical and physiological alterations with effects at populational levels. Freshwater planarians can be useful for the evaluation of contaminant stress on behavioural endpoints, given that many of their behaviours can be evaluated using simple protocols. Hence, in this work, we studied the sensitivity of the freshwater planarian *Girardia tigrina* in response to two model stressors (Hg and NaCl) by evaluating mortality, feeding rate and locomotion. To achieve this, a simple protocol for the feeding endpoint evaluation in *G. tigrina* was devised, and an automated tracking system was used to evaluate the locomotion endpoint. The calculated 96 h LC_{50}s were 176.8 µg L^{-1} of Hg and 6.79 g L^{-1} of NaCl. Acute effects of Hg also included the progressive disintegration of tissues starting in the cephalic region, and loss of pigments in body and photoreceptors. Acute effects of NaCl included the motionless of planarians and rupture of the tegument. Concentration-dependent effects were observed for the planarian feeding rate under both Hg and NaCl. Post-exposure locomotion showed a similar sensitivity as post-exposure feeding rate and were both more sensitive than mortality. Despite the distinct toxicity mechanisms of the studied stressors, behavioural endpoints showed similar sensitivities for Hg and NaCl. Still, in the case of Hg, the most sensitive endpoint was locomotion and for NaCl, the feeding endpoint. This study further demonstrates the usefulness of planarians for ecotoxicological research and shows that simple protocols that evaluate behavioural endpoints can provide important and relevant information on the sub-lethal impacts of stressors to freshwater invertebrates.

Keywords: Behaviour; Feeding rate; Locomotion; Planarian; Metal; NaCl.

1 Introduction

Behavioural endpoints have been gaining relevance for ecotoxicology, as these can be very sensitive to contaminants. Organismal responses such as avoidance, burrowing, feeding, locomotion or mating can be considered behavioural endpoints (Hellou, 2011). The relevance of studying behaviour in ecotoxicology is based on the principle that it may provide a link between physiological and biochemical effects and higher levels of biological organization (Amiard-Triquet, 2009; Peterson et al., 2017). This is because sub-organismal effects of xenobiotics, such as changes on neurotransmitters or hormone levels can manifest into behaviour, which in turn can affect population dynamics (Amiard-Triquet, 2009; Peterson et al., 2017). Ultimately, these effects could be translated in im-

pairments of ecosystem functions (Forrow and Maltby, 2000). Moreover, behavioural assays can be more sensitive than lethality, sometimes by several orders of magnitude, and can be faster than developmental and reproduction assays (Gerhardt, 2007; Hellou, 2011; Melvin and Wilson, 2013). Among behavioural endpoints, feeding activity has had some relevance in ecotoxicological studies, mostly because some feeding-related endpoints can be easily measured (such as feeding rate) and they are closely related to energy intake and expenditure (Melvin and Wilson, 2013). Moreover, xenobiotic exposure frequently leads to feeding impairment, reducing energy input, with potentially long-term consequences, such as reduced growth, development rates and reproduction or even leading to mortality (Allen et al., 1995; Maltby, 1999). Locomotor behaviour has also been widely used to evaluate sub-lethal impacts of xenobiotics in animals. Alterations on locomotor behaviour may be related to impacts on neurotransmitter systems, physical or metabolic condition of the organism, and have a high ecological importance, since many other behaviours depend on proper locomotion, such as feeding or escape from predators (Amiard-Triquet, 2009; Bayley, 2002). Moreover, locomotor behaviour can be relatively easy to measure using simple protocols, and can also be easily automated (Bayley, 2002; Peeters et al., 2009).

Among freshwater invertebrates, planarians are good candidates for behavioural studies, since a variety of simple behaviours can be easily evaluated. This is even more relevant, as they can be quite sensitive to a variety of pollutants (Best and Morita, 1991; Wu and Li, 2018). For instance, simple but sensitive protocols have been devised to measure differences in locomotor activity in planarians exposed to xenobiotics (Raffa et al., 2001; Rodrigues et al., 2016). In planarians, this endpoint seems to be quite sensitive in detecting effects when other classical endpoints such as mortality are not significantly affected (Alonso and Camargo, 2011; e. g. Horvat et al., 2005). Moreover, the usage of automated tracking systems makes the measurement of this endpoint easier, more reproducible and less prone to observer bias (Peeters et al., 2009; Rodrigues et al., 2016). Also, planarians are predators of other invertebrates including gastropods, oligochaetes, insects or small crustaceans (Lombardo et al., 2011; Pickavance, 1971). Their predation potential on several invertebrates reared in laboratory is well known and they have been proposed for pest control in the field (Perich et al., 1990; Perich and Boobar, 1990; Tranchida et al., 2009). Nevertheless, their feeding behaviour has seldom been used to evaluate toxicant effects, with the few works that do evaluate this endpoint, evidencing that it can be quite sensitive to xenobiotic exposure (e. g. Best and Morita, 1991; Ofoegbu, 2018; Ofoegbu et al., 2016; Rodrigues et al., 2016). Planarians are very effective predators; they detect prey through chemical cues, and once near it, they use their powerful muscles to wrap themselves around prey. Mucus is used to entangle and further immobilize the captured animals (Inoue et al., 2015; Kotpal, 2012; Thorp and Covich, 2010). Once a prey is captured, the pharynx is forced inside its body, ingesting tissues and body fluids. Digestion of food progresses in the gut lumen and inside cells of the gastrodermis (Forsthoefel et al., 2011; Jennings, 1962, 1957). Thus, chemicals that impair mucus secretion, affect muscular action and/or cilia, alter/impair chemical detection, affect secretion of proteolytic enzymes or affect the brain,

have the potential to impact feeding behaviour in planarians. Furthermore, in the case of feeding in a contaminated environment, the protrusion and sucking of food particles with the pharynx, may allow the ingestion of contaminants. This makes the evaluation of feeding in planarians a highly integrative endpoint, with potential to evaluate effects at sub-lethal levels for a wide range of chemicals with distinct modes of action.

This work investigated the effects of mercury (Hg) and sodium chloride (NaCl) on mortality and behavioural endpoints (post-exposure locomotion and feeding rate) of the freshwater planarian *Girardia tigrina*. For this, we used a previously described protocol to measure planarians locomotion with automated video tracking system (with slight modifications) (Rodrigues et al., 2016) and devised a simple protocol to measure planarian feeding rate. We chose Hg as a stressor, since Hg continues to be a widespread contaminant and can cause neurotoxicity (Mckinney et al., 2015; Pereira et al., 2016), thus likely affecting planarian behavioural endpoints. NaCl was chosen as stressor, since salinization of freshwaters is a major concern nowadays and salinity increases lead to changes in the osmotic relations of freshwater organisms and media (Cañedo-Argüelles et al., 2013), thus affecting planarians through different mechanisms compared to Hg. In this way, we aimed to evaluate the impacts of differently-acting stressors on behavioural endpoints of freshwater planarians.

2 Material and methods

2.1 Planarian and chironomid cultures

The used organisms belonged to a *G. tigrina* population reared in laboratory and reproducing sexually. Cultures were kept in plastic containers with ASTM hard water (ASTM, 2014), at a temperature of 20 ± 1 °C. Animals were maintained protected from direct light, except when feeding and when the medium was renewed. Animals were fed once a week either with cow liver or chironomid larvae. Water and containers were changed immediately after feeding and 2 – 3 days after. Prior to the experiments, planarians were starved for about 1 week to ensure a uniform metabolic state (Oviedo et al., 2008).

Chironomid cultures were maintained in plastic containers with a layer of inorganic sediment and ASTM hard water (ASTM, 2014). Sediments were sieved (< 1 mm) and burned at 500 °C during 4 h. Room temperature was maintained at 20 ± 1 °C and aeration was provided to the water. Animals were fed with ground Tetramin® every other day and media was replaced once every other week.

2.2 Feeding protocol development

Several factors that would likely affect planarian feeding were tested in order to devise a protocol to evaluate feeding rate in response to xenobiotic stress. These include size of planarian, size of chi-

ronomid larvae, feeding period and lighting conditions. Preliminary tests showed that vial size did not affect feeding rate (between 50, 80 or 115 mm Ø crystallizing dishes with 15, 50 or 200 mL of ASTM hard water, respectively), nor did fasting period (between 1 or 2 weeks without food). We chose the immature stages of *C. riparius* as food item, since chironomid larvae are *G. tigrina*'s natural prey. *C. riparius* larval stages seem to be suitable, given their benthic nature, making encounters with planarians likely to occur, as opposed to other insect larvae (Meyer and Learned, 1981). Also, *C. riparius* is a model for ecotoxicological studies and larvae can be easily obtained in high amounts from laboratory cultures. Moreover, they have been successfully used in feeding assays with other planarian species (Ofoegbu et al., 2016; Rodrigues et al., 2016).

In the first feeding trial, we compared feeding rates between planarians of differing sizes; 3 categories were chosen, small (0.5 ± 0.1 cm), medium (1.0 ± 0.1 cm) and large (1.5 ± 0.1 cm). Animals were measured while moving and fully extended in a transparent petri dish with a small amount of water placed above a millimetric sheet. For chironomid larvae, 2 sizes were chosen based on larval stage, either late 2nd (5 - 6 days) or 4th (9 days) larval instars (according to Domingues et al., 2007). To ensure planarians did not eat all the provided food items before the end of the test, 30 and 20 of 2nd and 4th instar larvae, respectively, were chosen for a 12 h assay. Twenty planarians of each size were selected and placed individually in medium-sized (80 mm Ø) crystallizing dishes with 50 mL of ASTM medium, containing the larvae. Ten replicates were made for each combination of planarian and larval sizes (3 planarian sizes × 2 larval sizes × 10 replicates). The number of unconsumed larvae was recorded every 3 h until the end of the test at 12 h, for a total of 4 observations. The assay was performed with ambient lighting (200 – 250 lux).

In a second trial, we tested the lighting conditions with the previously chosen planarian and larval sizes, i.e. medium sized planarians and *C. riparius* 2nd larval stage, respectively, along with the chosen time period, 3h. For this, 20 planarians were selected, and individually placed in crystallizing dishes with 50 mL of medium, containing 30 larvae. Two different conditions were tested: ambient light and darkness, with 10 replicates each. Darkness was achieved by completely covering a tray with aluminium foil, in a similar way as cultures are kept. The light environment was the standard room illumination provided by fluorescent lamps, with luminosity between 200 – 250 lux. After 3 h, the number of remaining larvae was counted.

After these assays, the devised protocol was used in the following experiments.

2.3 **Planarian exposure experiments**

All tests were conducted in ASTM hard water at a temperature of 20 ± 1 °C, with planarians being kept in a light-protected environment, similarly as cultures are kept.

2.3.1 Hg experiments

In the Hg experiments, mercury chloride ($HgCl_2$) was used to prepare the experimental solutions. $HgCl_2$ was of analytical grade (CAS: 7487-94-7; purity ≥ 99.5 %) and acquired from Merck (Darmstadt, Germany). A 96-h acute test was performed. The experimental solutions were obtained from a stock solution of 8 g L^{-1} Hg (in ultra-pure water). Seven experimental solutions (144.5, 192.2, 255.7, 340.0, 452.2, 601.5 and 800.0 µg L^{-1}) were prepared in ASTM hard water plus a control treatment (ASTM only). Planarians were exposed in plastic vials with 30 mL of solution, with 4 replicates of 5 animals being performed for each treatment. To prevent evaporation, the vials were covered throughout the exposure. The evaluated endpoint was mortality, every 24 h, with any morphological anomalies also being recorded. Only animals that where completely still and did not respond to stimuli were considered dead. A 3-mL sample of each replicate's solution was taken at 24 and 96 h for chemical analysis. These samples were kept at -20 °C until Hg quantification. For the sub-lethal test, animals were exposed during 8 days to 4 Hg concentrations (86.8, 104.2, 125.0 and 150.0 µg L^{-1} of Hg) and a control treatment (ASTM only). A stock solution of 1.5 g L^{-1} of Hg was performed in ultra-pure water and used to prepare the experimental solutions. Planarians were exposed in plastic vials, with 5 replicates of 4 animals per vial in 30 mL of test solution. To prevent evaporation, the vials were covered throughout the exposure. After exposure, animals were used to evaluate either the feeding or the locomotion endpoint (described in sections 2.5 and 2.6). A 3-mL sample of each replicate's solution was taken at 1 and 8 d for chemical analysis. These samples were kept at -20 °C until Hg quantification. Hg in experimental solutions was measured by atomic absorption using the mercury analyser AMA-254 (Altec, Czech Republic). The analytical procedure was validated using DORM-3 (fish protein) and TORT-2 (lobster hepatopancreas), two biological reference materials for trace metals certified by the National Research Council of Canada, at the beginning and end of each set, thereby ensuring the accuracy of the method (Costley et al 2000).

2.3.2 NaCl experiments

NaCl was of analytical grade (CAS: 7647-14-5; purity ≥ 99.0 %) and purchased from Merck (Darmstadt, Germany). For the 96-h acute test, a stock solution of 10 g L^{-1} of NaCl was performed by directly dissolving NaCl in the culture media. The experimental solutions were obtained by dilution of this stock solution in ASTM hard water. Seven concentrations were chosen, plus a control treatment (ASTM only). The chosen concentrations were 5.64, 6.21, 6.83, 7.51, 8.26, 9.09 and 10.00 g L-1 of NaCl. Planarians were exposed in glass crystallizing dishes with 30 mL of solution, with 4 replicates of 5 animals being performed. To prevent evaporation, the vials were covered throughout the exposure. The evaluated endpoints were mortality, every 24 h, and any morphological anomaly. Only animals that where completely still and did not respond to stimuli were considered dead. Solutions were replaced every other day to avoid increases in salt concentration because of evaporation. Conductivity and salinity were measured using a conductivity hand-held meter (WTW con-

ductivity Hand-Held Meter LF 330) at day 0, day 2 and at the end of the experiment (day 4). For the sub-lethal exposures, a stock solution of 4 g L^{-1} of NaCl was obtained by directly dissolving the salt in ASTM hard water. Four treatments were performed: 2.31, 2.78, 3.33 and 4.00 g L^{-1} of NaCl, by dilution of the stock solution in ASTM hard water plus a control treatment (ASTM only). To prevent evaporation, the vials were covered throughout the exposure. Moreover, solutions were replaced every other day, to avoid increases in salt concentration due to evaporation. Planarians were exposed in glass crystallizing dishes with 30 mL of solution in 5 replicates of 4 animals each. Conductivity and salinity were measured at day 0, day 4 and at the end of the experiment (day 8). Post-exposure feeding and locomotion were performed as described in the following sections (2.4 and 2.5).

2.4 **Post-exposure feeding**

After the sub-lethal exposures, 2 animals from each replicate were randomly selected, washed in clean media and individually transferred to vials with 50 mL of clean ASTM medium and 30 2nd stage larvae. After 3 h, the number of remaining larvae was counted. For each treatment, feeding was assessed for 10 individuals. This test was performed with ambient lighting (200 – 250 lux).

2.5 **Post-exposure locomotion**

Half of the animals used in the sub-lethal exposures were randomly selected, washed in clean media and used to evaluate locomotion. A total of 10 individuals were used per treatment. Planarian locomotion was evaluated in 24-well plates, with 1 planarian per well in 1 mL of clean media. A ZebraBox™ system with the ZebraLab® v3 software (Viewpoint, France) were used to track planarians. We based our protocol on a previous study that used this system with planarians (Rodrigues et al., 2016), with some modifications. Briefly, recordings were performed over a 5-min period, including a 1 min acclimation in bright light (approximately 550 lux), followed by 4 min with the same lighting conditions. Readings on the distance travelled for each planarian were recorded every min.

2.6 **Statistical analyses**

To compare the number of prey items eaten between the different planarian classes for the 0 – 3 h feeding period, one-way ANOVA was used to compare treatments, followed by Tukey's post-hoc test. Whenever normality (tested with Shapiro-Wilk's test) or homogeneity of variances could not be achieved, data was analysed with Kruskal-Wallis followed by Tukey's post-hoc test. To compare differences between the lighting conditions, a two-tailed t-test was performed. For the acute tests with Hg and NaCl, data of each replicate was transformed in % of survival and used to calculate the LC50 through a 4-parameter logistic regression, with x as Log (concentration). In the post-

exposure locomotion experiment, readings on distance travelled were pooled for the 4 min recordings and used to calculate total distance travelled. For the post-exposure locomotion and feeding, treatments were analysed with one-way ANOVA, followed by Dunnett's *post-hoc* test to evaluate differences in comparison to the control treatment. Whenever normality (tested with Shapiro-Wilk's test) or homogeneity of variances could not be achieved, data was transformed to comply with ANOVA requirements. Data from the locomotion endpoint was square root transformed, prior to ANOVA. In the cases where ANOVA requirements were still not met, data was analysed with Kruskal-Wallis followed by Dunn's post-hoc test. Analyses were performed with SigmaPlot v. 12.3 and with GraphPad Prism v. 6.01 for Windows.

3 Results

3.1 **Feeding inhibition protocol**

An average of 4.40 ± 2.22, 18.30 ± 2.87 and 24.60 ± 3.56 2nd stage larvae was consumed by the small, medium and large size planarians, respectively, after the 12-h period. The number of ingested prey items was higher for the first 3 hours for all planarian sizes, with an average of 3.10 ± 0.74, 14.40 ± 2.22, 16.5 ± 2.51 larvae eaten per planarian for small, medium and large sized planarians, respectively. Small planarians ate less prey in the first 3 hours, when compared to the medium and large planarians (one-way ANOVA: $F_{2, 27} = 132.559$; $p < 0.001$; Figure II-3– a).

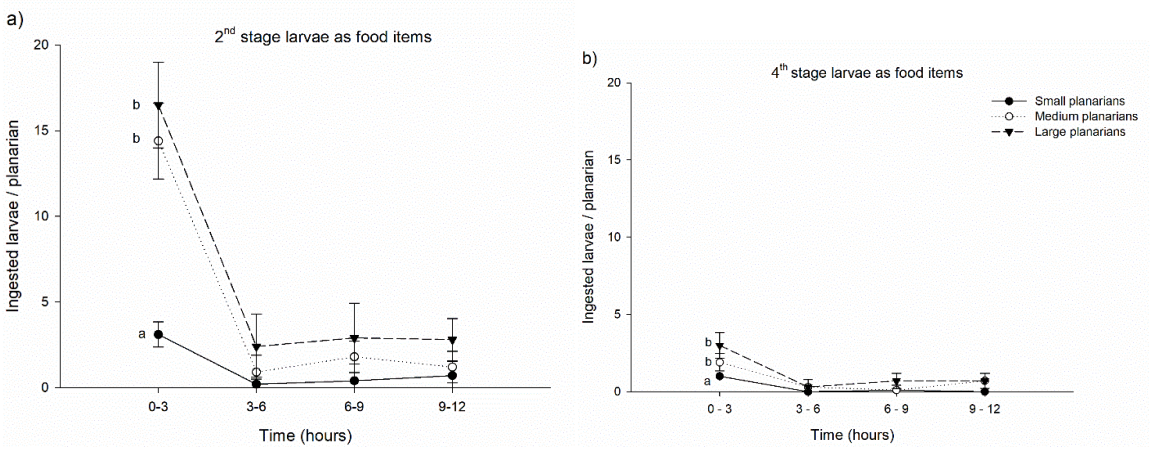

Figure II-3 – Number of ingested *C. riparius* larvae by small, medium and large *G. tigrina* individuals in the evaluated time periods. a) Number of 2nd stage larvae eaten by planarians. b) Number of 4th stage larvae eaten by planarians. Different letters denote significant differences in the number of ingested larvae between planarian sizes (ANOVA followed by Tukey's post-hoc test, $p < 0.05$). Data is presented as means ± SD.

The average 4th stage larvae consumed after 12 h was 1.10 ± 0.32, 3.00 ± 0.67 and 4.70 ± 0.82 for small, medium and large sized planarians, respectively. Most larvae were consumed in the first 3 h and amounted to 1.00 ± 0.00, 1.90 ± 0.57 and 3.00 ± 0.82 prey items eaten by planarians for

small, medium and large sized planarians, respectively. Small planarians ate less prey items than medium and large planarians (Kruskal-Wallis test: H = 22.032; 2 d. f.; p = < 0.001; Figure II-3 – b).

The number of consumed larvae was higher under light conditions (t -test: t = 2.178; 18 d.f.; p = 0.043;Figure II-4), with an average of 13.30 ± 2.41 and 10.70 ± 2.91 2nd stage larvae consumed after 3 h by medium sized planarians with light and with dark conditions, respectively.

Based on these results, the chosen protocol uses medium sized *G. tigrina* individually placed in vials with 30 2nd stage chironomid larvae with ambient lighting. Feeding period, is set as 3 h, after which the remaining larvae are counted. This protocol was used in the subsequent experiments.

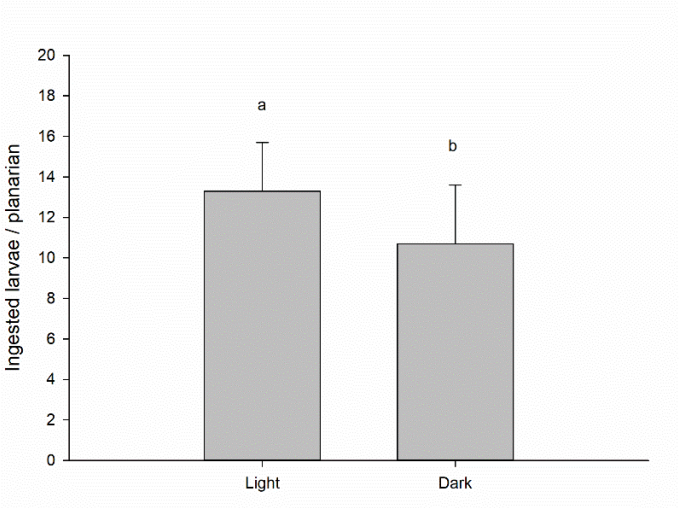

Figure II-4 – Number of 2nd stage *C. riparius* larvae ingested by medium sized *G. tigrina* under differing luminosity conditions, either light or dark. Letters above columns show significant differences in the number of ingested larvae by planarians under different lighting conditions (t-test, p < 0.05). Data is presented as means ± SD.

3.2 Mercury effects

The measured concentrations in test solutions after 24 h were below 60% and 65% of the nominal initial Hg concentrations, for the acute (Supplementary Table II-4) and sub-lethal tests (Supplementary Table II-5), respectively. From this point onward, the measured concentrations at 24 h will be used throughout the text.

The estimated 96 h LC50 for *G. tigrina* was 176.8 µg Hg L^{-1} (168.2 to 185.8 µg Hg L^{-1}). Loss of pigment on the body surface and of photoreceptors was observed, for all tested Hg concentrations. Disintegration of tissues progressed from the anterior to the posterior portion of the body, so that many of the animals lost their head portion. Disintegration of tissue did not result in the immediate death of the organism, which occurred only when half or more of the body was lost.

The 8-day sub lethal exposure to Hg impaired the locomotion of animals. Organisms exposed to 60.18 and 94.97 µg Hg L^{-1} travelled smaller distances than controls (Kruskal-Wallis: H = 13.394;4

d.f.; p = 0.010;Figure II-5– a). *Post-hoc* trend analysis showed a linear decrease in the distance travelled by planarians as the Hg concentration increased (slope = -4.698; r^2 = 0.148; p = 0.006). Feeding of animals exposed to 75.69 and 94.97 µg Hg L^{-1} was also significantly reduced (one-way ANOVA: $F_{4, 45}$ = 3.608, p = 0.012; Figure II-5 – b), for an inhibition in relation to control of 12 and 15%, respectively. *Post-hoc* trend analysis showed a linear decrease in the number of consumed prey items as concentration increased (slope = -0.660; r^2 = 0.239; p < 0.001).

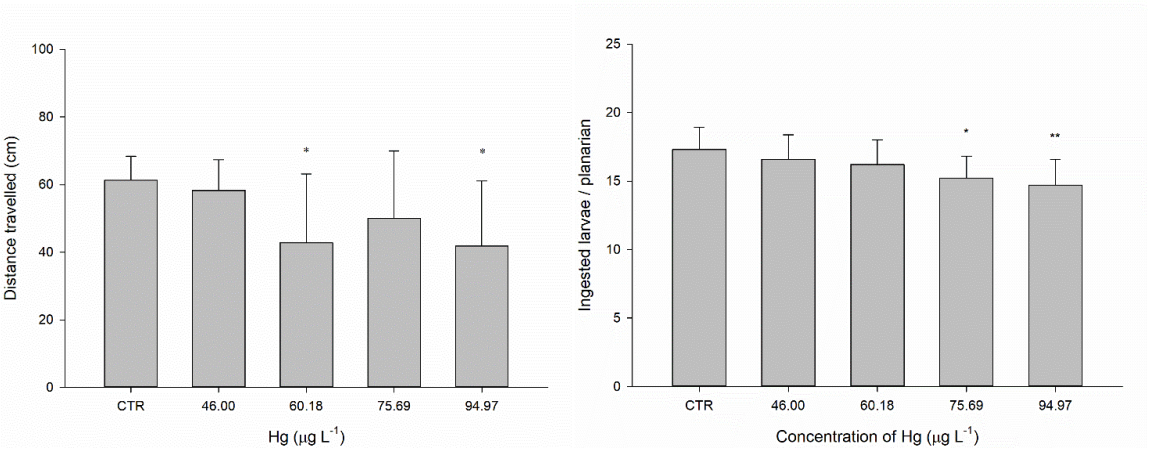

Figure II-5 – Effects of mercury in *G. tigrina.* a) Distance travelled by *G. tigrina* over 4 min. b) Mean larvae consumed by *G. tigrina* over 3 h. Data is presented as means ± SD. Treatments were compared with Kruskal-Wallis one-way ANOVA using a Dunn's post-hoc test (distance travelled) or one-way ANOVA using a Dunnett's post-hoc test (Ingested larvae/planarian). Asterisks above columns represent significant differences from the control treatment. * p<0.05, ** p<0.01.

3.3 NaCl effects

The conductivity and salinity of solutions were stable throughout the duration of both acute (Supplementary Table II-6) and sub-lethal (Supplementary Table II-7) exposures. Since the variation was slight and below 5%, nominal concentrations of NaCl are used throughout the text.

The estimated 96 h LC_{50} was 6.79 g L^{-1} of NaCl (6.73 to 6.84 g L^{-1} of NaCl). The salinity effects in the acute experiment were characterized by the motionless of exposed animals, and with increasing concentrations, by the rupture of the tegument, followed by disintegration of body parts and death. The rupture and disintegration did not progress from the snout to the tail, contrary to the Hg experiments.

The 8-day sub-lethal exposure impacted behaviour of animals. Locomotion was severely impaired in the 3.33 and 4.00 g L^{-1} of NaCl treatments (one-way ANOVA: $F_{4, 45}$ = 11.469; p < 0.001; Figure II-6 – a). Some animals could barely move, while others only moved for short periods of time. Post-hoc trend analysis showed a linear decrease in the distance travelled by planarians as concentration increases (slope = -13.860; r2 = 0.441; p < 0.001). Feeding was significantly reduced for the 2.78, 3.33 and 4.00 g L^{-1} of NaCl treatments (one-way ANOVA: $F_{4, 45}$ = 17.773; p < 0.001; Figure II-6 – b). Post-hoc trend analysis showed a linear decrease in the number of consumed prey items as

concentration increases (slope = -2.040; r^2 = 0.598; p < 0.001). Feeding inhibition followed a concentration-dependent response with an inhibition relative to control animals of 8.6%, 25.8%, 33.8% and 54.9%, from the lowest to the highest concentration, respectively.

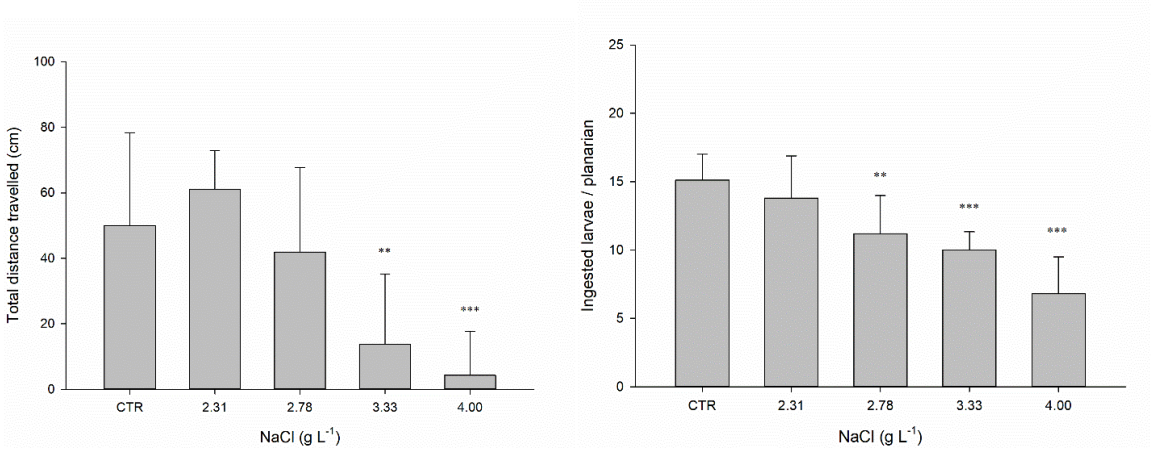

Figure II-6 – Effects of salinity in *G. tigrina*. a) Distance travelled by *G. tigrina* over 4 min. b) Mean larvae consumed by *G. tigrina* over 3 h. Data is presented as means ± SD. Treatments were compared using one-way ANOVA with a Dunnett's post-hoc test. Asterisks above columns represent significant differences from the control treatment. ** p<0.01, *** p<0.001.

4 Discussion

The present work shows that simple, but sensitive protocols that take advantage of planarian behaviour can be useful tools to evaluate sub-lethal effects of stressors. Here we show that post-exposure feeding and locomotion are useful and simple endpoints that can detect effects for differently acting stressors, shown by the observed impairments for both Hg and NaCl exposures. Additionally, this study further evidences the suitability of planarians for ecotoxicity studies.

After testing several factors that could affect planarian feeding, we observed that the ideal period to evaluate this endpoint is the first 3 h. In this period, consumption reaches its peak and decreases sharply in the subsequent periods. This was consistent for all planarian and prey size combinations. Possibly, the animals feed until their digestive system is full, continuing to eat more prey as food is being digested and excretion occurs, but at a slower pace than when starved. Also, the short time is ideal to evaluate effects prior to recovery, as invertebrates can recover feeding rates in a short period, seen for instance in Daphnia exposed to the insecticide paraoxon-methyl, recovering filtration rates to control levels after 4.5 h in clean media (Duquesne and Küster, 2010). Using this protocol, recovery could also be evaluated after the adequate time period. Moreover, the evaluation of feeding after exposure ceases may eliminate some confounding factors, such as avoidance or feeding depression caused by contaminated food particles (Taylor et al., 1998; Wilding and Maltby, 2006), thus enabling a better discrimination of toxicant effects in the studied organism.

Feeding rate of small planarians was much lower when compared to the other planarian sizes. Tranchida et al (2009), observed that small sized *G. anceps* (< 0.5 cm) consumed less 1st and 2nd

instar mosquito larvae than large planarians (> 0.5 cm), but the same amount for the 3rd and 4th instars. In our case, the differences were observed for all larval prey sizes, which may simply reflect the differences in the gut size of planarians and therefore the amount of food they can ingest. On the other hand, larger animals may also be more experienced in catching and handling prey, affecting the number of captured animals, as suggested by Martinez-Haro et al (2016) for *Echinogammarus marinus*. Nevertheless, medium and large planarians ingested similar amounts of prey in the first 3 h, suggesting that either planarian size is suitable for the feeding assay. Larval size also drastically affected planarian feeding rate, the larger the prey, the less items planarians captured and ingested. Probably, larger larvae are simply more nutritious than smaller larvae. Still, planarian size did not influence their ability to capture prey, since even small planarians could capture larger prey than themselves. Given these results, the ideal size of *C. riparius* larvae to use is the 2nd larval stage, since much more prey items can be consumed in the same amount of time, thus making possible differences in planarian feeding rate more noticeable.

The presence / absence of light during the feeding test affected the feeding rate of planarians. With ambient lighting, planarians captured more prey than when in dark conditions. Photoreceptors are believed to have no role in terms of capturing prey, as these structures can only detect light intensity and direction (Cebrià et al., 2015). Instead, chemical cues play a major role in the location of prey (Inoue et al., 2015). Despite the photonegative behaviour and nocturnal activity of *G. tigrina*, when presented with prey, they will still react and try to capture prey in daylight (Lombardo et al., 2011). Inoue et al (2015) have shown that when planarians are presented simultaneously with light (400 Lux) and chemicals from prey, they will still respond to the chemical stimulus, being drawn to it. Moreover, the ability to learn has been shown in planarians (Shomrat and Levin, 2013), so one possible explanation might be related to this ability, since our planarian cultures are fed in ambient lighting. Nevertheless, our initial concern was that the presence of light could inhibit the feeding of planarians, but it did not, so ambient lighting is adequate to evaluate this endpoint.

In the Hg experiment, *G. tigrina* (96h LC50 = 176.8 µg Hg L^{-1}) mortality endpoint showed a similar or lower sensitivity compared to other freshwater invertebrates, such as the crab *Oziotelphusa senex senex* (72 h LC_{50} = 443 µg Hg L^{-1}), *C. riparius* (96 h LC_{50} = 547 µg Hg L^{-1}) (Boening, 2000) or *Daphnia magna* (48 h LC_{50} = 251 µg Hg L^{-1}) (Meng et al., 2008). The sensitivity of *G. tigrina* was also similar to that of the planarian *Dugesia dorotocephala* (5-day LC50 159.78 – 399.45 µg Hg L^{-1}) (Best et al., 1981). Moreover, *G. tigrina* post-exposure feeding and locomotion endpoints were about 2.3 to 2.9 times, respectively, more sensitive to Hg exposure than mortality (LC_{50} / LOEC). Locomotion was slightly more sensitive than post-exposure feeding, which may be related to the specific mode of action of Hg. Hg is a known neurotoxic agent that can impact behaviour of animals (Korbas et al., 2012; Pereira et al., 2016), thus it is not surprising that planarian behaviour was impaired. In fact, Best and Morita (1991) observed that *D. dorotocephala* planarians regenerated in methyl mercuric chloride had a lower synaptic density and presented behavioural anomalies, such as delayed righting response, decreases in motility and reduced prey capture (LOEC - 15.97 µg Hg

L^{-1}), evidencing Hg neurotoxicity towards planarians. Moreover, the sensitivity of *G. tigrina* behavioural endpoints to Hg seems to be in the same range as for other freshwater invertebrates, such as *C. riparius* larvae, with altered locomotor behaviour after 4 and 10 d in contaminated sediments (LOECs = 149.35, 40.88 µg Hg L-1, respectively) (Azevedo-Pereira and Soares, 2010), reduced feeding rate of *Brachionus calyciflorus* after a 30 min exposure (NOEC = 0.10 mg/L of Hg) (Juchelka and Snell, 1994) and *Ceriodaphnia dubia* with reductions in feeding rate after 1 h exposure (NOEC = 0.01 mg/L of Hg) (Juchelka and Snell, 1995). Feeding depression / inhibition in freshwater crustaceans and rotifers has also been reported for other metals, such as cadmium, zinc, copper or vanadium (Allen et al., 1995; Juchelka and Snell, 1994; McWilliam and Baird, 2002; Pestana et al., 2007). These studies and the present one evidence that behavioural assays can be important and ecologically relevant as endpoints when evaluating metal toxicity in aquatic invertebrates.

Other interesting effects of Hg exposure were the loss of photoreceptor pigmentation to a point where it was almost unnoticeable, and depigmentation in the body region. Some metals were shown to cause depigmentation in the body region of Polycelis felina, namely aluminum (Kovačević et al., 2009) and zinc (Franjević et al., 2000). These studies attribute depigmentation of body parts due to the decomposition of parenchyma cells, visible under histological preparations. This could be a possible explanation for the depigmentation observed in our study. Nevertheless, loss of pigmentation has been observed in other animal models exposed to metals; Sánchez et al (2005) studied the crab *Chasmagnathus granulatus* and found that hatchlings exposed during embryonic development had abnormal pigment spots, loss of pigmentation in areas that are usually pigmented and hypopigmentation of the eyes. Other metals, such as copper, lead and zinc appear to cause similar pigmentation loss in the same crab species (Lavolpe et al., 2004). These studies suggest that eye hypopigmentation might be related to alterations in the secretion of neurotransmitters and neuromodulators that regulate the migration of pigments to the correct regions, as seen for cadmium in crustaceans (Fingerman et al., 1998). Lavolpe et al (2004) also suggest that this might be related to an inhibition of the melanin synthesis pathway elicited by Hg. In planarians it has been observed that inhibition of enzymes involved in the metabolism of melanin (the pigment in the planarian eye cup cells; Hase et al., 2006) resulted in loss of eye pigmentation. For instance, chemical inhibition of tyrosine hydroxylase results in progressive eye pigment loss in *D. dorotocephala* (Ness et al., 1996) and failure to regenerate eye pigmentation caused by RNA interference of tryptophan hydroxylase has been shown in *Schmidtea mediterranea* (Lambrus et al., 2015). Moreover, Hg is known to inhibit tyrosinase (also involved in the melanin metabolism) (Lerner, 1952) and has affinity for sulfhydryl groups in proteins (Stohs and Bagchi, 1995). The disruption of the melanin metabolism or affected migration of pigments might offer an alternative explanation for the observed depigmentation.

Salinity can have severe impacts on freshwater planarians, since their tegument is very permeable, with no hard or impermeabilized structures (Buttarelli et al., 2000; Rink, 2013), making them very

susceptible to dissolved chemicals (Kapu and Schaeffer, 1991). Thus, they are believed to be among the most susceptible groups to increased salinity (Hart et al., 1991; Kefford et al., 2003). The sensitivity of freshwater planarians to salinity, was studied by Rivera and Perich (1994). Among 4 freshwater planarian species, *Cura foremanii* was the most sensitive, with 100% mortality recorded at ≈ 4.88 g L^{-1} of NaCl, in a 14-day exposure period. The other tested species, *D. dorotocephala*, *G. tigrina* and *Dendrocoelopsis vaginata* had reduced survival rates at ≈ 6.38 g L^{-1} of NaCl and at ≈ 7.83 g L^{-1} of NaCl all presented 100% mortality within 14 d. Another study using *S. mediterranea*, determined a 96 h LC_{50} of 7.55 g L^{-1} of NaCl (Ofoegbu et al., 2019). Thus, our results indicate that *G. tigrina* lethality under NaCl exposure is in the same range as the above-mentioned studies. Moreover, post-exposure feeding and locomotion of *G. tigrina* were about 2.4 to 2.0 times, respectively, more sensitive than mortality (LC_{50} / LOEC) caused by NaCl addition to the media. The study by Ofoegbu et al. (2019) showed that locomotion decreased linearly up to a concentration of 3 g L^{-1} of NaCl, and post-exposure feeding was decreased at a LOEC of 0.75 g L^{-1} of NaCl for *S. mediterranea*. It is interesting to note that the behavioural endpoints evaluated in *S. mediterranea* were also impacted by salinity (Ofoegbu et al., 2019), and together with the results obtained in the present study, show that behavioural endpoints in planarians are sensitive to salinity increases. In cladocerans, feeding also seems to be a sensitive endpoint in response to increased salinity. In *D. longispina*, a 24 h EC_{50} for feeding inhibition of approximately ≈ 2.02 g L^{-1} of NaCl to around ≈ 1.01 g L^{-1} of NaCl was estimated, for the most and least resistant lineages tested, respectively (Venâncio et al., 2018). A genotype of *D. dentifera*, exposed to a rise in salinity levels achieved by addition of 0.6 g L^{-1} of NaCl to freshwater media, showed reduced feeding rates (Searle et al., 2016). All these results evidence that studies focused on adverse effects of salinity should also address behavioural endpoints, as behavioural effects can be expected at considerably lower salinity levels than mortality.

Under the current scenarios of climate change, both Hg and salinity might have increased negative effects on freshwaters. Climatic changes might have consequences for the biogeochemical cycle of Hg, and predictions indicate that the presence of this compound in aquatic environments will likely increase, for instance as a consequence of higher precipitation intensity (increasing runoff from nearby areas) and extreme weather events (leading to sediment resuspension) (Krabbenhoft and Sunderland, 2013; Nadal et al., 2015). Increased salinities in freshwaters are also expected to occur in some regions, since the higher temperatures, and the consequent water evaporation, as well as decreased precipitation patterns predicted, will likely increase the salinity of many waterbodies (Cañedo-Argüelles et al., 2013). These scenarios might pose an increased risk for freshwater planarian populations, since sub-lethal levels of Hg and NaCl were, in the present work, observed to impair important behaviours such as feeding and locomotion. The impairment of these behaviours in planarians inhabiting natural environments, can lead to decreased ability to escape from predators and to decreased energy inputs. Ultimately, this could lead to population-level effects, which when we consider the limited dispersal abilities of freshwater planarians, is even more concerning.

5 Conclusion

This work shows that planarian behavioural endpoints are simple but sensitive ways of evaluating xenobiotic effects on the freshwater planarian *G. tigrina*. For both tested stressors, Hg and NaCl, behavioural endpoints were effective in detecting effects below lethal levels. Moreover, through the testing of 2 differently acting stressors, we observed a consistency on effect detection, evidencing the integrative nature of behavioural endpoints. Also, given the simplicity and celerity of measuring behavioural endpoints in planarians, it would be simple to use these as post-exposure endpoints following in situ exposures. These endpoints increase the ecological relevance in ecotoxicity testing with planarian species and provide simple and useful tools to evaluate effects at sub-lethal levels of stressors.

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Supplementary material

Supplementary Table II-4 – Concentrations of mercury (Hg) measured in the experimental solutions at 24 and 96 h of the acute exposure. Concentrations are expressed as means ± SD (n = 4).

Nominal concentrations (µg Hg L^{-1})	Measured Hg concentrations at 24 h (µg Hg L^{-1})	Measured Hg concentrations at 96 h (µg Hg L^{-1})
0.00	0.00 ± 0.00	0.10 ± 0.13
144.5	74.41 ± 7.34	55.95 ± 5.98
192.2	112.50 ± 6.48	67.98 ± 3.02
255.7	151.07 ± 4.75	68.81 ± 10.32
340.0	164.54 ± 12.45	71.55 ± 20.31
452.2	186.19 ± 10.99	47.07 ± 7.80
601.5	296.81 ± 7.80	72.53 ± 18.21
800.0	357.13 ± 13.96	97.95 ± 70.92

Supplementary Table II-5 – Concentrations of mercury (Hg) measured in the experimental solutions at 1 and 8 days for the sub-lethal exposure. Concentrations are expressed as means ± SD (n = 4).

Nominal concentrations (µg Hg L^{-1})	Measured Hg concentrations at 1 d (µg Hg L^{-1})	Measured Hg concentrations at 8 d (µg Hg L^{-1})
86.81	46.00 ± 0.93	16.23 ± 14.31
104.17	60.18 ± 2.83	34.43 ± 2.83
125.00	75.69 ± 4.23	34.95 ± 0.61
150.00	94.97 ± 1.22	34.47 ± 19.19

Supplementary Table II-6 – Conductivity and salinity measured in the experimental solutions at 0, 2 and 4 days for the acute salinity test. Data are expressed as means ± SD (n = 4).

	Day 0		Day 2				Day 4	
			Before renewal		After renewal			
Treatment (g NaCl L^{-1})	Conductivity (mS cm^{-1})	Salinity	Conductivity (mS cm^{-1})	Salinity	Conductivity (mS cm^{-1})	Salinity	Conductivity (mS cm^{-1})	Salinity
CTR	0.580 ± 0.000	0.00 ± 0.00	0.594 ± 0.008	0.00 ± 0.00	0.591 ± 0.001	0.00 ± 0.00	0.595 ± 0.001	0.00 ± 0.00
5.64	10.915 ± 0.017	6.20 ± 0.00	10.913 ± 0.059	6.20 ± 0.00	10.865 ± 0.044	6.18 ± 0.05	10.878 ± 0.105	6.18 ± 0.05
6.21	11.945 ± 0.021	6.85 ± 0.06	11.970 ± 0.029	6.88 ± 0.05	11.958 ± 0.021	6.88 ± 0.05	12.020 ± 0.029	6.90 ± 0.00
6.83	13.008 ± 0.005	7.50 ± 0.00	13.038 ± 0.040	7.50 ± 0.00	13.080 ± 0.032	7.50 ± 0.00	13.143 ± 0.029	7.60 ± 0.00
7.51	14.278 ± 0.021	8.30 ± 0.00	14.313 ± 0.039	8.30 ± 0.00	14.433 ± 0.026	8.40 ± 0.00	14.495 ± 0.031	8.45 ± 0.06
8.26	15.475 ± 0.006	9.05 ± 0.06	15.528 ± 0.045	9.10 ± 0.00	15.560	9.10	15.620	9.10
9.09	16.975 ± 0.034	10.00 ± 0.00	17.025 ± 0.026	10.00 ± 0.00	a	a	a	a
10.00	18.528 ± 0.005	11.00 ± 0.00	18.510 ± 0.016	11.00 ± 0.00	a	a	a	a

a – values are missing because all planarians died during the test.

Supplementary Table II-7 – Conductivity and salinity measured in the experimental solutions at 0, 4 and 8 days for the sub-lethal salinity test. Values are expressed means ± SD (n = 5).

	Day 0		Day 4				Day 8	
			Before renewal		After renewal			
Treatment (g of NaCl L^{-1})	Conductivity (mS cm^{-1})	Salinity	Conductivity (mS cm^{-1})	Salinity	Conductivity (mS cm^{-1})	Salinity	Conductivity (mS cm^{-1})	Salinity
0.00	0.579 ± 0.001	0.00 ± 0.00	0.595 ± 0.002	0.00 ± 0.00	0.589 ± 0.001	0.00 ± 0.00	0.589 ± 0.000	0.00 ± 0.00
2.31	4.968 ± 0.011	2.60 ± 0.00	5.026 ± 0.021	2.68 ± 0.04	4.976 ± 0.009	2.60 ± 0.00	5.084 ± 0.089	2.68 ± 0.04
2.78	5.876 ± 0.005	3.20 ± 0.00	5.886 ± 0.021	3.18 ± 0.04	5.890 ± 0.007	3.20 ± 0.00	6.062 ± 0.029	3.28 ± 0.04
3.33	6.868 ± 0.004	3.80 ± 0.00	6.936 ± 0.011	3.80 ± 0.00	6.868 ± 0.004	3.80 ± 0.00	7.052 ± 0.026	3.88 ± 0.04
4.00	8.110 ± 0.007	4.50 ± 0.00	8.154 ± 0.015	4.50 ± 0.00	8.118 ± 0.008	4.50 ± 0.00	8.352 ± 0.041	4.68 ± 0.04

Chapter III – Toxicity of different PAHs to the freshwater planarian *Girardia tigrina*

Toxicity of different PAHs to the freshwater planarian *Girardia tigrina*

Abstract

Freshwater planarians have been gaining relevance as experimental animals for numerous research areas given their interesting features, such as high regeneration potential, shared features with the vertebrate's nervous system or the range of endpoints that can be easily evaluated in response to contaminants. Ecotoxicological research using these animals has been steadily increasing in the past decades, as planarians' potentialities for this research area are being recognized. In this work, we used polycyclic aromatic hydrocarbons (PAHs) as model contaminants and evaluated effects of exposure to phenanthrene, pyrene and benzo[a]pyrene (B[a]P) in planarians. The freshwater planarian *Girardia tigrina* was chosen and mortality, cephalic regeneration (during and post-exposure), behavioural endpoints and presence of PAHs in tissues, were evaluated. Mortality was only observed in planarians exposed to phenanthrene, with an estimated LC_{50} of 830.1 µg L^{-1}. Results indicate that planarian behavioural endpoints were very sensitive in response to sub-lethal concentrations of PAHs, showing a greater sensitivity towards B[a]P and pyrene. Briefly, post-exposure locomotion and post-exposure feeding were significantly impaired by sub-lethal concentrations of all compounds, whereas regeneration of photoreceptors was only significantly delayed in planarians exposed to pyrene. Moreover, levels of PAH-type compounds in planarian tissues followed a dose-dependent increase, showing uptake of compounds from experimental solutions. The present results highlight the importance of studying alternative and complementary endpoints, such as behaviour, not only because these may be able to detect effects at lower levels of contamination, but also due to their ecological relevance. The simplicity of evaluating a wide range of responses to contaminants further demonstrates the utility of freshwater planarians for ecotoxicological research.

Keywords: Planarians; PAHs; behaviour; feeding; locomotion; regeneration.

1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) constitute a class of persistent organic pollutants that can be produced naturally or anthropogenically. Common sources of PAHs include fossil fuels, forest fires, volcanoes, combustion engines or industrial production of asphalt and coal tar (Burgess et al. 2003a; Ball and Truskewycz 2013). The majority of inputs to the environment are, however, anthropogenic, mainly from industrial sources and burning of fossil fuels for energy generation (Ball and Truskewycz 2013). Many of the PAHs released to the environment can enter freshwaters, for instance, through urban runoff, wastewater effluents, atmospheric deposition or discharges from industries (Burgess et al. 2003b; Latimer and Zheng 2003; Ball and Truskewycz

2013). Concentration in surface waters are usually in the ng L^{-1} range (Latimer and Zheng 2003). However, in heavily populated or near heavily industrialized areas, these compounds can reach very high concentrations (Manzetti 2013), as observed in surface waters of rivers in the Hangzhou region in China, with PAH concentrations reaching the µg L^{-1} range (Zhu et al. 2004). Oil spills / leaks can lead to even higher water concentrations, with reported values of 1 – 6 ppm (mg L^{-1}) after the North Cape Barge Spill in Rhode Island, USA (National Research Council 2003). Nevertheless, in the freshwater environment PAHs tend to adsorb to organic matter on sediments or be uptook by organisms (Burgess et al. 2003b; Latimer and Zheng 2003). Very high concentrations can be found in sediments near heavily polluted areas, sometimes in the mg/kg range (Zhu et al. 2004; Barakat et al. 2011). Organisms that live in close contact with sediments or that ingest contaminated particles may be more vulnerable to PAH contamination (Meador et al. 1995; Burgess et al. 2003a). Hence, benthic and epibenthic animals may be exposed to high amounts of PAHs in such environments, not only from waters but also from sediments.

The evaluation of PAHs effects in natural environments has proven challenging, given the differing metabolizing abilities among species, variety of effects that these compounds can elicit, and the fact that these compounds occur mainly as complex mixtures (Meador 2008). Moreover, for some invertebrate groups, such as the Platyhelminthes, there is virtually no toxicological information. Nevertheless, members of this phylum, such as freshwater planarians will likely be exposed to these contaminants. Given their epibenthic and predatory nature, (Vila-Farré and Rink 2018), planarians can be exposed to a variety of chemicals that end up in the freshwater ecosystem; chemicals that remain in the water column, those that adsorb to sediments and those found in their prey (Meador et al. 1995; Burgess et al. 2003a). Planarians are simple animals, but already possess a centralized brain, that shares similarities with the nervous system of vertebrates (Buttarelli et al. 2008). Planarians also display a range of behaviours in response to external stimuli, such as, negative phototaxis, thermotaxis, chemotaxis, or thigmotaxis, which are processed by the central nervous system (CNS) (Inoue et al. 2015). However, their most striking feature is the ability to regenerate from almost any fragment. This ability is provided by the pluripotent stem cells – neoblasts – that are distributed throughout their bodies, being responsible for cell turnover and regeneration of missing body parts (Newmark and Sánchez Alvarado 2002). As laboratory animals, planarians are easily reared and obtained in large numbers (Oviedo et al. 2008) and several behavioural endpoints, such as feeding and locomotion, can be easily evaluated (Rodrigues et al. 2016; Ofoegbu et al. 2016). All of these features make planarians very attractive as experimental animals for several research areas, such as developmental biology, regeneration research (Newmark and Sánchez Alvarado 2002), neuropharmacology (Buttarelli et al. 2008), and ecotoxicology (Wu and Li 2018).

In this work, our objective was to investigate the effects of single exposures to phenanthrene, pyrene and benzo[a]pyrene (B[a]P), on the freshwater planarian *Girardia tigrina*. This species was chosen since it one of the most well studied planarian species (Wu and Li 2018) and has been previously used for the testing of environmental contaminants (e. g. Knakievicz and Ferreira 2008;

Córdova López et al. 2019), being one of the only two planarian species for which chemical carcinogenicity has been demonstrated (see Voura et al. 2017). Evaluated responses include survival, behavioural endpoints (locomotor activity and feeding rate) and presence of PAHs in tissues. Cephalic regeneration (after decapitation) was evaluated as a measure of developmental toxicity, since this process relies on the proliferation of stem cells (Wu and Li 2018). The choice of compounds was based on the lack of ecotoxicological data concerning freshwater planarians and since these are among the most common PAHs in environmental samples, also included in the EPA priority list due to their toxicity to mammals and freshwater organisms (EPA 1987).

2 Methods

2.1 Experimental organisms

Planarians belonged to a sexually reproducing *G. tigrina* population, maintained in the laboratory for several years. Animals were kept in non-toxic plastic containers with ASTM hard water (ASTM 2014) at 20 °C. Cultures were maintained in near darkness, except when feeding. Once a week either cow liver or chironomid larvae were provided as food items. Cow liver was ground and stored in aliquots at -20 ºC until needed. Media and containers were changed twice a week, immediately after feeding and 2 to 3 days after.

Prior to experiments, planarians were starved for 7 days to ensure a uniform metabolic state (Oviedo et al. 2008). Before tests, planarians were measured while moving and fully extended in a crystallizing vial (with a small amount of water) placed above a millimetric sheet. Tests were performed at 20 ± 1.5 °C with a photoperiod of 16 h / 8 h light /dark, but planarians were kept at low ambient lighting, except when solutions were changed.

Chironomus riparius larvae were used as live prey for planarians and were taken from laboratory cultures maintained in non-toxic plastic containers with a layer of inorganic sediments and ASTM hard water with aeration. Sediments were sieved (< 1mm) and burned at 500 ºC during 4 h. Cultures were maintained at 20 °C with a 16 h / 8 h light / dark cycle and fed every other day with a suspension of macerated fish food (Tetramin®). Media and containers were replaced every two weeks.

2.2 Tests

2.2.1 Lethality tests

Animals of 1.05 ± 0.15 cm were selected. Planarians were kept in glass crystallizing vials, with 50 mL of test solution. Four replicates of 5 animals each were performed for each concentration. Stock solutions were performed in dimethyl sulfoxide (DMSO; CAS Number 67-68-5; Fisher Chemical;

purity ≥ 99.7%). For every performed concentration, a stock solution was obtained by dilution of the most concentrated stock and total amount was kept at 0.01% (v/v) of final experimental solution volume. Solutions were renewed after 48 h and new stock solutions were freshly prepared. To prevent evaporation, the vials were also kept covered with glass lids. Test solutions were performed by adding the stock solutions to ASTM hard water. For phenanthrene (CAS Number 85-01-8; Sigma-Aldrich; purity 98%), 7 concentrations were used, 401.9, 482.3, 578.7, 694.4, 833.3, 1000.0, 1200.0 µg L^{-1} plus a control (CTR – ASTM only) and a solvent control (SCTR – ASTM plus DMSO). Stock solutions were kept at -20°C until quantification. Mortality was recorded by visual inspection every 24 h, over a 96-h period. Immobile animals that did not respond to light stimulus and gentle prodding were considered dead. Any disintegration of body parts and behavioural abnormalities were also evaluated by visual inspection using a Zeiss stereomicroscope (Stemi 2000C, Carl Zeiss™). Head loss was evaluated as an acute parameter and only considered when the entire head was absent, but animals were still alive.

Acute tests were not performed for pyrene and B[a]P, since preliminary experiments recorded no mortality over 96 h using extremely high concentrations (up to 4500 µg L^{-1}).

2.2.2 Sub-lethal exposures

The selected animals measured 1.0 ± 0.1 cm. Exposure of animals used for feeding, locomotion and post-exposure cephalic regeneration was performed in glass crystallizing vials with 50 mL of test solution with 6 animals per vial, for a total of 24 animals per treatment. For pyrene (CAS Number 129-00-0; Sigma-Aldrich; purity 98%) and B[a]P (CAS Number 50-32-8; Sigma-Aldrich; purity ≥ 96%), concentrations of 2.5, 37.5, 75 and 150 µg L^{-1} were used. For phenanthrene 37.5, 75, 150 and 300 µg L^{-1} treatments were performed. The exposure period was 8 days for all endpoints, with renewal of solutions every 2 days. To prevent evaporation, the vials were also kept covered with glass lids. Stock solutions were freshly prepared every time test solutions were renewed. Stock solutions were prepared in DMSO, 1 for every concentration, and total amount in test solutions was kept at 0.01% (v/v). Controls (CTR) and solvent controls (SCTR) were performed. Stock solutions were kept at -20°C until quantification. Exposure of animals used for feeding, locomotion and post-exposure cephalic regeneration was performed in glass crystallizing vials with 50 mL of test solution.

2.2.2.1 Post-exposure feeding

After exposure, 9 animals were selected from each treatment and used to evaluate the feeding endpoint. Animals were washed in clean media and feeding was evaluated as previously described (Chapter II). Briefly, each planarian was placed in a glass crystallizing vial with 50 mL of ASTM hard water containing 30 *C. riparius* larvae (2nd larval stage). Planarians were allowed to capture and feed on larvae for 3 h, after which planarians were removed from the vials and the number of

remaining food items was counted by visual inspection with a Zeiss stereomicroscope (Stemi 2000C, Carl Zeiss™).

2.2.2.2 Post-exposure locomotion

From each treatment, 15 animals were used to measure the locomotion. Animals were washed in clean media and each was transferred to a well of a 24 well plate with 1 mL of medium. A video was recorded in a ZebraBox™ device using the ZebraLab® v3 software (Viewpoint, France), with a protocol to measure planarian locomotion, based on a previous study (Rodrigues et al. 2016), with some modifications. Briefly, recordings were performed over a 13 min period, including a 1 min acclimation in light (approximately 550 lux), followed by 4 min in the same conditions, a 4 min period in the dark and another 4 min period in light. Similarly to studies using zebrafish (e. g. Irons et al. 2010), we used cycles of dark and light in an attempt to determine if PAH exposure could elicit distinct reactions to alternating light stimuli. Readings on distance travelled (cm) and time in activity (s) were recorded every second and pooled by minute. The software tracks the centre of mass of the animal, and when this is displaced, the animal is considered as moving, otherwise the animal is classified as immobile. Time in activity is obtained by the period (s) in which the animal is considered as moving.

2.2.2.3 Cephalic Regeneration

Post-exposure cephalic regeneration of planarians was evaluated in the same 15 animals used for the locomotion assay. After locomotion assessment, each animal was decapitated with a scalpel, just below the auricles and above the pharynx. Each planarian was placed in a small crystalizing vial with 10 mL of clean media and left to regenerate.

Cephalic regeneration in PAH-contaminated media was also evaluated; animals were decapitated and placed individually in vials with 10 mL of experimental solutions (see section 2.2.2 for controls and concentrations used). At the end of the 8-day exposure period, animals were weighed with an AND GH-202 analytical balance (A&D Company Ltd.) and kept at -20 ºC for analysis of PAH residues in tissues.

Cephalic regeneration was checked with a Zeiss stereomicroscope (Stemi 2000C, Carl Zeiss™) every 24 h and followed over the necessary period to allow for photoreceptor and auricle formation. The number of days until appearance of eyespots and auricles was recorded for every replicate.

2.3 Detection of PAHs in stock solutions and planarians

PAHs were detected by wavelength fluorescence (FF) in stock solutions and in planarian tissues. This method can be applied to detect PAHs, since these compounds are strong fluorophores, absorbing light in the UV/visible region and emitting light at longer wavelengths, in the same spectrum

region (Guilbault et al. 1990; Beyer et al. 2010). The excitation and emission wavelengths are dependent on structural properties, being possible to discriminate PAHs from other compounds (Beyer et al. 2010). However, closely related molecules, such as PAHs and respective metabolites, are difficult to distinguish and results are based on number of aromatic rings and expressed as amount of parent PAH and associated compounds (Aas et al. 2000; Beyer et al. 2010). For detection of phenanthrene-, pyrene-, and B[a]P-type compounds, the following excitation and emission wavelength pairs were used, respectively: 256/380 nm; 341/383 nm; 380/430 nm (Aas et al. 2000; Oliva et al. 2010). Slit widths were set at 2.5 nm. Standard curves of known concentrations of the parent PAH were performed and used to calculate limit of detection (LOD) and limit of quantitation (LOQ). Stock solutions and tissues were analysed in a similar way as previously described, with slight modifications (Aas et al. 2000; Dissanayake and Galloway 2004; Almeida et al. 2012; Silva et al. 2013). Briefly, stock solutions were diluted at 1:30 in 50% methanol (CAS Number: 67-56-1; Fisher Chemical; ≥ 99.8%). For detection in tissues, 15 planarians were used per treatment and each animal was individually homogenized in 400 µL of ultra-pure water using a sonic homogenizer (Branson Ultrasonics™ Sonifier 250) and 150 µL of the whole homogenate were diluted in 1450 µL of methanol at 50%. Before measurements, all samples (stock solutions and planarian tissues) were homogenized using ultrasonic vibration (J. P. Selecta, Ultrasonic Bath) for 1 min. Samples were read in 96-well plates, with each sample performed in quadruplicates and running blanks in every plate for calibration of samples. Readings were performed at 20°C. Concentrations were estimated by using the standard curves of known parent PAH concentrations. The results for stock solutions were expressed in g L^{-1} of PAH and tissue results were expressed in ng of PAH equivalents/mg of tissue (wet weight). All fluorescence measurements were performed with a Hitachi F-7000 Fluorescence Spectrophotometer (Hitachi High-Technologies Corporation).

2.4 **Statistical analyses**

For the acute test, data for each replicate was transformed in % of survival and used to calculate the LC_{50} through a 4-parameter logistic regression, with x as Log (concentration). Similarly, an EC_{50} for head loss was obtained, and data was presented as % of head loss.

For the post-exposure feeding, treatments were analysed with one-way ANOVA, followed by Dunnett's post-hoc test to evaluate differences from the control treatment (SCTR). Whenever normality (tested with Shapiro-Wilk's test) or homogeneity of variances could not be achieved, data was analysed with Kruskal-Wallis followed by Dunn's post-hoc test. EC_{50s} for this endpoint were calculated, similarly as described above.

For the post-exposure locomotion, a linear mixed effects model was used to investigate the effect of PAHs on distance travelled. PAH concentration and light period were included as fixed effects, as well as the interaction between the two; to account for the repeated measures, planarian identity was included in the model as a random effect (R software, lme4 package; Bates et al. 2015). The

significance of fixed effects was calculated via Satterthwaite's degrees of freedom method (R software, LmerTest package; Kuznetsova et al. 2017). Total distance travelled and time spent in activity during the 12 min was analysed with one-way ANOVA, followed by Dunnett's post-hoc test to evaluate differences in comparison to the control treatment (SCTR). Whenever normality (tested with Shapiro-Wilk's test) or homogeneity of variances could not be achieved, data was analysed with Kruskal-Wallis followed by Dunn's post-hoc test.

For the cephalic regeneration endpoint, treatments were analysed with Kruskal-Wallis one-way analysis of variance, with a Dunn's post-hoc test ($P < 0.05$) to detect differences from the solvent control.

For calculations on PAHs in planarian tissues, measurements that fell below LOQ were set as 0. Treatments were analysed with Kruskal-Wallis one-way analysis of variance, with a Dunn's post-hoc test ($P < 0.05$) to detect differences from the solvent control.

Analyses were performed with SigmaPlot v. 12.3, GraphPad Prism v. 6.01 and R v. 3.5.1 software.

3 Results

3.1 Analysis of stock solutions

The analysis of stock solutions revealed that concentrations were, generally, close to nominal concentrations in both lethal (Supplementary Table III-11) and sub-lethal exposures (Supplementary Table III-12).

3.2 Lethality test

A 96 h LC_{50} of 830.1 (780.4 – 883.0) µg L^{-1} and 96 h EC_{50} of 483.2 (469.0 – 497.9) µg L^{-1} for head loss were estimated for planarians exposed to phenanthrene (Supplementary Figure III-10). Surviving exposed animals presented behavioural anomalies and disintegration of tissues. Disintegration of tissues progressed gradually from the snout, with many animals losing the entire head. This progressed further in some animals, resulting in mortality. Animals with no apparent disintegration of tissues, generally presented screw-like behaviour (sensu Buttarelli et al. 2008) or stillness with lack of response to stimuli, but this was not quantified. Animals presenting disintegration of tissues, had abnormal behaviours in the form of convulsions and spasms.

Preliminary tests with pyrene and B[a]P did not reveal mortality within 96 h. However, abnormal behaviour was observed in exposed animals to both PAHs, mainly contractions of the body and spasms. Some of the animals exhibited head loss.

3.3 Sub-lethal exposures

3.3.1 PAHs in planarian tissues

All PAHs were detected in planarian tissues. For phenanthrene, there was a dose-dependent increase of fluorescence, starting from the 75 µg L^{-1} treatment (H = 56.322; 4 d. f.; p = < 0.001; Figure III-7 – a). In the pyrene and B[a]P experiments, there was a significant dose-dependent increase in the PAH-type compounds detected starting from the 37.5 µg L^{-1} treatments (Pyrene: H = 69.217; 4 d. f.; p < 0.001. B[a]P: H = 57.575; 4 d. f.; p < 0.001. Figure III-7 – b, c).

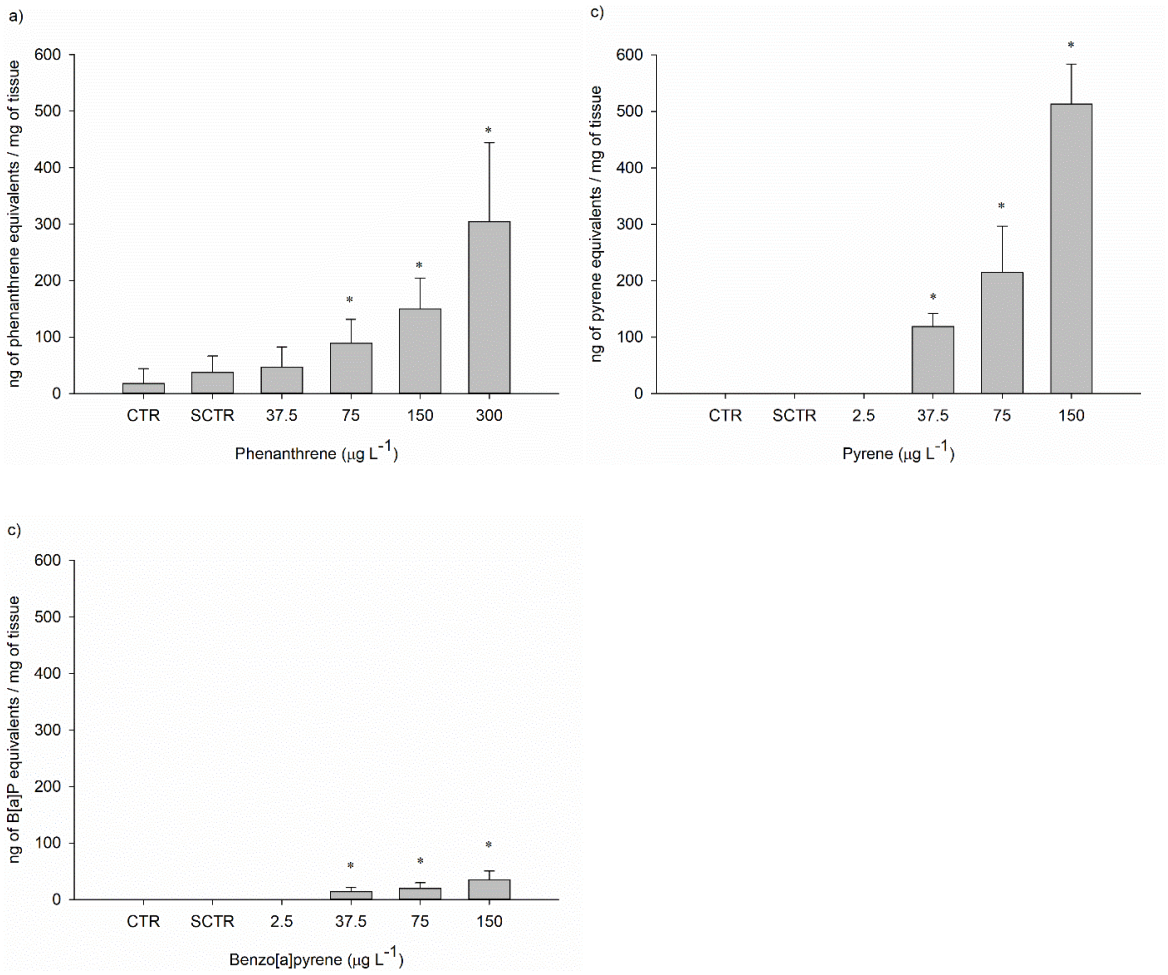

Figure III-7 – Presence of PAH-type compounds in tissue homogenates of *Girardia tigrina*, after exposure to: a) Phenanthrene; b) Pyrene; c) B[a]P. Data is presented as mean (± SD) ng of PAH-type compounds per mg of planarian tissue. Treatments were compared using Kruskal-Wallis one-way analysis of variance followed by Dunn's post-hoc test. * P ≤ 0.05.

3.3.2 Post-exposure feeding

All PAHs inhibited the feeding of planarians. Phenanthrene exposed animals ate fewer prey items at 300 and 150 µg L^{-1} ($F_{4, 40}$ = 11.285; p < 0.001; Figure III-8 – a). The EC_{50} for feeding inhibition

was 154.0 µg L^{-1} (41.830 to 566.6; r^2 = 0.523; Supplementary Figure III-13 – a). Pyrene decreased feeding of planarians at 75 and 150 µg L^{-1} ($F_{4, 40}$ = 96.021; p < 0.001; Figure III-8 – b). The EC_{50} for feeding inhibition was 85.56 µg L^{-1} (62.06 to 118.00; R^2 = 0.906; Supplementary Figure III-13 – b). In the B[a]P exposure, planarians ate fewer larvae at 37.5, 75 and 150 µg L^{-1} (H = 35.330; 4 d.f.; p < 0.001; Figure III-8 – c), with 100% of feeding inhibition at 150 µg L^{-1}. The EC_{50} for feeding inhibition was 13.14 µg L^{-1} (5.636 to 30.630; R^2 = 0.915; Supplementary Figure III-13 – c).

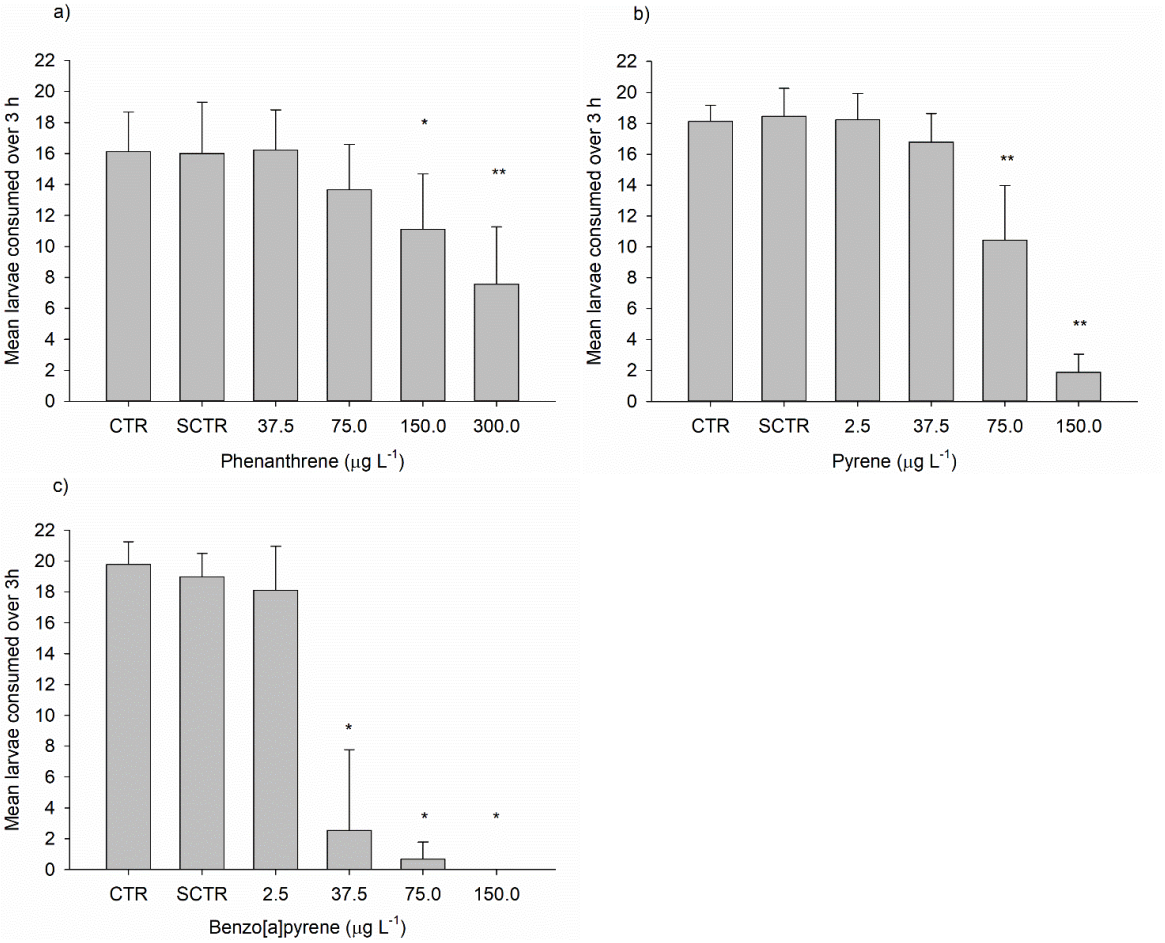

Figure III-8 – Effects of PAHs of the post-exposure feeding of *Girardia tigrina*. Data is presented as mean (± SD) *Chironomus riparius* larvae consumed over a 3 h period after exposure to: a) Phenanthrene; b) Pyrene; c) B[a]P. Treatments were compared using one-way ANOVA with a Dunnett's post-hoc test (phenanthrene and pyrene) or with Kruskal-Wallis one-way analysis of variance followed by Dunn's post-hoc test (B[a]P). * P ≤ 0.05; ** P ≤ 0.01.

3.3.3 Post-exposure locomotion

Locomotion of planarians was impaired by PAH exposure (Figure III-9). The mixed model analysis indicated no differences in the distance travelled between light and dark periods, but there was a significant decrease in the travelled distance with increasing concentrations for all tested PAHs. No significant interactions were found between lighting conditions and PAH concentrations (Table III-8). Total distance travelled (H = 4.642; 4 d. f.; p = 0.326; Supplementary Figure III-11 – a) and total

time spent in activity (H = 7.498; 4 d. f.; p = 0.112; Supplementary Figure III-12), were not significantly affected in the phenanthrene exposure. Pyrene affected the total distance travelled ($F_{4, 68}$ = 8.617; p < 0.001; Supplementary Figure III-11 – b) and total time spent in activity (H = 26.667; 4 d. f.; p < 0.001; Supplementary Figure III-12 – b) in animals exposed to 75 and 150 µg L^{-1}. In the B[a]P experiment, planarians exposed to 75 and 150 µg L^{-1}, travelled smaller distances (H = 17.761; 4 d. f.; p = 0.001; Supplementary Figure III-11 – c) and spent less time in activity (H = 27.398; 4 d. f.; p < 0.001; Supplementary Figure III-12 – c) compared to the control treatment.

Table III-8 – Estimates of the fixed effects from the linear mixed model applied to distance travelled in the locomotion endpoint.

	Parameters:	**Estimate**	**Std. Error**	**df**	**t value**	**Pr (>|t|)**
Phenanthrene	Intercept	61.082	4.548	101.688	13.432	< 0.001
	Light 1	-1.327	3.154	146	-0.421	0.674
	Light 2	-3.671	3.154	146	-1.164	0.246
	Treatment	-0.067	0.029	101.688	-2.262	0.026
	Treatment * Light 1	-0.028	0.020	146	-1.381	0.169
	Treatment * Light 2	0.017	0.020	146	0.815	0.417
Pyrene	Intercept	51.398	2.643	129.679	19.443	< 0.001
	Light 1	2.486	2.461	142	1.010	0.314
	Light 2	-2.086	2.461	142	-0.848	0.398
	Treatment	-0.153	0.034	129.679	-4.503	< 0.001
	Treatment * Light 1	-0.009	0.031	142	-0.279	0.780
	Treatment * Light 2	-0.015	0.031	142	-0.480	0.632
Benzo[a]pyrene	Intercept	51.189	4.031	91.793	12.698	< 0.001
	Light 1	1.350	2.334	146	0.578	0.564
	Light 2	1.450	2.334	146	0.621	0.535
	Treatment	-0.201	0.052	91.793	-3.851	< 0.001
	Treatment * Light 1	-0.030	0.030	146	-1.006	0.316
	Treatment * Light 2	-0.024	0.030	146	-0.796	0.427

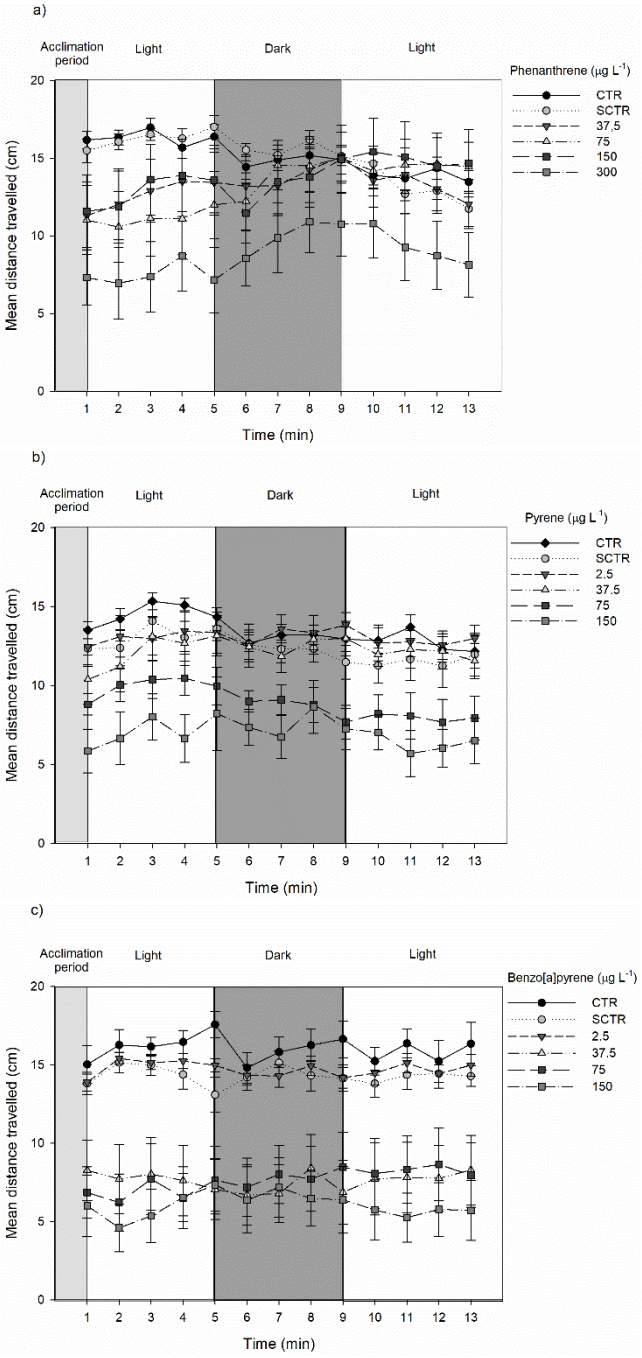

Figure III-9 – Distance travelled over 12 min by *Girardia tigrina* individuals exposed to: a) Phenanthrene; b) Pyrene; c) Benzo[a]pyrene. Data is presented as mean (± SD) distance travelled per minute.

3.3.4 Cephalic Regeneration

Post-exposure regeneration of photoreceptors (H = 2.070; 4 d. f.; p = 0.839, Table III-9) and auricles (H = 0.000; 4 d. f.; p = 1.000; Table III-9) was unaffected by phenanthrene exposure. Pyrene delayed the post-exposure regeneration of photoreceptors in animals exposed to 150 and 75 µg L^{-1} (H = 25.427; 4 d. f.; p < 0.001; Table III-9), but not of auricles (H = 0.000; 4 d. f.; p = 1.000; Table

III-9). B[a]P affected post-exposure regeneration of photoreceptors (H = 11.998; 4 d. f.; p = 0.017; Table III-9), but Dunn's *post-hoc* test ($p < 0.05$) could not detect differences between treatments and control group (SCTR). Regeneration of auricles was not affected (H = 5.458; 4 d. f.; p = 0.243; Table III-9).

Regeneration of photoreceptors (H = 4.223, 4 d. f.; p = 0.377; Table III-9) and auricles (H = 4.000; 4 d. f.; p = 0.406; Table III-9) was unaffected in phenanthrene-contaminated solutions, although exposed animals presented screw-like behaviour. Also, 1 animal exposed to 300 µg L^{-1} lost the tail portion on day 3 of exposure but started regenerating and had a small tail by the end of the exposure period. 2 other animals had small injuries by day 5, 1 exposed to 37.5 and other to 75 µg L^{-1}, but by the end of the test, they were almost unnoticeable. Pyrene contaminated media did not delay regeneration of photoreceptors (H = 4.765; 4 d. f.; p = 0.312; Table III-9) or auricles (H = 7.749; 4 d. f.; p = 0.101; Table III-9), but animals exposed to the 150 and 75 µg L^{-1} presented abnormal behaviour, such as twisting and contractions of the body. Cephalic regeneration in B[a]P-contaminated media was unaffected, with animals regenerating photoreceptors between the 4^{th} and 5^{th} days (H = 6.787; 4 d. f.; p = 0.148; Table III-9) and auricles between the 5^{th} and 6^{th} days (H = 4.203; 4 d. f.; p = 0.379; Table III-9). One animal in the 2.5 µg L^{-1} treatment lost the posterior part of its body, including the pharynx, but still regenerated both cephalic structures after 5 days. Behaviour was severely affected at all concentrations, especially when media renewals were performed. Some abnormal behaviours included locomotion only by muscular contraction (as opposed to usual locomotor behaviour by ciliary beating), spasms, snake-like movement (sensu Buttarelli et al. 2008) and severe contraction behaviour in avoidance of bright lights.

Table III-9 – Regeneration of *Girardia tigrina*'s cephalic structures (photoreceptors – P, and auricles - A) in post- and during exposures to phenanthrene, pyrene, and benzo[a]pyrene. Data is presented as mean number of days (± SD) until the regeneration of structures for each treatment. Treatments were compared with Kruskal-Wallis followed by Dunn's post-hoc test to detect differences from the control treatment (SCTR). Statistical differences from the control group are represented by asterisks (* P ≤ 0.05).

		Phenanthrene (µg L^{-1})					Pyrene (µg L^{-1})					Benzo[a]pyrene (µg L^{-1})				
		SCTR	37.5	75.0	150.0	300.0	SCTR	2.5	37.5	75.0	150.0	SCTR	2.5	37.5	75.0	150.0
Post-exposure regeneration	P	4.0 ± 0.0	4.1 ± 0.3	4.1 ± 0.3	4.1 ± 0.3	4.1 ± 0.3	4.1 ± 0.3	4.1 ± 0.3	4.3 ± 0.5	**4.7 ± 0.5***	**4.7 ± 0.5***	3.8 ± 0.4	3.9 ± 0.4	4.1 ± 0.4	4.1 ± 0.4	4.2 ± 0.4
	A	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	4.6 ± 0.5	4.6 ± 0.5	4.7 ± 0.5	4.7 ± 0.5	4.9 ± 0.3
Regeneration in contaminated media	P	4.1 ± 0.3	4.1 ± 0.3	4.2 ± 0.4	4.3 ± 0.5	4.3 ± 0.5	4.0 ± 0.0	3.9 ± 0.4	4.0 ± 0.4	4.0 ± 0.0	4.1 ± 0.4	4.1 ± 0.4	4.1 ± 0.4	4.3 ± 0.5	4.4 ± 0.5	4.5 ± 0.5
	A	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.1 ± 0.3	5.0 ± 0.0	5.3 ± 0.5	5.2 ± 0.4	5.2 ± 0.4	5.2 ± 0.4	5.6 ± 0.6	5.4 ± 0.5	5.1 ± 0.4	5.2 ± 0.4	5.1 ± 0.4	5.3 ± 0.5

Table III-10 – Summary of the tested endpoints and respective LOECs (lowest observed effect concentration) and / or EC_{50}s (median effective concentration) for the freshwater planarian *Girardia tigrina* exposed to phenanthrene, pyrene and benzo[a]pyrene.

		PAHs (µg L^{-1})		
Endpoint	Exposure period (days)	Phenanthrene	Pyrene	Benzo[a]pyrene
Mortality	4	EC_{50} - 830.1	EC_{50} > 4500	EC_{50} > 4500
Head disintegration	4	EC_{50} - 483.2	nc	nc
Regeneration	8	nd	nd	nd
Regeneration (post-exposure)	8	nd	LOEC: 75	nd
Feeding	8	LOEC: 150 / EC_{50}: 154	LOEC: 75 / EC_{50}: 85.56	LOEC: 37.5 / EC_{50}: 13.14
Total distance travelled	8	nd	LOEC: 75	LOEC: 75

nc: not calculated

nd: no statistical differences detected

4 Discussion

In this work we explore and highlight the potential of planarians for ecotoxicological research by evaluating a variety of endpoints in response to PAH exposure. Behavioural endpoints were especially sensitive and fluorescence analysis evidenced uptake of compounds from the solutions in a dose-dependent fashion.

The detection of PAH-type compounds was an important factor in the evaluation of impacts on planarians. The FF technique has been shown to be a sensitive, fast and simple method to detect PAH-type compounds in bile and liver fractions (Aas et al. 2000), brain and muscle (Almeida et al. 2012) of fish and also in eye, digestive gland, muscle (Silva et al. 2013) and fluids (Dissanayake and Galloway 2004) of crustaceans. In our study, detection of PAH-type compounds in planarian tissues was especially relevant, since it clearly shows that there was an uptake from solutions into planarian tissues in a dose-dependent way.

A 96 h LC_{50} was only possible to estimate for phenanthrene (830.1 µg L^{-1}), but not for pyrene and B[a]P, since the latter compounds did not cause any mortality even at very high concentrations (up to 4500 µg L^{-1}). This result is not surprising, since many studies show that PAHs with low number of aromatic rings (2- and 3-rings) are generally more acutely toxic than PAHs with higher number of

rings (≥ 4-rings), with the latter frequently inducing mutagenic and/or carcinogenic effects (Manzetti 2013). It seems that the sensitivity of *G. tigrina* towards phenanthrene is in the same range as for *Daphnia*, [48 h LC_{50}s – 730.67 µg L^{-1} and 0.96 – 1.28 mg L^{-1} (Geiger and Buikema Jr. 1982; Verrhiest et al. 2001)], and slightly higher than *Chironomus* [96 h LC_{50s} - 1.21 mg L^{-1} and 1.60 mg L^{-1} (Morais et al. 2014; Richardi et al. 2018)], at least considering mortality. However, effects of phenanthrene in sub-lethal endpoints for *Daphnia* [7 d EC_{50} for growth: 349 ± 19 µg L^{-1} (Olmstead and LeBlanc 2005); reproduction: LOEC 0.36 mg L^{-1} (Geiger and Buikema Jr. 1982)] and *Chironomus* [body size: LOEC: 0.12 mg L^{-1} (Richardi et al. 2018); DNA damage LOEC: 0.16 mg L^{-1} (Morais et al. 2014)] seem to be in the same range as for *G. tigrina*. On the other hand, pyrene exposure can lead to mortality in daphnids at lower concentrations than phenanthrene [48 h LC_{50s}: 135.8 µg L^{-1} (Brausch and Smith 2009), 67.9 ± 7.90 µg L^{-1} (Clément et al. 2005)]. However, the sensitivity of sub-lethal endpoints [7 d EC_{50} for growth: 72.7 ± 7.8 µg L^{-1} (Olmstead and LeBlanc 2005); feeding after 24 h exposure: reduction of ≈ 50 % at 101.13 8 µg L^{-1} (Perminova et al. 2001)] seems to be in the same range as for *G. tigrina*. Chironomids seem to be the least sensitive, with the only study evaluating pyrene effects, evidencing no effects on survival and dry weight of larvae exposed at 200 µg/g of spiked sediments over a 10-day period (Clément et al. 2005). Finally, Chironomids seem to possess a high tolerance towards B[a]P, with mortality being recorded at very high concentrations [24 h LC_{50} 9.873 (7.426–14.61) mg L^{-1} (Ha and Choi 2008)], as opposed to daphnids that seem to be much more sensitive [24 h EC_{50} for immobilization - 29.3 (13.32–242.2) µg L^{-1} (Ha and Choi 2009); 48 h LC_{50} - 0.25 ± 0.04 mg L^{-1} (Atienzar et al. 1999)]. Similarly, sub-lethal impacts of B[a]P on chironomids [larval weight: LOEC 1 mg/L (Ha and Choi 2008)] seem to occur at much higher concentrations than in daphnids [14 d number of neonates: LOEC 0.025 mg L^{-1}; 21 d number of neonates: LOEC 0.02 µg L^{-1} (Atienzar et al. 1999; Ha and Choi 2009)] and planarians.

Information regarding toxicity of PAHs to planarians is very scarce, although a recent study evidenced a low degree of toxicity of these 3 PAHs to *Dugesia japonica*, with pyrene affecting regeneration rate and thermotaxis, but only at very high concentrations (2 - 20 mg L^{-1}) (Zhang et al. 2018). It seems that, similarly to other aquatic invertebrates, *G. tigrina* can survive under short-term exposures to pyrene or B[a]P at high levels (Clément et al. 2005; Silva et al. 2013). It is also interesting to note that, although we could only estimate an LC_{50} for phenanthrene and not for the other PAHs, planarians were more sensitive to pyrene and B[a]P in the sub-lethal endpoints. This evidences that, in planarians, the mortality endpoint may not be the most suitable to evaluate effects of some compounds, since they may be able to survive and endure long periods of starvation. Our experiments illustrate this issue, for instance considering B[a]P exposure; it did not cause any mortality over 96 h at > 4500 µg L^{-1}, but the feeding endpoint was severely impacted, with an estimated EC_{50} of 13.14 µg L^{-1}. Although still alive, many of the exposed planarians would probably die in continued exposure over a long period, because they would not feed. These results emphasize the importance of evaluating alternative endpoints to mortality, as is the case of behavioural endpoints.

During lethality exposure, phenanthrene induced the disintegration of planarian tissues, with the severity of this disintegration being dependent on the concentrations tested. Severe xenobiotic stress in planarians can cause a progressive disintegration starting on the head region and progressing caudally, and in more extreme cases it can lead to complete body disintegration (e. g. Calevro et al. 1998). Some authors have argued that disintegration of the head region can be related to accumulation of xenobiotics in this portion, such as Wu and co-workers (2011) that found that *D. japonica* accumulated cadmium (Cd^{2+}) in the head portion and inhibited acetylcholinesterase activity. Other authors have also observed both head disintegration and neurotoxicity in planarians caused by organophosphorus insecticides (Villar et al. 1994) or head disintegration caused by a known neurotoxic agent (TBT) (Ofoegbu et al. 2016). In the case of PAHs, they have previously been shown to be neurotoxic to some aquatic invertebrates (Han and Wang 2009; e. g. Gauthier et al. 2016). Although the above-mentioned works might suggest a connection between accumulation of xenobiotics and disintegration of the head region, other studies have shown that different stress types may also lead to disintegration of tissues in planarians starting from the head and progressing caudally. Examples include heat stress and radiations (Brøndsted 1955). Further studies are needed to uncover the mechanisms behind this phenomenon that is still not clearly elucidated.

An intriguing result arises from the regeneration endpoint, since only slight or no delays were observed in response to PAH exposure. In planarians, regeneration is accomplished by a complex process that relies on neoblasts and their specialized progeny to replace the missing tissues (Newmark and Sánchez Alvarado 2002). In this way, it was expected that PAHs could be harmful for the process of planarian regeneration, since, at least B[a]P is a potent pro-carcinogen known to induce DNA damage and adducts in mammals, fish or invertebrates (Ball and Truskewycz 2013). When testing impacts of xenobiotics on planarians, regeneration may be a very sensitive endpoint, with delays or even malformations being often observed (Ofoegbu et al. 2016). Nevertheless, some studies have detected impacts of compounds on behavioural endpoints, but not on regeneration (Hagstrom et al. 2015) or regeneration in the presence of neurotoxic agents, such as cadmium (Calevro et al. 1998). Moreover, a recent study supports the results here obtained, showing that only pyrene at very high concentrations [100 µM (20.2 mg L^{-1})], but not phenanthrene or B[a]P, resulted in regeneration delays on *D. japonica* (Zhang et al. 2018). Also, Best and Morita (1991), observed lethality, but no signs of neoplasia or malformations in *D. dorotocephala* exposed to B[a]P up to 12 weeks, concluding that B[a]P is not a pro-carcinogen or teratogen to planarians or may be only slightly so. Nevertheless, contrasting results were observed in others studies; a high percentage of *Bdellocephala brunnea* individuals has been observed to regenerate with malformations after introduction of B[a]P crystals (5 µg) in their pharyngeal cavities (Hoshina and Teshirogi 1991), and a study by Foster (1963) found that 5% of *D. dorotocephala* individuals exposed to B[a]P developed neoplastic growths in 3-month exposures. Hence, the subject of regeneration and carcinogenicity under PAH exposure merits further study and probably requires knowledge on the metabolization of these compounds by planarians, since their carcinogenicity

and mutagenicity is related to metabolic activation resulting in more reactive and toxic metabolites (Ball and Truskewycz 2013). While planarians possess several phase I and phase II enzymes of xenobiotic metabolism (Wu and Li 2018), there is no information regarding planarian's specific metabolism towards PAHs. Even so, the fact remains that planarians have a high-fidelity regeneration system (Newmark and Sánchez Alvarado 2002) and, as suggested by Hagstrom et al. (2015), some toxicants might target very specific pathways, thus not affecting the regeneration dynamics of planarians. Still, it was intriguing that regeneration delays were observed only for the post-exposure regeneration experiment (in clean media) but not for the regeneration while in contaminated media. Yet, Hagstrom et al. (2015) have also observed that regenerating planarians were more resilient to several toxicants when compared to intact animals, with the LC_{50} for regenerating planarians under permethrin exposure being almost 3 times higher than for intact planarians. These authors propose that the differences in activity might reflect a difference in metabolism, since regenerating individuals are usually more immobile than intact planarians. Moreover, Balestrini et al. (2014) observed that regenerating *D. japonica* planarians exposed to berberine had considerably lower concentrations in their bodies when compared to exposed intact animals, indicating a lower uptake by the regenerating animals. These authors propose that the observed differences might be due to the absorption of berberine being only through the skin and not through the pharynx in non-feeding regenerating animals, as opposed to intact animals. Hence, these studies offer possible explanations for the observed differences in our regeneration experiments, although future studies should investigate this phenomenon.

The behaviour of planarians was highly affected by PAH exposure. This was consistent with the observed increases if PAH-type compounds in planarian tissues. Planarian feeding and locomotion were impaired, and other behavioural responses were also observed (but not quantified), including uncoordinated movements, stereotypical behaviours (screw-like behaviour and snake-like movement), lethargy and delayed or absent reactions to stimuli. Other studies have previously reported an impact of PAHs on feeding and/or locomotor behaviour of aquatic animals such as daphnids (Perminova et al. 2001) or prawns (Silva et al. 2013). In planarians, locomotion is achieved mainly by ciliary beating (gliding), but muscle contraction can also be employed. For normal locomotor behaviour, the correct functioning of the nervous system is required (Nishimura et al. 2007). Moreover, the feeding behaviour requires the coordination of several aspects by the nervous system: detection of chemical cues released from prey, movement toward prey, muscular action and mucus secretion to subdue prey, eversion of pharynx, secretion of digestive enzymes and suction of prey tissues (Jennings 1957; Inoue et al. 2015). As hydrophobic compounds, PAHs can accumulate and disrupt cellular membranes causing narcosis (Ball and Truskewycz 2013), which could be related to the observed motionless and delayed response to stimuli observed in the locomotor assay, that could help explain the decreases in distance travelled and feeding rate. Moreover, stereotypical behaviours have been related to disruption of the normal functioning of the neural pathways of planarians (Buttarelli et al. 2008) and evidence suggests that PAHs can be neurotoxic to poly-

chaetes and amphipods (Han and Wang 2009; Gauthier et al. 2016). Thus, a combination of both narcosis and disruption of neural pathways could help explain the observed behavioural responses. Also, it has been reported that in the amphipod *Corophium volutator* exposed to naphthalene (2-ring PAH), male ability to detect females was impaired, although females still produced hormones (Krång 2007), which suggests that chemical detection may be affected by PAHs. If so, feeding behaviour could also be impacted by the altered detection ability towards chemical cues released from food items. In terms of consequences for planarians populations, behavioural impairments can have serious impacts in natural environments. Sub-lethal effects can potentially lead to mortality in the long term or severely affect fitness. A reduced feeding activity has obvious impacts in growth and reproduction, while a reduction of locomotor activity can potentially result in vulnerability toward predators, in escaping from unfavourable situations and in the capture of prey, thus illustrating the ecological relevance of evaluating behavioural endpoints.

Regarding the evaluation of locomotion with the automated tracking system, it is noteworthy that although light and dark cycles were used, no differences were detected when light conditions were alternated. This was somewhat surprising, since planarians usually exhibit a very marked response of increased velocity when exposed to bright lights and usually move more slowly when in darkened environments (Davidson et al. 2011). Nevertheless, Zhang and colaborators (2018), observed that, after a 30 s light stimulus in the phototactic assay with *D. japonica*, the activity of animals in the following 30 s dark period was much higher than activity when in darkness for a long period. Probably in our experimental setup, the exposure to the light stimulus first, was responsible for the increased activity in the subsequent dark period, thus leading to the absence of differences between lighting conditions. Apparently, this seems to indicate that 4 min are not enough for *G. tigrina* to return to activity levels in darkness. Still, the automated assay was sensitive and able to detect effects elicited by PAHs, showing that the lighting conditions did not interact with PAH toxicity to shape locomotor responses and proving to be a useful tool for the evaluation of effects on locomotor activity of planarians.

5 Conclusions

Overall, planarians were severely affected by PAHs. Behavioural endpoints proved to be sensitive, detecting effects at much lower levels than mortality. Surprisingly, cephalic regeneration was not a very useful parameter to evaluate the toxicity of these compounds, since only slight delays were observed. Feeding was the most sensitive endpoint, clearly evidencing the toxic effects of these PAHs in a dose-dependent way. The toxicity to *G. tigrina* appears to be higher for B[a]P, followed by pyrene and lastly, phenanthrene, similarly as found for other freshwater organisms such as *Daphnia*. In terms of sensitivity, *G. tigrina* appears to be overall more sensitive than *Chironomus* and slightly less than Daphnia, when sub-lethal endpoints are considered. This study emphasizes

the usefulness of planarians for ecotoxicological studies, while evidencing the importance and relevance of studying behavioural endpoints.

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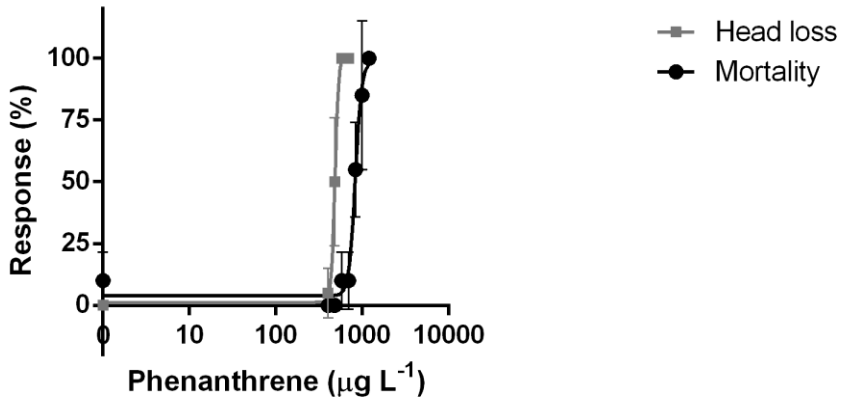
Supplementary material

Supplementary Table III-11 – Estimated concentrations for stock solutions of phenanthrene from the acute experiment. Concentrations are expressed as means (± SD) of both renewal days (days 0 and 2).

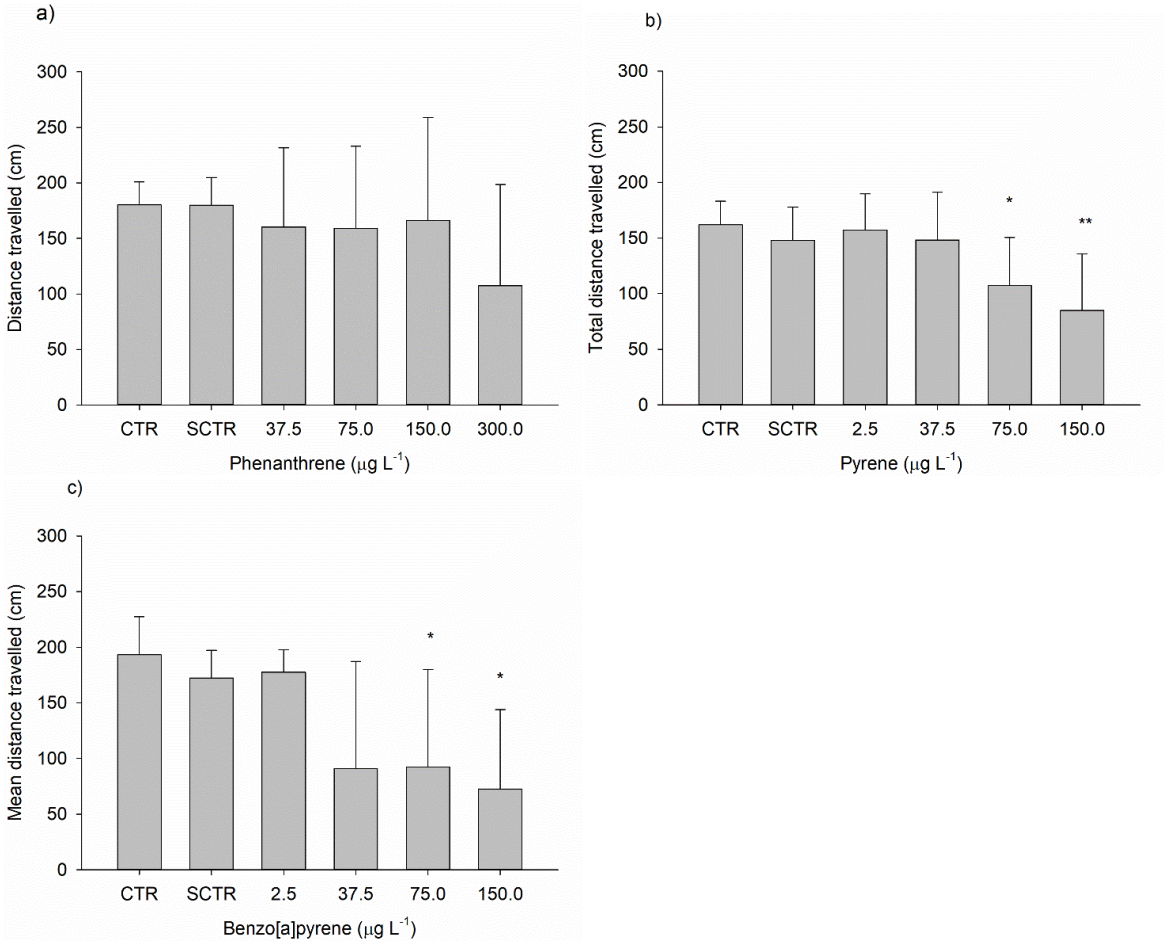
Phenanthrene nominal concentration (g L^{-1})	Estimated phenanthrene concentration (g L^{-1})	% of nominal concentration
12.000	12.061 ± 0.554	100.507
10.000	9.898 ± 0.496	98.977
8.333	8.626 ± 0.303	103.509
6.944	7.412 ± 0.414	106.741
5.787	5.952 ± 0.097	102.847
4.823	5.108 ± 0.050	105.919
4.019	4.058 ± 0.253	100.974

Supplementary Table III-12 – Estimated concentrations for PAH stock solutions from the 8-day sub-lethal exposures. PAHS: phenanthrene (Phe), pyrene (Pyr) and benzo[a]pyrene (B[a]P). Concentrations are expressed as means (± SD) of all renewal days (0, 2, 4 and 6).

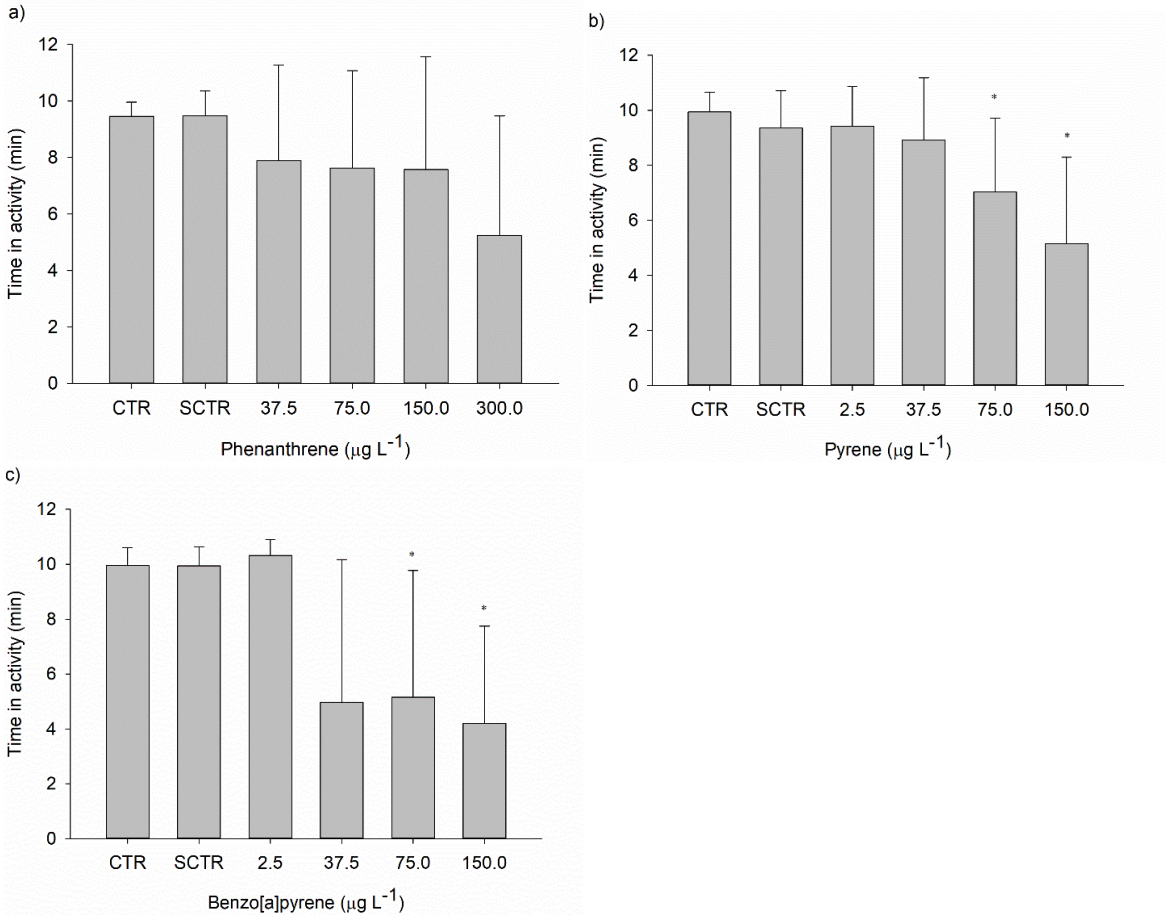
PAH	nominal concentration (g L^{-1})	Estimated concentration (g L^{-1})	% of nominal concentration	Estimated concentration (g L^{-1})	% of nominal concentration
Phe	3.000	2.802 ± 0.171	93.392	2.859 ± 0.09	95.298
	1.500	1.543 ± 0.034	102.881	1.522 ± 0.057	101.489
	0.750	0.792 ± 0.062	105.612	0.775 ± 0.006	103.361
	0.375	0.334 ± 0.015	89.167	0.334 ± 0.011	88.976
Pyr	1.500	1.645 ± 0.081	109.659	1.506 ± 0.085	100.419
	0.750	0.894 ± 0.037	119.135	0.811 ± 0.020	108.170
	0.375	0.449 ± 0.017	119.620	0.412 ± 0.017	109.816
	0.025	0.037 ± 0.003	146.283	0.032 ± 0.002	129.529
B[a]P	1.500	1.752 ± 0.137	116.785	1.735 ± 0.157	115.669
	0.750	0.920 ± 0.101	122.621	0.882 ± 0.081	117.651
	0.375	0.417 ± 0.036	111.319	0.445 ± 0.041	118.658
	0.025	0.030 ± 0.003	119.169	0.029 ± 0.003	117.694



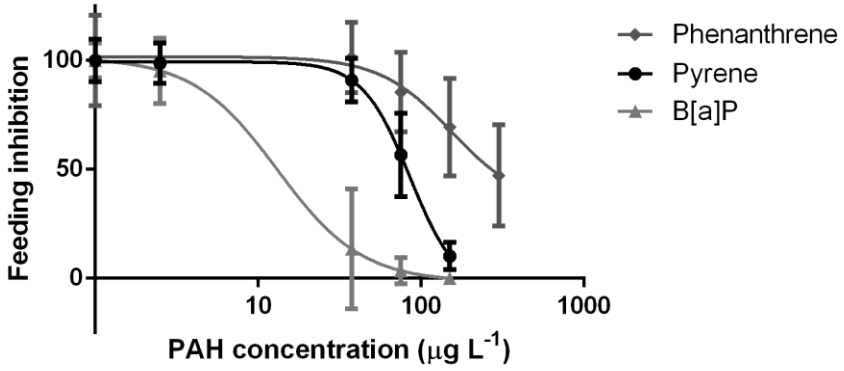
Supplementary Figure III-10 – Mortality and head loss in *Girardia tigrina* in the phenanthrene acute test at 96 h. Data points are presented as mean (±SD) for each tested concentration, fitted with a 4-parameter logistic regression (with x as Log (concentration)). The 96 EC_{50}s for mortality and head loss were calculated as 830.1 and 483.2 µg L^{-1}.



Supplementary Figure III-11 – Total distance travelled in 12 minutes by *Girardia tigrina* exposed to: a) Phenanthrene; b) Pyrene; c) B[a]P. Data is presented as mean (± SD) distance travelled (cm). Treatments were compared using one-way ANOVA with a Dunnett's post-hoc test (pyrene) or with Kruskal-Wallis one-way analysis of variance followed by Dunn's post-hoc test (Phenanthrene and B[a]P). Differences from the control group (SCTR) are represented by asterisks. * P ≤ 0.05; ** P ≤ 0.01.



Supplementary Figure III-12 – Total time spent in activity by *Girardia tigrina* in the locomotion test for the a) phenanthrene, b) pyrene and c) benzo[a]pyrene exposures. Data is presented as mean (± SD) time in activity (s). Treatments were compared using Kruskal-Wallis followed by Dunn's post-hoc test. Differences from the control group (SCTR) are represented by asterisks above columns. * P ≤ 0.05.



Supplementary Figure III-13 – Feeding inhibition in *Girardia tigrina* in the 8-day sublethal tests with phenanthrene, pyrene and benzo[a]pyrene (B[a]P). Data points are presented as mean (±SD) for each tested concentration, fitted with a 4-parameter logistic regression (with x as Log (concentration)). The 8-day EC_{50}s for feeding inhibition were calculated as 154.0, 85.56 and 13.14 µg L^{-1} for phenanthrene, pyrene and B[a]P, respectively.

Chapter IV – Biochemical responses of *Girardia tigrina* exposed to phenanthrene, pyrene and benzo[a]pyrene

Biochemical responses of *Girardia tigrina* exposed to phenanthrene, pyrene and benzo[a]pyrene

Abstract

Planarians are promising experimental animals for ecotoxicological studies and are increasing in relevance for this research area. However, for many freshwater contaminants, such as polycyclic aromatic hydrocarbons (PAHs), there is scarce to no information regarding their specific toxicity towards planarians. Hence, we aimed to uncover how some important physiological functions, such as, neurophysiology, cellular energy allocation and consumption, as well as, detoxification and oxidative stress status, are affected on the freshwater planarian *Girardia tigrina* by low and high molecular weight PAHs [phenanthrene, pyrene and benzo[a]pyrene (B[a]P)]. Phenanthrene affected planarian antioxidant defences and detoxification biomarkers, observed as increases in levels of total glutathione (TG) and activity of glutathione-S-transferase (GST), the latter in a dose-dependent way (from 75 to 300 µg L^{-1}). Moreover, phenanthrene also decreased carbohydrate levels in planarians exposed to 150 and 300 µg L^{-1}. Pyrene caused an increase in electron transport system activity (ETS), and a decrease in cellular energy allocation (CEA), revealing higher oxygen consumption and decreased energy budget in planarians exposed to 75 and 150 µg L^{-1}. Added to this, lipid peroxidation (LPO) was lower in planarians exposed to 2.5 and 75 µg L^{-1} of pyrene. In the B[a]P experiment, planarian GST activity decreased in a dose-dependent way and LPO levels were decreased at 37.5, 75 and 150 µg L^{-1} of B[a]P. The present data indicate that each of these PAHs seems to elicit somewhat different biochemical responses in planarians, possibly pointing to distinct biochemical interactions and / or metabolization of each compound. This study is the first report on the biochemical effects of PAHs on freshwater planarians and evidences that these compounds can affect planarian oxidative defences, detoxification mechanism and energy budget, raising concerns on their potential impacts in environmental scenarios.

Keywords: Planarians; PAHs; oxidative stress; neurotoxicity; energy allocation; lipid peroxidation.

1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a class of widespread compounds in the environment (Alegbeleye et al., 2017; Burgess et al., 2003). This is because they occur in fossil fuels, are formed through incomplete combustion of organic matter and are released as a result of many anthropogenic activities, hence assuring their ubiquity in all environmental compartments (Burgess et al., 2003; Manzetti, 2013). Many of the inputs of PAHs to the aquatic environment are resultant of spills and leaks of petroleum products, effluent discharges, or deposition of airborne particles resulting from combustion processes (Albers, 2003; Alegbeleye et al., 2017; Burgess et al., 2003). Hence, watercourses near heavily industrialized or populated areas will generally contain higher

PAH loads than areas of low human impact (Beasley and Kneale, 2002; Egardt et al., 2018). For instance, in areas of high human impact, such as, the Jiulong River Estuary and Western Xiamen Sea in China, concentrations of 2.56 – 26.1, 2.74 – 37.4 and 12.2 – 96.8 µg L^{-1}, were registered in pore water for phenanthrene, pyrene and B[a]P, respectively. Moreover, five-ringed PAHs, such as B[a]P (8 – 163 ng g^{-1}), were predominant in sediment samples, with ΣPAHs being found at very high concentrations (59 – 1177 ng/g) (Maskaoui et al., 2002). Events such as accidental spills can also lead to increased environmental concentrations, as for instance after the Gulf War, along the Kuwait coast, where ΣPAHs were estimated as 21.14 to 320.5 µg L^{-1} on surface waters (Bu-Olayan et al., 1998). Aquatic organisms can be exposed to these compounds via absorption through body surfaces or ingestion of contaminated particles or prey (Altenburger et al., 2003; Meador et al., 1995). Once inside organisms, PAHs can exert deleterious effects, with studies reporting impairments on survival, behaviour, reproduction or biochemical biomarkers of invertebrate species (Feldmannová et al., 2006; Jensen et al., 2008; Silva et al., 2013). Still, mortality elicited by PAHs, is often only recorded at (very) high exposure levels (Altenburger et al., 2003). In this sense, biochemical biomarkers can be invaluable by potentially detecting effects at sub-lethal exposure levels and simultaneously provide important information regarding specific effects of compounds inside organisms (van der Oost et al., 2003).

Animals, such as epibenthic freshwater planarians, can be exposed to both PAHs present in water and sediments, as well as through the ingestion of contaminated prey (Altenburger et al., 2003; Vila-Farré and Rink, 2018). Freshwater planarians are non-parasitic flatworms that belong to the Platyhelminthes phylum and constitute a widespread group that occurs in all continents except Antarctica (Noreña et al., 2014; Vila-Farré and Rink, 2018). They are predators of other invertebrates and can even be top predators in some habitats (Vila-Farré and Rink, 2018). Planarians have long been in the spotlight given their extraordinary regeneration abilities provided by a population of stem cells (Newmark and Sánchez Alvarado, 2002). Their unique features, such as apparent immortality and a nervous system sharing similarities with the vertebrate brain, have made these important experimental animals for several research areas such as regeneration research, developmental biology, ageing research and neuropharmacology (Buttarelli et al., 2008; Karami et al., 2015; Mouton et al., 2011). They are currently increasing in relevance for ecotoxicological research, due to their sensitivity towards toxicants and the vast range of endpoints that can easily be measured in response to chemical stress (Wu and Li, 2018). Still, there is almost no information regarding PAH toxicity towards planarians (or other members of the Platyhelminthes phylum) and no study (that we know of) has evaluated the impacts of these compounds at sub-cellular levels in freshwater planarians.

In this work, we tried to uncover how some important physiological functions are affected in freshwater planarians in response to low and high molecular weight PAHs by evaluating neurotoxicity, biotransformation, oxidative stress and energy-related biomarkers. For this, 3 of the most common PAHs, phenanthrene, pyrene and benzo[a]pyrene (B[a]P) were selected and the common freshwa-

ter planarian *Girardia tigrina* was chosen as test species. PAHs, like many pollutants in the aquatic environment, can lead to the formation of reactive oxygen species (ROS) that have the potential to damage cells, and many invertebrates possess the ability to biotransform them (Altenburger et al., 2003; Meador, 2008; van der Oost et al., 2003). Hence, we evaluated the activity of phase II biotransformation enzyme, glutathione-S-transferase (GST) and some of the cell's antioxidant defences, the activity of the enzyme catalase (CAT), and the levels of the non-enzymatic antioxidant glutathione (TG). Moreover, the levels of lipid peroxidation (LPO) were also investigated as a measure of oxidative damage. Furthermore, PAHs have been shown to impact the activity of acetylcholinesterase (AChE) on some invertebrate species (Gauthier et al., 2016; e. g. Han and Wang, 2009) and apparently can inhibit the activity of eel and human AChE (Jett et al., 1999; Kang and Fang, 1997). So, we measured the activity of planarian ChEs, to evaluate potential neurotoxic impacts of these compounds. Moreover, we determined the energy reserves (lipids, carbohydrates, proteins), the energy consumption [through measurement of the electron transport system (ETS) activity] and the cellular energy allocation (CEA) of planarians exposed to these PAHs, since sub-lethal stress can lead to compensatory adjustments that affect organisms' energy budget and has potential impacts on the long-term (De Coen and Janssen, 2003; Sokolova et al., 2012). With this approach, we hope to shed some light on specific biochemical effects of these compounds and gain a better understanding on their toxicity towards freshwater planarians.

2 Methods

2.1 Planarian cultures

The chosen *G. tigrina* individuals belong to a culture of sexually reproducing individuals, maintained for several years in laboratory. Planarians were kept in plastic containers with reconstituted hard water (ASTM, 2014), at a temperature of 20 °C. Animals were maintained in near darkness conditions, except when feeding. Room photoperiod was set at 16 h light / 8 h dark. Animals were fed once a week with either cow liver or chironomid larvae. Cow liver was ground and stored at -20 °C until needed and chironomids were supplied from laboratory cultures maintained as previously described (Rodrigues et al., 2015). Media and containers were changed immediately after feeding and 2 – 3 days after. Prior to experiments, planarians were starved for 10 days to ensure a uniform metabolic state (Oviedo et al., 2008).

2.2 Planarian exposures for biomarker analysis

Planarians of 1.0 ± 0.1 cm were selected by measuring them in a crystalizing vial placed above a millimetric sheet, after which they were visually inspected using a stereomicroscope. Total exposure time was 8 days, with solution renewal every 2 days. To avoid evaporation, the vials were kept covered with glass lids. The concentrations tested were 37.5, 75, 150 and 300 µg L^{-1} for phenan-

threne (CAS Number 85-01-8; purity 98%; Sigma-Aldrich) and 2.5, 37.5, 75 and 150 µg L^{-1} for pyrene (CAS Number 129-00-0; purity 98%; Sigma-Aldrich) and B[a]P (CAS Number 50-32-8; purity ≥ 96%; Sigma-Aldrich). Solvent controls [ASTM + dimethyl sulfoxide (DMSO) at 0.01%] were also performed. Every time solutions were renewed, stock solutions were freshly prepared, with DMSO (CAS Number 67-68-5; purity ≥ 99.7%; Fisher Chemical) being kept at 0.01% of final solution volume (v/v). For 250 mL of final solution volume, 25 µL of stock solution were added. Animals were kept in glass vials with 30 mL of experimental solution. Seven replicates of 3 animals were performed for each treatment. At the end of exposure, animals were manipulated with a soft brush, dried in filter paper, and each replicate (3 animals) was weighed and frozen in liquid nitrogen. Whenever animals were accidentally damaged by manipulation, these were excluded to avoid any influence on analysis. This resulted in a few replicates of only 2 animals. Animal samples were kept at -80 ºC until further processing. Stock solutions were kept at -20 °C until analysis.

2.3 **Quantification of PAHs in stock solutions**

PAH concentrations in stock solutions were measured using the fixed wavelength fluorescence method (FF). Experimental solution samples were also analysed, but fluorescence levels were very low, and we were unable to estimate concentrations. Stock solutions were diluted in methanol (CAS Number: 67-56-1; ≥ 99.8%; Fisher Chemical) at 50% (1:30). For detection of phenanthrene-, pyrene-, and B[a]P-type compounds, the following excitation and emission wavelengths pairs were used, respectively: 256 / 380 nm; 341 / 383 nm; 380 / 430 nm. Slit widths were set at 2.5 nm. Concentrations were calculated by using standard curves of known parent PAH concentrations. These measurements were performed with a Hitachi F-7000 Fluorescence Spectrophotometer (Hitachi High-Technologies Corporation).

2.4 **Homogenization of planarian samples**

Each replicate was homogenized in ultra-pure water with an ultrasonic homogenizer (Branson Ultrasonics™ Sonifier 250). After homogenization, the following aliquots were separated: 200 µL for lipid peroxidation determination (LPO fraction), 300 µL for electron transport system activity (ETS fraction), 300 µL for lipid and 300 µL for protein and carbohydrate content. The remaining was diluted with an equal volume of K-phosphate buffer (0.2 M; pH = 7.4) and centrifuged for 20 min at 10000 g and 4 °C to isolate the post mitochondrial supernatant (PMS). The PMS was further divided in aliquots to estimate activities of cholinesterase (ChE), catalase (CAT), glutathione-S-transferase (GST) and total glutathione (TG) content. All aliquots were stored at -80 °C until further processing.

2.5 **Oxidative stress and neurotoxicity biomarkers**

Protein quantification of the PMS (in a 100 µL fraction) was performed using the Bradford's method (Bradford, 1976) adapted to microplate (Guilhermino et al., 1996), using bovine γ-globulin as a standard and measured at 600 nm. Cholinesterase (ChE) activity was measured (in a 250 µL fraction) at 414 nm using acetylthiocholine as a substrate, according to the Ellman's (1961) method. Catalase (CAT) activity was measured (in a 100 µL fraction) by the decrease of H_2O_2 at an absorbance of 240 nm (Clairborne, 1985). Glutathione S-transferase (GST) activity was measured (in a 250 µL fraction) by the absorbance increase at 340 nm, following the conjugation reaction of GSH with 1-chloro-2,4-dinitrobenzene (Habig et al., 1974). Total glutathione (TG) content was measured (in a 250 µL fraction) by following the formation of a chromogen at 412 nm, resulting from the reaction of reduced glutathione with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). The assay was performed in the presence of excess glutathione reductase (GR), that recycles the oxidized glutathione (GSSG) to reduced glutathione (GSH) after reaction with the DTNB, so that the rate of chromophore formation was dependent on total glutathione levels (Baker et al., 1990; Tietze, 1969). Total Glutathione levels were expressed as µM per mg of protein, with a curve with known GSH concentrations being used as a standard to calculate concentrations. Lipid peroxidation (LPO) was measured by the amount of thiobarbituric acid reactive substances (TBARS) present in homogenate samples (in a 200 µL fraction) at an absorbance of 535 nm (Bird and Draper, 1984; Ohkawa et al., 1979). All absorbance readings were performed using a Microplate reader MultiSkan Spectrum (Thermo Fisher Scientific, USA) at 25 °C.

2.6 **Energy-related biomarkers**

The measurement of energetic reserves and consumption was performed based on De Coen and Janssen (1997), with some adaptations according to (Rodrigues et al., 2015). The electron transport system activity (ETS) assay was used to estimate the energy consumption (E_c), and consists in adding an excess of electron donors (NADH and NADPH) and an artificial electron acceptor (INT - Iodonitrotetrazolium chloride) to the samples. The resulting production rate of the chromogen (formazan) was measured kinetically at 490 nm and used to calculate the oxygen consumption rate (based on the stoichiometric relationship: for 2 µmol of INT-formazan produced, 1 µmol of oxygen is consumed). The oxygen consumption values were used to calculate the energy consumption (E_c) by using the specific oxyenthalpic equivalent for an average mixture of lipids, protein and carbohydrates of 480 kJ/mol O_2.

The lipid content was extracted (from a 300 µL fraction) by adding chloroform and methanol and centrifuging at 1000 g for 5 min. The organic phase was then separated and incubated in a glass vial with sulfuric acid (H_2SO_4) at 200 °C for 15 min. After cooling to room temperature, ultra-pure water was added, and samples were measured at 375 nm, with tripalmitin used as a standard. Carbohydrate and protein measurements were performed using a 300 µL fraction. Trichloroacetic

acid (at 15%) was added to samples and, after incubation, these were centrifuged for 10 min at 1000 g. The supernatant contains the carbohydrate fraction and the pellet contains the protein fraction. After removal of the carbohydrate fraction, phenol and H_2SO_4 were added, followed by incubation at room temperature. Absorbance was measured at 492 nm, using glucose as a standard. The protein fraction was resuspended using sodium hydroxide (NaOH), incubated at 60 °C during 30 min, followed by the addition of hydrochloric acid (HCl). These samples were then measured at 592 nm with Bradford's reagent and using bovine γ-globulin as a standard, according to Bradford's method (1976). Absorbance readings were performed using a Microplate reader MultiSkan Spectrum (Thermo Fisher Scientific, USA) at 25 °C. The calculation of energetic equivalents was performed by using the combustion energy of each reserve type: 17500 mJ / mg glycogen, 24000 mJ / mg protein and 39500 mJ / mg lipid (De Coen and Janssen, 1997). The energy available (E_a) was calculated as the sum of energy derived from the carbohydrates, proteins and lipid fractions and used to compute the cellular energy allocation (CEA) through the equation: $CEA = E_a / E_c$.

2.7 **Statistical analyses**

Treatments were analysed with one-way ANOVA, followed by a Dunnett's post-hoc test to evaluate differences from the solvent control (SCTR). Whenever normality (tested with Shapiro-Wilk's test) or homogeneity of variances could not be achieved, data were analysed with Kruskal-Wallis, followed by a Dunn's post-hoc test ($P < 0.05$) to detect difference from the solvent control (SCTR). All analyses were performed with SigmaPlot v. 12.3 and GraphPad Prism v. 6.01 for Windows.

3 Results

The analysis of stock solutions revealed that concentrations were generally close to nominal values (Table IV-13). Phenanthrene exposure induced a dose-dependent increase in planarian GST activity, significantly so in the 75, 150 and 300 µg L^{-1} treatments (Figure IV-14– a; one-way ANOVA; $F_{4, 29} = 7.425$; $p < 0.001$), while TG levels were increased for the 150 µg L^{-1} treatment (Figure IV-15 – a, one-way ANOVA; $F_{4, 28} = 7.387$; $p < 0.001$). On the other hand, for planarians under phenanthrene exposure, sugar content was decreased in the 150 and 300 µg L^{-1} treatments (Table IV-14; Kruskal-Wallis; $H = 17.984$; 4 d. f.; $p = 0.001$). No differences were found for ChE (Table IV-14; Kruskal-Wallis; $H = 7.527$; 4 d. f.; $p = 0.111$) and CAT (Figure IV-16 – a; one-way ANOVA; $F_{4, 28} = 1.990$; $p = 0.123$) activities, LPO levels (Figure IV-17 – a; one-way ANOVA; $F_{4, 29} = 1.744$; $p = 0.167$), energy consumption (ETS) (Table IV-14; one-way ANOVA; $F_{4, 30} = 1.921$; $p = 0.133$), lipid (Table IV-14; one-way ANOVA; $F_{4, 30} = 2.466$; $p = 0.066$) and protein (Table IV-14; one-way ANOVA; $F_{4, 30} = 0.336$; $p = 0.852$) contents nor energy budget (CEA; Table IV-14; one-way ANOVA; $F_{4, 30} = 2.514$; $p = 0.062$) of planarians exposed to phenanthrene.

Table IV-13 –Measured concentrations for stock solutions used in exposures. Concentrations are expressed as means (± standard deviation, SD) of all stock solutions performed (days 0, 2, 4 and 6).

PAH	Measured concentration (mg mL^{-1})	SD (mg mL^{-1})	% of nominal concentration	Nominal concentration (mg mL^{-1})
Phenanthrene	2.980	0.085	99.333	3.000
	1.568	0.044	104.506	1.500
	0.816	0.019	108.849	0.750
	0.356	0.006	101.720	0.350
Pyrene	1.263	0.046	84.196	1.500
	0.664	0.079	88.517	0.750
	0.354	0.019	94.516	0.375
	0.028	0.001	112.349	0.025
B[a]P	1.643	0.053	109.543	1.500
	0.844	0.015	112.516	0.750
	0.478	0.079	127.409	0.375
	0.028	0.001	111.856	0.025

In the pyrene exposures, planarian LPO levels were decreased for the 2.5 and 75 µg L^{-1} treatments (Figure IV-17 – b; Kruskal-Wallis; H = 17.215; 4 d. f.; p = 0.002). Regarding the energy-related endpoints, a dose-dependent increase in energy consumption (ETS), significant at the 75 and 150 µg L^{-1} treatments (Table IV-15; one-way ANOVA; $F_{4, 30} = 8.697$; $p < 0.001$) and the CEA values were significantly decreased at the 75 and 150 µg L^{-1} treatments (Table IV-15; one-way ANOVA; $F_{4, 30} = 5.410$; $p = 0.002$). There were no differences for ChE (Table IV-15; one-way ANOVA; $F_{4, 30} = 1.043$; $p = 0.402$), CAT (Figure IV-16 – b; Kruskal-Wallis; H = 5.882; 4 d. f.; p = 0.208) and GST (Figure IV-14 – b; one-way ANOVA; $F_{4, 30} = 0.481$; $p = 0.749$) activities, TG levels (Figure IV-15 – b; Kruskal-Wallis; H = 5.362; 4 d. f.; p = 0.252), nor sugar (Table IV-15; one-way ANOVA; $F_{4, 30} = 1.733$; $p = 0.169$), lipid (Table IV-15; one-way ANOVA; $F_{4, 30} = 1.054$; $p = 0.396$) or protein (Table IV-15; one-way ANOVA; $F_{4, 30} = 0.439$; $p = 0.779$) contents in planarians exposed to pyrene.

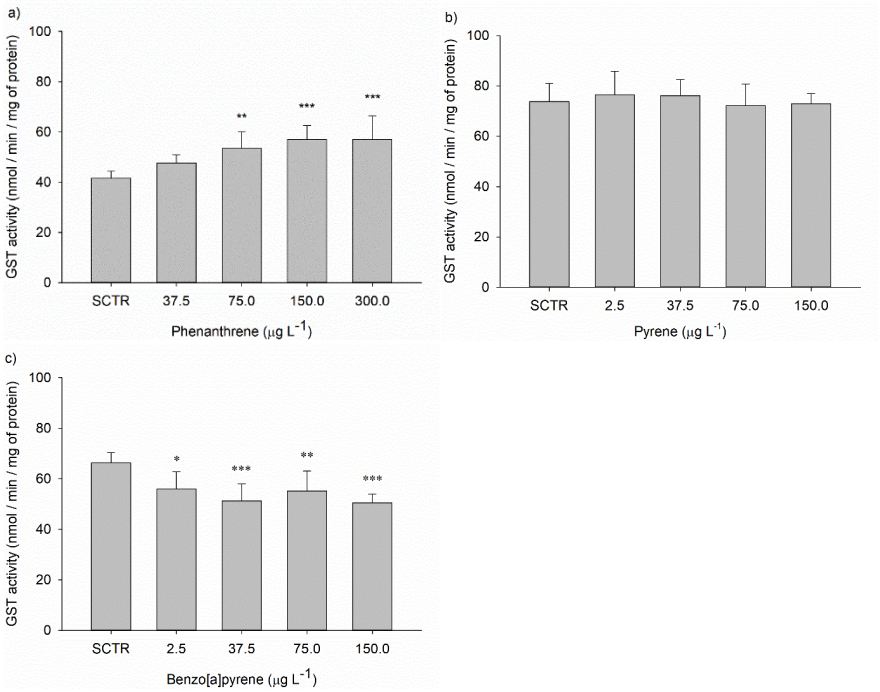


Figure IV-14 – Glutathione-S-transferase activity in *Girardia tigrina* planarians exposed during 8-days to the PAHs: a) phenanthrene; b) pyrene; c) benzo[a]pyrene. Data is presented as mean ± SD. Treatments were compared using one-way analysis of variance (ANOVA) with Dunnett's post-hoc test to detect differences from the solvent control (SCTR). * P < 0.05; ** P < 0.01; *** P < 0.001.

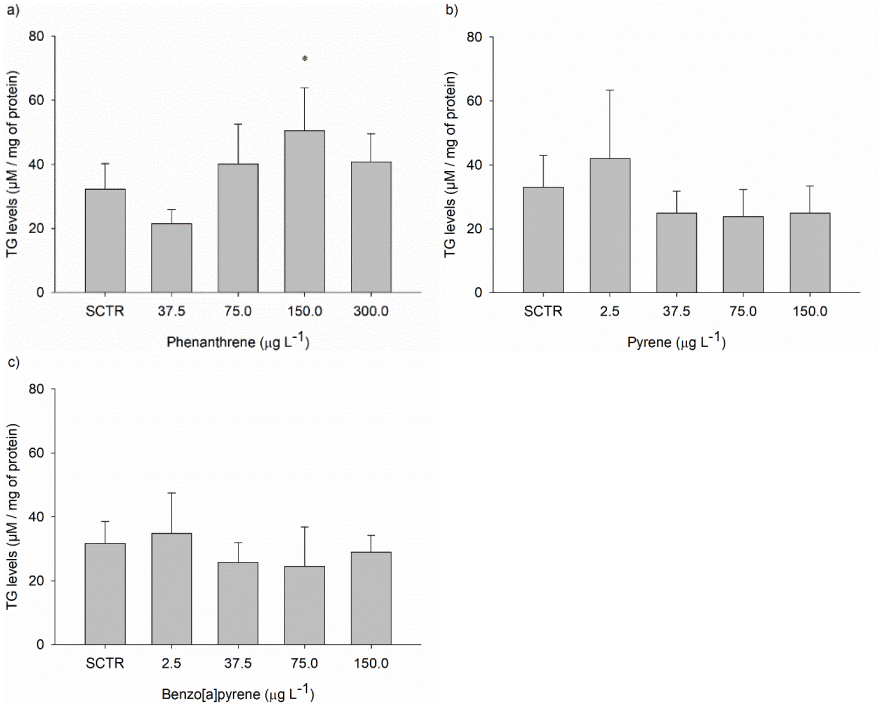


Figure IV-15 – Total glutathione levels (TG) in *Girardia tigrina* planarians exposed during 8-days to the PAHs: a) phenanthrene, b) pyrene and c) benzo[a]pyrene. Data is presented as mean ± SD. Treatments were compared using one-way analysis of variance (ANOVA) with Dunnett's post-hoc test or with Kruskal-Wallis followed by a Dunn's post-hoc tests to detect differences from the solvent control (SCTR). * P < 0.05; ** P < 0.01.

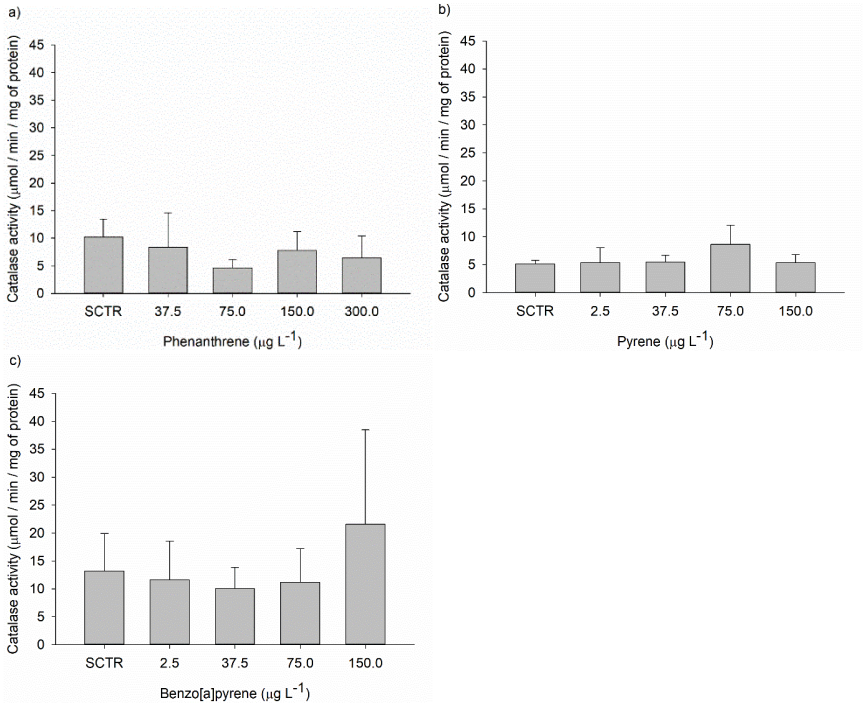


Figure IV-16 – Catalase activity in *Girardia tigrina* planarians exposed during 8-days to the PAHs: a) phenanthrene, b) pyrene and c) benzo[a]pyrene. Data is presented as mean ± SD. Treatments were compared using one-way analysis of variance (ANOVA) with Dunnett's post-hoc test or with Kruskal-Wallis followed by a Dunn's post-hoc tests to detect differences from the solvent control (SCTR).

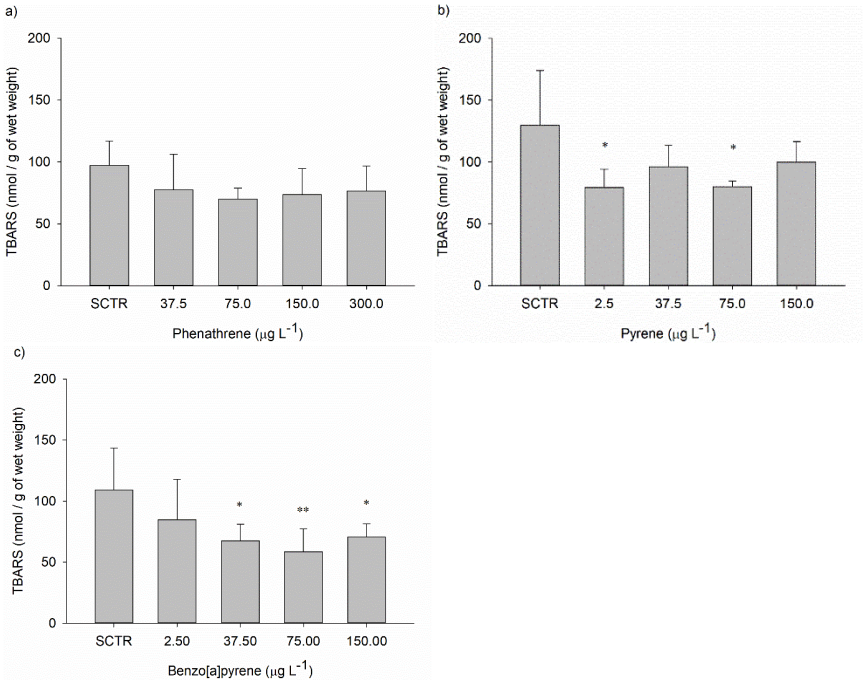


Figure IV-17 – Lipid peroxidation levels (LPO) in *Girardia tigrina* planarians exposed during 8-days to the PAHs: a) phenanthrene, b) pyrene and c) benzo[a]pyrene. Data is presented as mean ± SD. Treatments were compared using one-way analysis of variance (ANOVA) with Dunnett's post-hoc test or with Kruskal-Wallis followed by a Dunn's post-hoc tests to detect differences from the solvent control (SCTR). * P < 0.05; ** P < 0.01.

In the B[a]P experiment, there was a decrease in the activity of planarian GST for all tested concentrations (Figure IV-14 – c; one-way ANOVA; $F_{4, 30} = 7.647$; $p < 0.001$). LPO levels were significantly decreased when compared to the control treatment, starting from the 37.5 and up to the 150 µg L^{-1} treatment (Figure IV-17 – c; one-way ANOVA; $F_{4, 30} = 4.696$; $p = 0.005$) in planarians under B[a]P exposure. Significant differences were detected in the energy consumption (ETS) (Table IV-16; one-way ANOVA; $F_{4, 30} = 3.858$; $p = 0.012$) of planarians exposed to B[a]P, but Dunn's test did not detect differences between the treatments and control group (SCTR), although a significant increasing trend was detected with increasing concentrations (slope = 5.771; $r^2 = 0.256$; $p = 0.002$). No differences were found in the activities of ChE (Table IV-16; one-way ANOVA; $F_{4, 30} = 2.042$; $p = 0.114$) or CAT (Figure IV-16 – c; Kruskal-Wallis; H = 2.307; 4 d. f.; $p = 0.679$) nor TG levels (Figure IV-15 – c; one-way ANOVA; $F_{4, 30} = 1.487$; 4 d. f.; $p = 0.231$) of planarians exposed to B[a]P. Regarding planarian energy reserves in the B[a]P experiment, significant differences for the lipid content were found (Table IV-16; Kruskal-Wallis; H = 12.131; 4 d. f.; $p = 0.016$), but the Dunn's *post-hoc* test did not detect differences between treatments and the control group (SCTR). Protein (Table IV-16; one-way ANOVA; $F_{4, 28} = 2.496$; $p = 0.065$), and sugar (Table IV-16; one-way ANOVA; $F_{4, 30} = 0.643$; $p = 0.636$) contents were not affected by B[a]P exposure. Significant differences were found in the CEA values (Table IV-16; Kruskal-Wallis; H = 20.129; 4 d. f.; $p < 0.001$), although Dunn´s *post hoc* test did not detect differences between treatments and the control group (SCTR).

Table IV-14 – Summary of biomarker responses in *Girardia tigrina* exposed to phenanthrene for 8 days. Values are presented as mean ± SD. Treatments were compared using one-way analysis of variance (ANOVA) with Dunnett's post-hoc test or Kruskal-Wallis followed by a Dunn's post-hoc tests to detect differences from the solvent control (SCTR); * P < 0.05.

		Phenanthrene (µg L^{-1})				
Type of biomarker	Biomarker	SCTR	37.5	75.0	150.0	300.0
Neurotoxicity	ChE (nmol / min / mg of protein)	78.95 ± 15.36	79.10 ± 8.06	83.63 ± 10.56	91.41 ± 12.33	89.36 ± 12.87
Energy consumption (E_c)	ETS (mJ / mg organism / h)	179.45 ± 6.50	188.65 ± 8.78	190.25 ± 9.47	178.49 ± 15.32	185.98 ± 8.71
Energy reserves (E_a)	Carbohydrates (mJ / mg organism)	446.47 ± 110.53	351.20 ± 54.24	353.37 ± 35.31	**296.36 ± 29.39***	**291.06 ± 38.50***
	Proteins (mJ / mg organism)	1049.30 ± 148.37	1046.73 ± 145.82	1030.42 ± 150.96	1001.89 ± 93.84	982.89 ± 112.69
	Lipids (mJ / mg organism)	2397.19 ± 1019.70	2165.97 ± 1066.12	1718.69 ± 378.37	3221.51 ± 1406.39	2816.57 ± 693.36
Cellular energy allocation	CEA (E_a / E_c)	21.73 ± 6.05	18.83 ± 5.719	16.38 ± 2.23	25.46 ± 8.77	22.04 ± 4.04

Table IV-15 – Summary of biomarker responses in *Girardia tigrina* exposed to pyrene for 8 days. Values are presented as mean ± SD. Treatments were compared using one-way analysis of variance (ANOVA) with Dunnett's post-hoc test or Kruskal-Wallis by a Dunn's post-hoc tests to detect differences from the solvent control (SCTR); * P < 0.05, *** P < 0.001.

		Pyrene (µg L^{-1})				
Type of biomarker	Biomarker	SCTR	2.5	37.5	75.0	150.0
Neurotoxicity	ChE (nmol / min / mg of protein)	81.74 ± 5.68	80.15 ± 7.82	79.71 ± 5.48	75.24 ± 6.40	78.34 ± 6.03
Energy consumption (E_c)	ETS (mJ / mg organism / h)	154.83 ± 8.20	161.58 ± 7.17	167.88 ± 12.28	**176.63 ± 13.86***	**191.31 ± 18.60*****
Energy reserves (E_a)	Carbohydrates (mJ / mg organism)	264.90 ± 101.92	306.08 ± 102.79	383.16 ± 84.16	440.00 ± 262.57	469.05 ± 233.23
	Proteins (mJ / mg organism)	1883.53 ± 125.07	1913.34 ± 171.60	1922.70 ± 168.46	1844.41 ± 185.00	1942.97 ± 98.565
	Lipids (mJ / mg organism)	3187.19 ± 409.53	3037.79 ± 637.62	2794.11 ± 486.46	2883.10 ± 296.14	2656.40 ± 724.91
Cellular energy allocation	CEA (E_a / E_c)	34.53 ± 3.07	32.58 ± 4.48	30.54 ± 3.89	**29.24 ± 1.19***	**26.40 ± 3.19*****

Table IV-16 – Summary of biomarker responses in *Girardia tigrina* exposed to benzo[a]pyrene for 8 days. Values are presented as mean ± SD. Treatments were compared using one-way analysis of variance (ANOVA) with Dunnett's post-hoc test or Kruskal-Wallis followed by a Dunn's post-hoc tests to detect differences from the solvent control (SCTR).

		Benzo[a]pyrene (µg L^{-1})				
Type of biomarker	Biomarker	SCTR	2.5	37.5	75	150
Neurotoxicity	ChE (nmol / min / mg of protein)	102.64 ± 9.35	89.97 ± 12.41	96.77 ± 20.01	99.52 ± 19.23	82.70 ± 9.31
Energy consumption (E_c)	ETS (mJ / mg organism / h)	161.79 ± 16.59	153.59 ± 21.30	169.23 ± 8.62	175.82 ± 9.90	179.53 ± 10.03
Energy reserves (E_a)	Carbohydrates (m J / mg organism)	294.97 ± 36.10	287.71 ± 39.09	288.83 ± 53.48	294.67 ± 43.72	265.50 ± 19.34
	Proteins (mJ / mg organism)	1272.23 ± 55.98	1204.02 ± 199.71	1161.75 ± 75.42	1098.79 ± 190.73	1012.93 ± 194.94
	Lipids (mJ / mg organism)	2335.17 ± 610.41	3905.75 ± 1700.17	2217.32 ± 596.11	3641.67 ± 995.19	2127.27 ± 232.12
Cellular energy allocation	CEA (E_a / E_c)	25.75 ± 4.55	35.11 ± 11.13	20.46 ± 2.85	28.54 ± 4.68	19.03 ± 2.44

4 Discussion

This work evidenced that, in planarians, the biotransformation enzyme GST was affected by the PAHs, with its activity being induced by phenanthrene and inhibited by B[a]P. Also, there were some alterations on energy-related biomarkers, possibly pointing to increased energetic demand in PAH-exposed planarians. Unexpectedly, LPO levels were lower in PAH-exposed planarians, significantly so for pyrene and B[a]P. The present results seem to suggest that each of the tested PAH causes slightly distinct effects on planarians, pointing to possible different biochemical interactions and / or differences in metabolization. The observed effects raise concerns over potential fitness costs on natural populations of planarians in PAH-polluted environments.

Previous studies have evaluated the biochemical effects on planarians in response to chemical exposure. The comparison of control levels observed in the present study with others from the literature indicate that these are in the same range as those previously obtained by other authors and other planarian species (e.g. Li, 2008; Li, 2012; Villar et al., 1994; Zhang et al., 2011). The majority of the biochemical studies in planarians has evaluated oxidative stress responses and / or (A)ChE activity. These studies indicate that oxidative stress biomarkers can be useful in detecting xenobiotic effects in planarians, given that many contaminants can elicit these responses, as also evidenced by the changes in oxidative stress biomarkers in the present work. The evaluation of planarian (A)ChE was also able to detect deleterious effects of organophosphorus insecticides (Villar et al., 1994), surfactants (Li, 2008) and cadmium (Wu et al., 2014), indicating that these planarian enzymes can be affected by (some) environmental contaminants, although contrasting with the lack of changes observed in the present work for PAHs. On the other hand, we found no study dealing with energy-related biomarkers in planarians exposed to contaminants. However, this will probably be an interesting subject to investigate, given the developmental plasticity of planarians, that allows them to survive through the consumption of their own tissues when conditions are unfavourable. Further studies would be useful to better understand the energy dynamics of planarians under chemical stress.

The exposure of planarians to phenanthrene led to a concentration-dependent increase in GST activity (at 75, 150 and 300 µg L^{-1}). GSTs are phase II biotransformation enzymes, that act by conjugating electrophilic metabolites with glutathione, but are also important in the detoxification of ROS-damaged molecules (Hayes et al., 2005; van der Oost et al., 2003). Hence, the increased GST activity in phenanthrene-exposed planarians suggests the induction of these enzymes to detoxify electrophilic metabolites of phenanthrene and / or ROS-damaged molecules. The altered TG levels further suggest the presence of ROS and / or reactive metabolites, with the increase at 150 µg L^{-1} suggesting a compensatory glutathione production to cope with xenobiotic stress. This is consistent with the work of Zhang et al. (2014), which evidences that exposure to phenanthrene (2 – 50 µg L^{-1}) leads to increased activities of GST, superoxide dismutase and higher levels of GSH in

the clam *Venerupis philippinarum*, although other studies using invertebrate species have not detected an increased ROS production nor activation of antioxidant defences in response to phenanthrene (Feldmannová et al., 2006; Gauthier et al., 2016). These contrasting evidences possibly point to interspecies differences in PAH metabolization or even in effect concentrations. However, this emphasizes the need to further investigate the metabolization of phenanthrene (and other PAHs) by planarians, as well as by other invertebrates. Still, the upregulation of antioxidant defences is a mechanism to deal with increased levels of oxidative stress elicited by ROS (Monaghan et al., 2009) and, in this case, seemed to prevent oxidative damage (at least in lipids), since lipid peroxidation was unaffected by phenanthrene exposure. Added to this, the energy metabolism seemed to be affected by phenanthrene as well, since carbohydrate reserves were decreased in exposed planarians. The carbohydrate decreases (at 150 and 300 µg L^{-1}) point to an expenditure of these energetic resources, possibly due to higher metabolic costs elicited by the activation of defence mechanisms or other toxicant-induced costs. Other works have shown that chemical stress can lead to decreases in carbohydrate reserves (Campos et al., 2016; De Coen and Janssen, 2003), although in these studies either increases of energetic demands for defence mechanisms and decreases in food intake or assimilation efficiency are possible explanations. In this case, since no food intake occurred during the test, the most probable explanation is the expenditure of carbohydrate reserves to cope with xenobiotic stress. In fact, carbohydrates represent the smallest energy reserve in planarians, that during periods of stress, such as starvation, are rapidly utilized to fuel metabolic processes (Calow and Woollhead, 1977; Jennings, 1957). In a previous work (Chapter III) we tested the same range of phenanthrene concentrations using *G. tigrina* and found that phenanthrene-type compounds were increased in planarian tissues in a concentration dependent way, indicating increasingly higher body burdens. Moreover, locomotion was decreased with increasing concentrations and feeding was impaired at 150 and 300 µg L^{-1}, hinting that behavioural impairments may be, at least partly, explained by the increased energetic demands and defence costs elicited by this PAH.

In the pyrene experiment, there was no obvious alteration on oxidative stress-related and biotransformation biomarkers, since neither the enzymatic activities of CAT and GST nor the glutathione levels (TG) were altered. Moreover, lipid peroxidation was not increased, indicating no oxidative damage in lipids (but see discussion below). Contrary to our observations, Xie et al. (2017) observed that the oyster *Pinctada martensii* exposed to pyrene (4 to 64 µg L^{-1}), had reduced glutathione levels (GSH) and higher LPO levels (both endpoints from 8 to 64 µg L^{-1}), indicating oxidative stress and damage. On the other hand, Luís & Guilhermino (2012) observed that the prawn *Palaemon serratus* exposed to pyrene (6 to 400 µg L^{-1}) presented an increased GST activity (only at 0.40 mg/L), while CAT activity remained unaffected, and LPO levels were increased at intermediate concentrations (13 and 25 µg L^{-1}). It is interesting to note that, at similar concentrations as the ones tested in the present study (2.5 to 150 µg L^{-1}), GST and CAT activities in *P. serratus* were not affected by pyrene exposure in the Luís & Guilhermino (2012) study, in accordance with our re-

sults. Still, the increased LPO levels that these authors observed are indicative of oxidative damage and disagree with the reductions that were observed in the present study. Nevertheless, as mentioned above, metabolic differences might account for disparities between species, but also exposure time, concentrations and other experimental conditions. Regarding planarian GST activity in the present study, it is possible that the similar activity found in control and exposed planarians might indicate that planarian GSTs play no role in the biotransformation of pyrene. In fact, several studies have identified phase II metabolites of pyrene in invertebrate species, including conjugates with sulphate, glucuronic acid or glucose (Beach et al., 2010; Carrasco Navarro et al., 2013; Stroomberg et al., 2004), which potentially indicates that phase II enzymes other than GSTs might be responsible for the metabolization of these compounds. Regarding energy-related endpoints, pyrene exposure increased the energy consumption (estimated by the ETS assay) and reduced the energy budget (evaluated by the CEA), indicating higher energetic demands in exposed planarians. Increases in energetic demands have been observed for instance, in *C. riparius* in response to UV-filters (Campos et al., 2017) or *Neomysis integer* exposed to chlorpyrifos (Verslycke et al., 2004). Increased energetic demands can be a consequence of a stressful situation, in which the organism needs more energy to cover the costs of activating defence mechanisms (Sokolova et al., 2012). Moreover, the increased energy consumption might imply potential consequences on the long-term for growth and reproduction (Verslycke et al., 2004). It is interesting to analyse these results considering our previous study that evaluated other sublethal endpoints in planarians exposed to the same pyrene concentrations (Chapter III). Cephalic regeneration delays, decreased feeding rate and decreases in locomotor activity were observed at 75 and 150 µg L^{-1}, the same concentrations at which ETS activity was significantly increased and CEA decreased in the present study, while pyrene-type compounds in tissues were elevated in relation to controls starting from the 37.5 µg L^{-1} in a concentration-dependent way. This seems to suggest that planarians reduced their behavioural activities to conserve energy for detoxification mechanisms (Sokolova et al., 2012). Possibly, this may also account for the delays on cephalic regeneration, although other mechanisms, such as cellular arrest to repair damages in DNA or other macromolecules prior to cell division, cannot be excluded (Barghouth et al., 2018; Sokolova et al., 2012).

Under B[a]P exposure, a decrease in GST activity was observed. This result agrees with the study by Vicentini et al. (2017), that observed a reduction in GST activity in *Chironomus sancticaroli* exposed to B[a]P (2.13 to 4.73 µg L^{-1}). Nevertheless, Cunha et al. (2005), observed an increase in GST activity in the sea-urchin *Paracentrotus lividus* exposed to B[a]P (at 100 µg L^{-1}), while Silva et al. (2013) observed no changes in GST activity in the prawn *P. serratus* exposed to B[a]P (16 to 4096 µg L^{-1}). On the other hand, B[a]P was observed to inhibit eel GST activity (above 0.1 mg of B[a]P / kg of fish) (Regoli et al., 2003) and in there is evidence that a B[a]P metabolite is able to inhibit GST activity in humans (Regoli et al., 2003), so one possible explanation to our observations might be the inhibition of planarian GSTs by B[a]P metabolites. Besides conjugating target molecules with glutathione, other functions are known to be performed by GSTs, such as hormone syn-

thesis, tyrosine catabolism, or the direct binding and transport of compounds by acting non-enzymatically as ligandins (Boušová and Skálová, 2012; Frova, 2006; Hayes et al., 2005). The ability of GSTs to bind and transport xenobiotics has been described (Boušová and Skálová, 2012) and some studies using fish species have proposed that a decrease in GST activity might be a consequence of the direct scavenging of PAHs or their metabolites by these enzymes (Almeida et al., 2012a; Vieira et al., 2008), which might offer an explanation for the GST inhibition observed in the present study. Still, regardless of the inhibition cause, a decreased activity of these enzymes might be deleterious to cells, since biotransformation of xenobiotics as well as detoxification abilities towards ROS-damaged molecules might be reduced. Interestingly, the oxidative stress biomarkers, CAT and TG, were not affected, while oxidative damage (as measured by LPO) was not increased under B[a]P exposure (but see discussion below). This seems to suggest that B[a]P did not induce oxidative stress nor damage, contrary to several studies that observed changes in oxidative stress biomarkers and / or oxidative damage in invertebrates (Silva et al., 2013; Wen and Pan, 2016) and fish (Almeida et al., 2012b; Gravato and Guilhermino, 2009). It is possible that B[a]P did not elicit appreciable amounts of ROS in exposed planarians, thus explaining our observations, although the inverse cannot be excluded, since the levels of ROS were not directly measured in the present study. Regarding the energy-related biomarkers, B[a]P induced a tendency of increased energy consumption with increasing concentrations. This seems to suggest increased energetic demands in planarians exposed to B[a]P. In a previous experiment (Chapter III), we observed that B[a]P exposure (at the same concentrations tested in the present chapter) led to feeding inhibition and locomotion impairment. The trend of increased energy consumption and GST inhibition with increasing concentrations of B[a]P, suggests that planarians were under a considerable stress and might help explain the reductions in behavioural activities.

Unexpectedly, the peroxidation levels (LPO) decreased under pyrene and B[a]P exposure, while in the case of phenanthrene, non-significant reductions (10.0 to 25.8%) were observed. Many studies investigating the effects of phenanthrene, pyrene or B[a]P have observed increases in LPO levels of invertebrate (e. g. Silva et al., 2013; Xie et al., 2017; H. Zhang et al., 2014) and fish (e. g. Almeida et al., 2012a; Gravato and Guilhermino, 2009; Oliveira et al., 2008) species exposed to these compounds. In fact, although not a specific biomarker for PAH exposure, the increase in LPO levels seems to be a common response in fish and invertebrate species at relatively high PAH concentrations (Kasiotis and Emmanouil, 2015; Santana et al., 2018). On the other hand, Martins et al. (2013) observed reductions in LPO levels in clams exposed to sediments contaminated with phenanthrene or benzo[b]fluoranthene and reductions in LPO levels have been observed for aquatic species exposed to other xenobiotics, such as carbendazim (Novais et al., 2014), herbicides (Pretto et al., 2011), CuO nanoparticles (Buffet et al., 2013) or nickel (Wang and Wang, 2010). Martins et al. (2013) propose that the decrease in LPO levels in clams exposed to PAHs might be related to an “over-compensatory antioxidant response”, in which antioxidants are synthesized in response to contamination, and their increased levels lead to a decrease in LPO. Hence, a similar

phenomenon could help explain our observations, although this study's results regarding oxidative stress biomarkers do not seem to hint this, since neither CAT activity nor TG levels were increased in response to pyrene or B[a]P. Nevertheless, other non-measured antioxidant agents might still be responsible for the observed LPO decreases. On the other hand, Novais et al. (2014) hypothesize that the reduction of LPO levels could be related to the accumulation of ROS (due to inhibition of antioxidant enzymes) and consequent triggering of cell apoptosis. This widespread cell death would indirectly lead to LPO reduction by decreasing the amount of measurable cellular lipids. Interestingly, a bell-shaped response in LPO levels (with significant increases at intermediate but not at higher concentrations) is not uncommon under PAH exposure, and, for instance, in fish species exposed to pyrene was found in combination with bell-shaped responses in the activities of antioxidant enzymes (Oliveira et al., 2012), or with lack of induction and / or reduction in the activities of antioxidant enzymes with increasing concentrations (Almeida et al., 2012a), being the above-mentioned hypothesis interesting to consider in light of these observations. Since the present study's observations seem to indicate that exposed planarians were under a considerable amount of stress, the hypothesis of cell lysis due to oxidative stress might be a possible explanation for the observed reductions in LPO levels. Moreover, it is relevant to note that planarian neoblasts are very resilient to stress. They possess a very efficient set of repair mechanisms that allows them to cope with stressors, including high levels of thiol antioxidants efficiently maintained in the reduced forms, allowing survival to environmental insults (Barghouth et al., 2018; Natarajan et al., 2015; Sahu et al., 2017). Also, autophagic processes are a key aspect of planarian survival, allowing for the recycling and elimination of damaged or old cells, acting as a protective response (González-Estévez and Saló, 2010). So, it is possible that the specific features of planarians might help explain these observations. Nevertheless, further study is needed to better understand this phenomenon.

Planarian ChE activity was not affected by any of the tested PAHs, although we previously observed that these compounds could lead to abnormal behaviours that are consistent with disruption of neurochemical pathways (Chapter III). Some studies have shown that AChE in invertebrates can be inhibited by these PAHs, while others have shown no inhibition of these enzymes (Cunha et al., 2005; Han and Wang, 2009). Two studies have shown that PAHs can inhibit the activity of eel and human AChE *in vitro*, although they have reached conflicting conclusions on the type of inhibition (competitive vs noncompetitive inhibition) (Jett et al., 1999; Kang and Fang, 1997). Nevertheless, these studies have shown that inhibition of AChE occurs at very high levels of PAHs (lowest for B[a]P inhibition of human AChE at EC_{50}: 0.38 ± 0.13 mg L^{-1}), which might indicate that the concentrations used in the present study may be too low to detect significant enzymatic inhibition. On the other hand, planarian ChE has intermediate features of vertebrate AChE and butyrylcholinesterase (BChE) (Hagstrom et al., 2018, 2017), which may imply differences in inhibition when compared to vertebrate AChE. Still, the stereotypical behaviours observed in planarians could be related with

inhibition of different enzymes or alteration of neurotransmitter levels, with these issues meriting further investigation.

5 Conclusion

Phenanthrene seem to mainly lead to induction of detoxification, antioxidant defences and expenditure of carbohydrates in planarians, the latter possibly to cope with the increased metabolic costs of antioxidant defences. On the other hand, the planarians exposed to pyrene mainly increased energy consumption and presented reduced energy budgets, also pointing to increased metabolic costs. Finally, B[a]P lead to decreased detoxification abilities and induced a trend of increased energy consumption in planarians. Unexpectedly, LPO levels decreased under PAH exposure, significantly so for pyrene and B[a]P. The decreased LPO levels could be related with a compensatory antioxidant response or, alternatively, with promotion of cell lysis and loss of damaged components due to extreme stress. The differences found between 3-, 4-, and 5-ringed PAHs seem to suggest metabolic differences and / or biochemical interactions for each compound. This study demonstrates that freshwater planarians can be affected at sub-cellular levels by PAHs, with the biochemical biomarkers allowing for a better understanding of PAH effects upon these organisms.

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Chapter V – Effects of pyrene and benzo[a]pyrene on the reproduction of the freshwater planarian *Girardia tigrina*

Effects of pyrene and benzo[a]pyrene on the reproduction of the freshwater planarian *Girardia tigrina*

Abstract

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous pollutants of aquatic ecosystems. Because they can persist for long periods of time, there is great potential for chronic toxicity to aquatic species and the evaluation of reproductive effects is fundamental to understand potential impacts on natural populations. In this context, planarians are interesting experimental animals, since they can be sensitive to environmental pollutants and a wide range of reproductive-related endpoints can be assessed. In this work we evaluated fecundity, fertility, newborn anomalies, adult weight, regenerative abilities and PAH-residues in tissues of the freshwater planarian *Girardia tigrina*, exposed to either pyrene or benzo[a]pyrene (B[a]P). Fecundity was affected by both PAHs, but fertility was only affected by pyrene exposure. After the exposure period, adults showed a trend of smaller size with increasing concentration and increased levels of PAH-type compounds in planarian tissues indicated uptake from the media. Cocoons were kept in clean media and newborns were evaluated after hatching for behavioural and morphological anomalies. Some morphological defects were observed, but not significantly higher than controls. Nevertheless, many of the newborns resulting from the B[a]P experiment revealed behavioural anomalies, such as spasms and uncoordinated movements. These results evidence that exposure to PAHs can impact planarian populations by decreasing their reproductive output. Moreover, the exposure of adults to B[a]P affected the fitness of their offspring, raising concern on the possible long-term consequences of these compounds for planarian populations.

Key words: Planarians; fertility; fecundity; PAHs; newborn anomalies.

1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are classified as persistent organic pollutants (POPs), since they can stay in the environment and organisms for long periods, may be transported over long distances and are toxic (Douben, 2003; Nadal et al., 2015). PAHs are produced by several natural and anthropogenic processes and are released to the environment as a mixture of chemicals (Burgess et al., 2003). Moreover, environmental inputs of these chemicals are constant, with major contributes from extraction and transport of fossil fuels (partially constituted by PAHs), as well as their combustion (Albers, 2003; Burgess et al., 2003). Aquatic environments can receive considerable inputs of these pollutants, from spills and leaks, wastewater effluents, surface runoff or deposition from the atmosphere (Albers, 2003). PAHs can affect organisms, with factors such as feeding behaviour, trophic level, preferred habitat, metabolism or elimination processes influencing the uptake and body burden (Altenburger et al., 2003; Baumard et al., 1998). For aquatic inverte-

brates, impacts such as mortality, narcosis, behavioural impairments or genetic damage have been reported (Bellas and Thor, 2007; Canova et al., 1998; Lotufo, 1997; Paumen et al., 2008). Once inside the organism, the metabolic activity, along with the tissue lipid content seem to be major influencing factors on the distribution of PAHs in tissues (Meador et al., 1995; Xu et al., 2011). Moreover, lipid-rich tissues can act as deposits for PAHs, given their hydrophobicity (Wen and Pan, 2016; Xu et al., 2011). Gonads and gametes, as lipid-rich tissues, may accumulate PAHs and in some species, body burdens decrease significantly after spawning events and may be transferred to zygotes (Ellis et al., 1993; Kaag et al., 1997; Rossi and Anderson, 1977). Therefore, studying effects of PAH's exposure on reproduction and offspring is fundamental to understand their potential long-term impacts for the maintenance of natural populations (Amiard-Triquet, 2009; Payne et al., 2003).

Information on PAH toxicity to some invertebrate groups, such as planarians (and Platyhelminthes in general) is very scarce (Best and Morita, 1991; see Foster, 1963; Hoshina and Teshirogi, 1991; Zhang et al., 2018), and impacts on reproduction are unknown. Planarians are important components of freshwater habitats, both in number of species and abundance, and can even be top predators in some habitats (Noreña et al., 2014; Schockaert et al., 2007; Vila-Farré and Rink, 2018). Despite their ecological importance, these animals have been overlooked by ecotoxicological research, but are currently increasing in relevance for this field (see Wu and Li, 2018). To evaluate chronic impacts of contaminants on planarians, sexual reproduction is highly relevant from the ecological point of view, and studies show that it can be sensitive to environmental pollutants (Knakievicz and Ferreira, 2008; Ribeiro and Umbuzeiro, 2014). In planarians, both asexual and sexual reproduction modes are possible (Newmark and Sánchez Alvarado, 2002; Ramm, 2017). In sexually reproducing planarians, reproductive organs and germ cells are formed post-embryonically, with animals developing these when they attain a certain size, being hermaphrodites with crossed fertilization (Newmark and Sánchez Alvarado, 2002; Peters et al., 1996; Vreys et al., 1997). After mating, several eggs with numerous yolk cells are encased in a protective spherical cocoon, deposited though the gonopore (Corso et al., 2006; Kotpal, 2012). Cocoons are attached to a substrate by a stalk and become progressively darker as they harden (Rouhana et al., 2017). The development of embryos is dependent on the temperature and species, usually taking a few weeks, after which newborns emerge measuring just a few millimetres (Noreña et al., 2014; Tachet et al., 2006; Vila-Farré and Rink, 2018).

In this study, we aimed to uncover the chronic impacts of 2 of the most common PAHs (pyrene and B[a]P) on planarians by evaluating reproduction-related endpoints, such as fecundity (number of deposited cocoons) and fertility (number of newborns). After hatching, morphological and behavioural alteration in newborns were assessed. By the end of the exposure period, adults were also weighed and presence of PAHs in tissues was evaluated. Moreover, the regeneration ability of adults was assessed, to investigate possible impairments on tissue regeneration over relatively long-term exposures.

2 Methods

2.1 Planarian cultures

Girardia tigrina specimens belonged to a sexually reproducing population, kept in laboratory for several years. Planarians were kept in non-toxic plastic containers with ASTM hard water (ASTM, 2014) at a temperature of 20 °C. Animals were maintained in a dark environment, except when feeding. Once a week either cow liver or *Chironomus riparius* larvae were given as food (see Chapter II for details on *C. riparius* culturing and maintenance). Media and containers were changed twice a week, immediately after feeding and 2 to 3 days after.

2.2 Experimental procedures

Prior to the experiments, planarians were starved for 7 days to ensure a uniform metabolic state (Oviedo et al., 2008). Sexually mature animals of 1.7 ± 0.3 cm were selected. Moreover, close inspection of the culture containers provided evidence of the egg-laying ability of the animals. Planarians were measured while moving and fully extended in a transparent crystallizing vial with a small amount of medium placed above a millimetric sheet.

Bioassays were performed with 5 replicates, each with 10 animals. This number of animals per replicate was chosen, since fertility and fecundity do not seem to be affected by population density in *G. tigrina* (Knakievicz et al., 2006). Moreover, preliminary experiments indicated this to be an adequate density for this experimental design.

Planarians were maintained in glass crystallizing vials with 300 mL of test solution for 21 days. For each PAH, 4 concentrations were used plus a solvent control treatment (SCTR). The highest concentration tested for each compound was based on the observed LOECs for feeding inhibition (Chapter III), to ensure that feeding would still occur. The used concentrations were: for pyrene (CAS Number 129-00-0; Sigma-Aldrich; purity 98%) 75.00, 18.75, 4.69 and 1.17 µg L^{-1} and for B[a]P (CAS Number 50-32-8; Sigma-Aldrich; purity ≥ 96%) 37.50, 9.37, 2.34, 0.59 µg L^{-1}. Stock solutions were freshly prepared in dimethyl sulfoxide (DMSO; CAS Number 67-68-5; Fisher Chemical; purity ≥ 99.7%) every time solutions were renewed. Total amount of solvent was kept at 0.01% (v/v) of final solution volume (30 µL of stock solution in 300 mL of test solution). Stock solution samples were kept at -20 °C until further analysis. Tests were performed at a temperature of 20.0 ± 1.5 °C, with animals in a darkened environment, similarly as cultures are kept, except when feeding and whenever solutions were renewed. Tests were renewed twice a week, once immediately after feeding and three days after. Animals were fed once a week with chironomid larvae *ad libitum* for 2-3 hours. Chironomids were chosen as food items since preliminary experiments showed that animals that were fed only with liver produced few viable cocoons, in comparison with animals fed chironomid larvae or a mixed diet (chironomid larvae and liver). Preliminary experiments also indi-

cated that food should be provided once per week, since animals reproduce less frequently when fed twice a week, an observation in line with a study by Dunkel and co-workers (2011). Whenever solutions were renewed, cocoons were removed from the vials.

At the end of the exposure period, 5 animals from each replicate were dried in filter paper, weighed and kept at -20 ºC for analysis of PAH-type compounds in tissues. The remaining 5 planarians were decapitated and kept in clean media. Each decapitated planarian was kept in the dark, in a glass crystalizing vial with 20 mL of clean media (ASTM hard water) at 20.0 ± 1.5 °C. The regeneration of photoreceptors was evaluated daily, by observation with a stereomicroscope (Stemi 2000C, Carl Zeiss™). Each animal was followed until the regeneration of photoreceptors and the number of days until reappearance of these structures was recorded.

Fecundity was evaluated as the number of deposited cocoons and fertility as the number of emerged newborns. Each cocoon resulting from the reproduction experiment was kept separately in a well of a 24-well plate with clean media (ASTM hard water) at a temperature of 20.0 ± 1.5 °C in a room with a 16h/8h light/dark photoperiod, but protected from light. Twice a week, cocoons were visually inspected to evaluate frequency of hatched cocoons (fecundity) emergence, number of newborns (fertility) and frequency of anomalies in newborns. Cocoons were inspected for up to 1 month after laying, since *G. tigrina* cocoons (kept at ≈ 20 °C) typically hatch after 2 to 3 weeks (Knakievicz et al., 2006; Vara et al., 2008). Each newborn planarian was visually inspected for any morphological or behavioural anomalies using a stereomicroscope (Stemi 2000C, Carl Zeiss™). Any observable alteration in body symmetry, misshaped, absent or duplicated body parts, changes in pigmentation, alterations in photoreceptors (such as reduction, absence, or extra photoreceptors) or presence of abnormal growths was recorded and categorized as a morphological anomaly. Alterations to normal gliding behaviour, such as sudden twists of the body, uncontrolled spasms, vomiting, or immobilization and absence of response to stimuli (light or touch) were recorded and categorized as a behavioural anomaly.

2.3 **Measurement of PAHs in solutions and tissues**

For the detection of pyrene and B[a]P in stock solutions and in planarian tissues, the fixed fluorescence (FF) method was used. Experimental solutions were not evaluated with this method, since PAH fluorescence in the media matrix was low. This method uses the fluorescent properties of PAHs to identify the compound in solution, based on the number of aromatic rings. The excitation / emission wavelengths used were 341 / 383 nm; 380 / 430 nm for pyrene and B[a]P-type compounds, respectively (Aas et al., 2000). Samples were analysed as previously described, with slight modifications (Aas et al., 2000; Amorim et al., 2011; Dissanayake and Galloway, 2004). Briefly, planarians were homogenized using an ultrasonic homogenizer (Branson Ultrasonics™ Sonifier 250) in 1200 µL of ultra-pure water. From each tissue homogenate, an aliquot of 150 µL was mixed with 1450 µL of 50 % methanol (CAS Number: 67-56-1; Fisher Chemical; ≥ 99.8%). Each stock

solution was diluted (1:30) in 50% methanol. Prior to fluorescence readings, samples (stock solutions and tissues) were well mixed using ultrasonic vibration (J. P. Selecta, Ultrasonic Bath – 1 min) and then vortexed. 96-well plates were used to measure samples (in quadruplicates), performing blanks in every plate. To estimate concentrations, standard curves of parent PAHs were performed and used to calculate the (LOQ) (Armbruster and Pry, 2008; Shrivastava and Gupta, 2011). Tissue measurements were expressed as ng of PAH equivalents / mg of tissue (wet weight) and stock solution measurements were expressed as mg L^{-1} of PAH. Fluorescence readings were performed with a Hitachi F-7000 Fluorescence Spectrophotometer at 20°C (Hitachi High-Technologies Corporation).

2.4 **Statistical analyses**

The number of deposited cocoons, the number of newborns per cocoon, the total number of newborns and the weight of adults were analysed with one-way ANOVA, followed by a Dunnett post-hoc test to evaluate differences between treatments and solvent control (SCTR). Both time for photoreceptor regeneration and amount of PAHs in planarian tissues were analysed with Kruskal-Wallis One Way Analysis of Variance on Ranks followed by a Dunn's post-hoc test to detect differences from the solvent control treatment (SCTR), since normality (tested with Shapiro-Wilk's test) and homogeneity of variances could not be achieved. For the fluorescence analysis of PAHs in planarian tissues, all measurements below LOQ were set as 0. A logistic model was used to investigate the effects of pyrene and B[a]P on cocoon fertility, by using PAH concentration as a response predictor. For the analysis of newborn anomalies, a mixed effects logistic model was used to investigate the effect of pyrene and B[a]P on newborn anomalies (morphological and behavioural); PAH concentration was included as a fixed effect, while replicate was included in the model as a random effect (R software, lme4 package; Bates et al., 2015). These analyses were performed with SigmaPlot v. 12.3 and R v. 3.5.3 software.

3 Results

The analysis of stock solutions revealed that the measured concentrations were close to nominal concentrations (Supplementary Table V-19). Planarian tissue samples presented pyrene- and B[a]P-type compounds in their tissues, respectively, indicating uptake of compounds form solutions. Nevertheless, SCTR, 1.17 and 4.69 µg L^{-1} treatments of pyrene and SCTR, 0.59 and 2.34 µg L^{-1} treatments of B[a]P were below LOQ (165.85 and 30.84 µg L^{-1} for pyrene and B[a]P, respectively). In the pyrene experiment, significantly higher amounts of pyrene-type compounds were detected in the 18.75 and 75.00 µg L^{-1} treatments when compared to solvent control treatment ($H = 118.583$; 4 d. f. $p < 0.001$; Figure V-18 – a). For the B[a]P exposed animals, significantly higher amounts of B[a]P-type compounds were found for the 37.50 µg L^{-1} treatment when compared to solvent control treatment ($H = 88.039$; 4 d. f. $p < 0.001$; Figure V-18 – b).

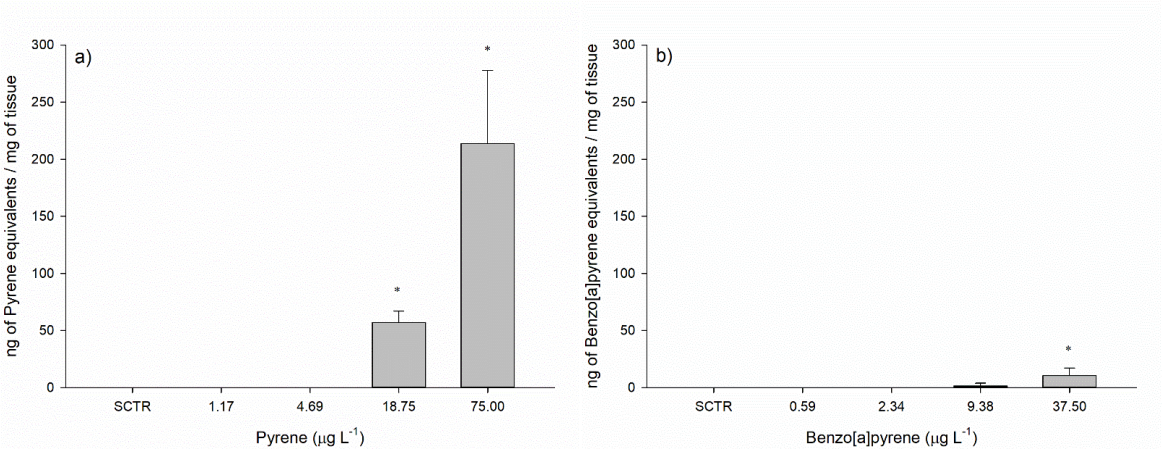

Figure V-18 – PAH-type equivalents in tissues of *Girardia tigrina* breeding adults at the end of the experiment, as determined by the Fixed fluorescence (FF) method. Data is presented as means ± SD. Treatments were compared using Kruskal-Wallis One Way Analysis of Variance on Ranks followed by a Dunn's post-hoc test to detect differences from the solvent control (SCTR); * P < 0.05.

The total number of deposited cocoons (fecundity) was significantly decreased by exposure to PAHs. In the pyrene experiment, the number of deposited cocoons was significantly lower for the 18.75 and 75.00 µg L^{-1} treatments ($F_{4,20}$ = 25.413; p < 0.001; Figure V-19 – a) in comparison with the solvent control treatment . In the B[a]P exposure, the number of deposited cocoons was lower at 37.50 µg L^{-1} ($F_{4,19}$ = 4.509; p = 0.010; Figure V-19 – b) in comparison with the solvent control treatment.

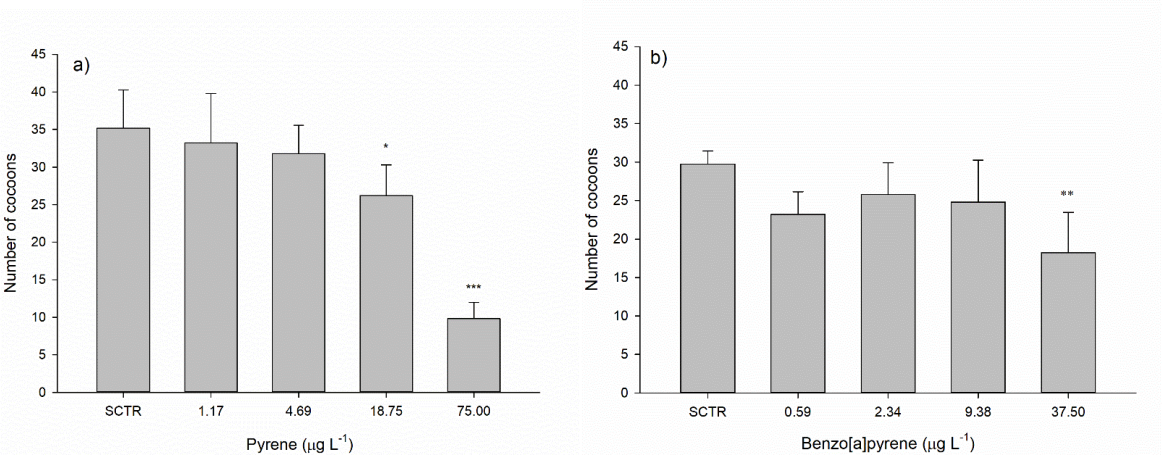

Figure V-19 – Total number of cocoons deposited by *Girardia tigrina* in the 21 days of exposure. Data is presented as means ± SD. Treatments were compared using one-way analysis of variance (ANOVA) with Dunnett's post-hoc test. * P < 0.05; ** P < 0.01; *** P < 0.001.

The percentage of fertile cocoons was affected by pyrene and B[a]P exposures (Table V-17). For pyrene, there was a decrease in the percentage of fertile cocoons with increasing concentrations (p = 0.035) and in the case of B[a]P, there was an increase in the percentage of fertile cocoons with increasing concentrations (p = 0.035) (Figure V-20). The emergence of newborns occurred mostly 2 to 3 weeks after cocoon laying. The mean number of newborns emerged per (fertile) cocoon was

unaffected by PAH exposure (Figure V-21. pyrene: $F_{4,20} = 0.473$; $p = 0.755$. B[a]P: $F_{4,19} = 0.927$; $p = 0.469$).

The realized offspring (fertility) was decreased under pyrene exposure, with reductions of 6.86%, 14.31%, 33.08% and 79.13% in relation to the solvent control (SCTR), for the 1.17, 4.69, 18.75 and 75.00 µg L^{-1} treatments, respectively ($F_{4,20} = 42.230$; $p < 0.001$; Figure V-22 - a). For the B[a]P exposure, reductions in fertility were 21.55%, 8.70%, 8.25% and 33.74% for the 0.59, 2.34, 9.37 and 37.50 µg L^{-1} treatments, respectively, as compared to the control, but no significant differences were detected ($F_{4,19} = 2.641$; $p = 0.066$; Figure V-22 - b).

Table V-17 – Estimates of the Logistic model applied to frequency of hatched cocoons.

PAH	Parameters:	Estimate	Std. Error	z value	Pr (>|z|)
Pyrene	Intercept	1.385	0.107	12.913	<2e-16
	Concentration	-0.018	0.009	-2.113	0.035
Benzo[a]pyrene	Intercept	1.225	0.121	10.130	<2e-16
	Concentration	0.020	0.009	2.111	0.035

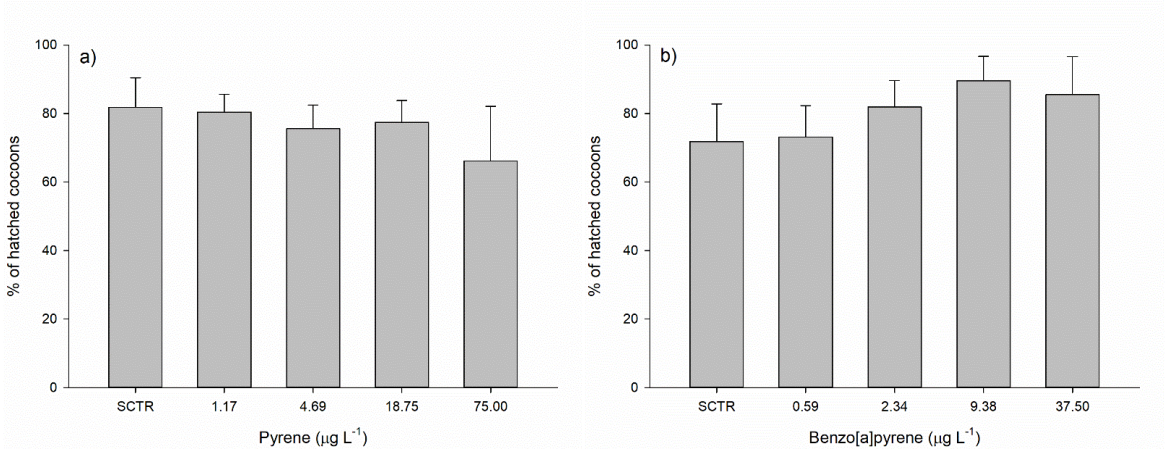

Figure V-20 – Percentage of *Girardia tigrina* cocoons producing offspring. Data is presented as means ± SD.

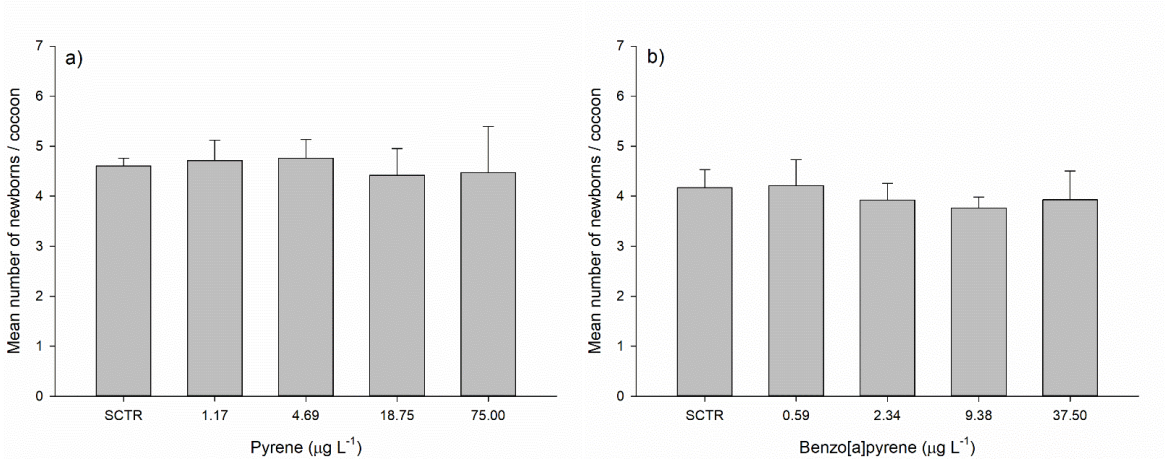

Figure V-21 – Mean number of newborns per hatched cocoon deposited by *Girardia tigrina* adults. Data is presented as means ± SD. Treatments were compared using one-way analysis of variance (ANOVA).

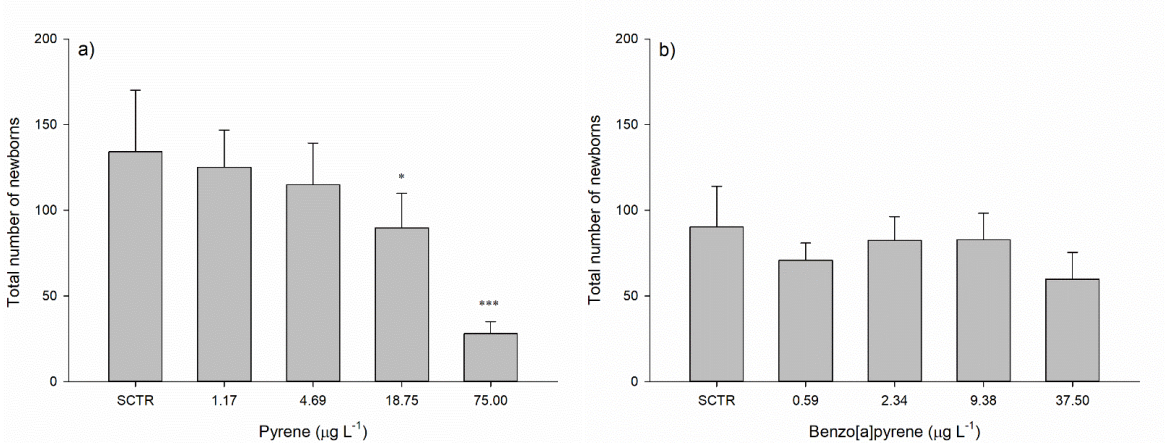

Figure V-22 – Total number of *Girardia tigrina* newborns emerged from the cocoons deposited over the course of the test. Data is presented as means ± SD. Treatments were compared using one-way analysis of variance (ANOVA) with Dunnett's post-hoc test. * P < 0.05; *** P < 0.001.

Table V-18 – Estimates of the fixed effects from the mixed effects logistic model applied to frequency of newborn anomalies.

Anomalies	PAH	Parameters:	Estimate	Std. Error	z value	Pr (>|z|)
Morphological	Pyrene	Intercept	-4.629	0.288	-16.088	<2e-16
		Concentration	-0.004	0.013	-0.325	0.745
	Benzo[a]pyrene	Intercept	-4.174	0.299	-13.964	<2e-16
		Concentration	0.008	0.015	0.539	0.59
Behavioural	Pyrene	Intercept	-6.662	0.908	-7.335	2.21e-13
		Concentration	0.015	0.018	0.883	0.377
	Benzo[a]pyrene	Intercept	-4.201	0.347	-12.117	< 2e-16
		Concentration	0.105	0.015	6.818	9.23e-12

The visual inspection of newborns revealed some behavioural (Supplementary Table V-20) and morphological (Supplementary Table V-21) anomalies. Most of the behavioural disorders observed were in the form of uncoordinated movements and spasms, while most of the morphological defects were in the form of missing or extra body parts, altered number of photoreceptors or deformed body parts. For pyrene, the mixed model analyses showed that, there was no significant increase on the frequency of behavioural or morphological anomalies with increasing concentrations. In the B[a]P experiment, there was a significant increase in the frequency of behavioural anomalies with increasing concentrations ($p < 0.001$), but not of morphological anomalies (Table V-18). The mixed model indicated that, for each unit of concentration, there was an increase of 1.64% in the behavioural anomalies induced by B[a]P.

The weight of adults did not differ between treatments in the pyrene experiment ($F_{4,20} = 2.747$; $p = 0.057$; Figure V-23 – a). In the B[a]P experiment, animals of the 9.37 and 37.50 µg L^{-1} treatments weighed less than control animals ($F_{4,19} = 6.292$; $p = 0.002$; Figure V-23 – b). Regeneration of photoreceptors was unaffected by the pyrene ($H = 9.253$; 4 d. f.; $p = 0.055$; Supplementary Figure V-24 – a;) and B[a]P exposures ($H = 5.014$; 4 d. f. $p = 0.286$; Supplementary Figure V-24 – b).

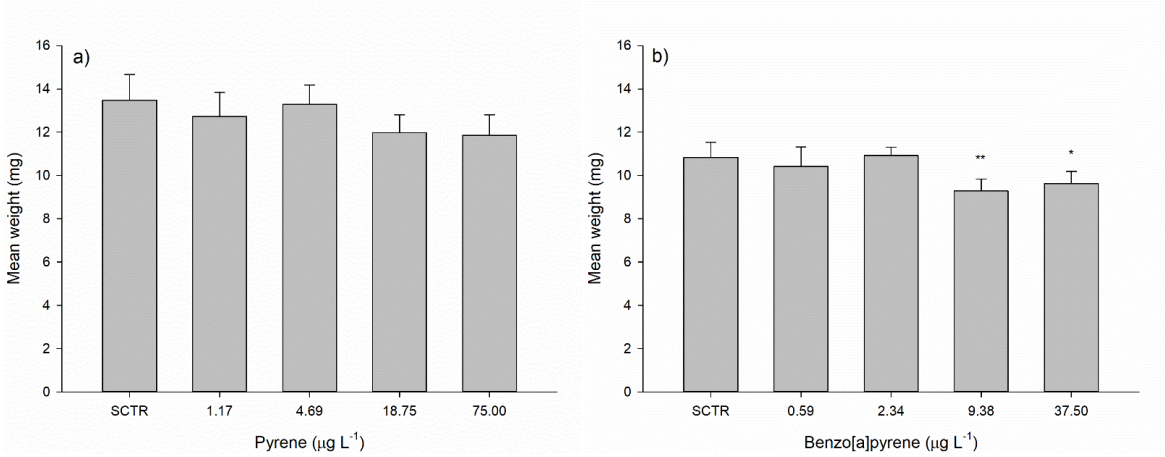

Figure V-23 – Weight of *Girardia tigrina* breeding adults by the end of the exposure period. Data is presented as means ± SD. Treatments were compared using one-way analysis of variance (ANOVA) with Dunnett's post-hoc test. * P < 0.05; ** P < 0.01.

4 Discussion

This work emphasizes the relevance of studying reproductive endpoints in planarians, illustrating that these can be sensitive while being ecologically relevant. Also, it is evidenced that planarians may be impacted by PAHs on the long-term, with reductions in fecundity and fertility potentially having negative consequences for natural populations. Moreover, there were visible negative effects on offspring from progenitors exposed to B[a]P, indicating a decrease in newborn fitness.

The concentrations at which endpoints measured in this study were affected, that is ≥ 18.75 and ≥ 9.38 µg L^{-1} for pyrene and B[a]P respectively, are in line with levels found in PAH-contaminated areas. Generally, PAH loads are higher in waterbodies near areas of increased human impact, such as cities, industrial complexes or harbours (Latimer and Zheng, 2003). Superficial waters can have high PAH levels, but these compounds will quickly be deposited in sediments, as a result of their physicochemical properties (Burgess et al., 2003). For instance, in the Gomti River, in India, during the winter season, superficial waters were estimated to contain up to 0.42, 3.41 and 75.57 µg L^{-1} of pyrene, B[a]P and total PAHs, respectively. On the other hand, sediments contained up to 60.08, 231.35 and 3365.13 ng g^{-1} dw of pyrene, B[a]P and total PAHs, respectively (Malik et al., 2011). Moreover, in the Yellow River in China, pyrene, B[a]P and total PAHs were found in sediments at 18.5–169, 59.3–180 and 464–2621 ng g^{-1}, respectively. In pore water, concentrations were estimated as 0.69–5.05, 1.16–3.90 and 48.2–206 µg L^{-1} for pyrene, B[a]P and total PAHs, respectively (Yu et al., 2009). The high PAH loads in sediments can have important implications for freshwater planarians, since their epibenthic nature may imply exposure through this route, added to waterborne exposure and transfer from contaminated prey. Moreover, the fact that PAHs do not occur as isolated substances, means that organisms will be exposed to a combination of several PAHs and related compounds. In areas with high human impact, freshwater planarian populations will likely be impacted by these contaminants.

The observed reduction in fecundity and fertility upon exposure to PAHs is highly relevant, since it indicates that natural populations might be at risk because of a decreased reproductive output. It is interesting to note that the concentrations causing reproduction impairments in the present study are much lower than those eliciting mortality (Chapter III). One possible explanation might be a reduced energy available for reproduction elicited by decreases in feeding. This is in line with previous studies, in which *G. tigrina* feeding was reduced under exposure to the same PAHs (Chapter III). Other studies have observed a decreased food intake elicited by PAHs and consequent decreases in reproductive output. For the copepod *Calanus finmarchicus* exposed to waterborne pyrene, reductions in grazing (measured by faecal pellet production rate) and egg production were observed (20.23 µg L^{-1}) (Jensen et al., 2008), while for the copepod *Schizopera knabeni* a reduction in grazing rate and number of offspring per female were detected in response to phenanthrene (≥ 56 mg kg^{-1}) exposure (Lotufo, 1997). Moreover, exposure to PAHs may lead to an increase in energy expenditure associated with metabolic costs, seen, for instance, as an increase in respiration rate in the amphipod *Hyalella azteca* exposed to phenanthrene (Gauthier et al., 2016), in the reduced energy reserves of the crab *Eriocheir sinensis* exposed to B[a]P (Yu et al., 2018) and in the increased respiration rates and decreased scope for growth in the oyster *Crassostrea gigas* exposed to a combination of several PAHs (Jeong and Cho, 2007). In a previous study we observed that pyrene increases the energy consumption (measured through electron transport system activity) (Chapter IV). Moreover, Vandenbrouck et al. (2010) observed decreased lipid reserves (at 35 µg L^{-1}) and reproductive output (measured as intrinsic population growth at 70 µg L^{-1}) in *Daphnia magna* exposed to pyrene. Hence, it is possible that the observed decreased reproductive output is related to disturbances in energy intake, metabolism and allocation, which might also help explain the weight differences in the planarians from the B[a]P experiment. Nevertheless, other factors, such as the frequency and / or success of mating events could also have played a role. Some studies have shown that mating success can be decreased in response to diesel oil or naphthalene, with these chemicals possibly altering chemical detection or decreasing the amount of pheromones released by females (Krång, 2007; Seuront, 2011) and, in planarians the number of successful mating events seems to be related to the number of deposited cocoons (Vreys et al., 1997). However, we cannot know if this was the case, since we did not quantify mating events. There is also the possibility that these compounds interfere with neurosecretions / neuromodulators involved in the control of sexual reproduction, since PAHs have been found to alter hormone levels in fish (Sun et al., 2015) or crabs (Wen and Pan, 2016). Still, the chemical substances involved in the control of planarian sexual reproduction are currently being uncovered (e. g. Collins et al., 2010; Rouhana et al., 2017) and there is no information available on toxicant effects on hormones involved in planarian sexual reproduction.

The development time for cocoons was around 2 to 3 weeks for all treatments, and in accordance with previously observed data for this species at 20 °C (Knakievicz et al., 2006; Vara et al., 2008). However, hatching success was differently affected by the tested PAHs, decreasing in the pyrene

exposure and increasing in the B[a]P experiment, with increasing concentrations. The lower hatching success observed in the pyrene exposures is in accordance with other works. For instance, egg production, potential recruitment rate and hatching success were affected in the copepod *Acartia tonsa* exposed to phenanthrene (≥ 320.8 µg L^{-1}) and pyrene (≥ 64.72 µg L^{-1}) (Bellas and Thor, 2007). Also, for the copepod *Calanus hyperboreus* exposed to pyrene, there was a decrease in the number of eggs produced per female per day and a decline in hatching success and number of nauplii per clutch (Nørregaard et al., 2014). In the B[a]P exposure, hatching success was increased, but the number of cocoons deposited was lower with increasing B[a]P concentrations. Hence, it is possible that B[a]P exposure lead to a selection of the better-quality embryos / eggs in the planarians, thus increasing hatching success of the fewer deposited cocoons. Nevertheless, more studies are needed to uncover the specific effects of contaminants on the sexual reproduction process of planarians to uncover these issues.

Although PAHs did not seem to induce appreciable amounts of morphological defects, many of the newborns resulting from the B[a]P experiment presented behavioural anomalies. These were mainly sudden twists, contractions and uncoordinated movements, being possible symptoms of neurotoxicity (see Buttarelli et al., 2000). This agrees with previously observed behavioural symptoms in *G. tigrina* exposed to PAHs (Chapter III). Since cocoons were transferred to clean media, it is possible that B[a]P was somewhat acquired through maternal transfer, for instance in the numerous yolk cells that are usually encased in the cocoons (Newmark and Sánchez Alvarado, 2002; Vila-Farré and Rink, 2018). The present results evidence that there was uptake of both PAHs from solutions to planarian tissues and previous studies have observed that PAHs accumulate in lipid-rich tissues and cells, as for instance, reproductive structures and gametes (Ellis et al., 1993; Hale, 1988; Hansen et al., 2017). In fact, some authors have argued that the incorporation of PAHs and / or PAH metabolites could account for lower viability of copepod embryos (Hansen et al., 2017; Jensen et al., 2008). Nevertheless, it is also possible that B[a]P could have been transferred through direct exposure after cocoon laying (until removal from vials), or that small amounts of B[a]P could have been adsorbed to the outer surface of cocoons and affected animals once they emerged. In fact, Best and Morita (1991) observed that the presence of ethanol in cocoon-incubation water can lead to decreases in hatching success and on size of newborns, evidencing the permeability of cocoons, at least to ethanol. These explanations might account for the observed effects on newborns of the B[a]P experiment, however, the same was not verified in the pyrene experiment. Some explanations can be proposed for these observations. For instance, B[a]P might have higher toxicity to developing / newborn planarians, also, higher amounts of B[a]P could be transferred to the embryos / cocoons, or metabolic differences may lead to distinct accumulation or elimination of the different PAHs. Further studies dealing on the specific metabolism and distribution of PAHs in planarian tissues and offspring may help uncover these issues.

The post-exposure regeneration endpoint was unaffected, with animals regenerating normally and without delays in clean media. In planarians, the neoblasts (stem cells) give rise to all other cellular

types, enabling even regeneration of the nervous system (Agata and Umesono, 2008). In *G. tigrina*, cephalic regeneration is relatively fast, with photoreceptors reappearing in about 4 days (Knakievicz et al., 2006; Medvedev and Komov, 2005). In a previous study we observed that planarian regeneration was only slightly or not affected by PAHs after relatively short exposure periods (8 days) (Chapter III) and it seems that the longer exposure still did not affect this intricate process, despite the detection of PAH-type equivalents in planarian tissues. Nevertheless, B[a]P exposure has been shown to lead to tumours in several animals (Baird et al., 2005), while in planarians, the few studies that exist, seem to point in opposite directions. On one hand, exposure of planarians to B[a]P has been seen to cause neoplasia and malformations (Foster, 1963; Hoshina and Teshirogi, 1991), on the other hand, no malformations or neoplasia were detected (Best and Morita, 1991; Zhang et al., 2018). In our study we show that there is uptake of B[a]P (and of pyrene) into tissues, but no developmental toxicity, malformations or neoplasia were observed in intact or regenerated adults.

5 Conclusion

Planarian reproduction was affected by PAH exposure, namely the number of deposited cocoons (fecundity) and, in the case of pyrene, also the number of emerging newborns (fertility). Reproducing adults in the B[a]P experiment also evidenced a trend of decreased size with increasing PAH concentration. Additionally, in the B[a]P experiment, behavioural anomalies were detected in unexposed newborns, indicating possible symptoms of neurotoxicity. These effects are evident at concentrations much lower than those causing lethality (Chapter III). Also, the sensitivity of reproduction-related endpoints in *G. tigrina* towards pyrene and B[a]P, appears to be in the same range than for *D. magna*. The observed reproduction-related effects may have serious consequences for populations in natural contexts, since reductions in reproductive output and newborn fitness may affect population dynamics and maintenance. Moreover, this study provides the first ecotoxicological data regarding PAH effects on reproductive endpoints of animals belonging to the Platyhelminthes phylum.

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Supplementary material

Supplementary Table V-19 – Measured concentrations of stock solutions for the pyrene and B[a]P experiments. Concentrations are presented as mean (± SD) (solutions prepared on days 0, 4, 7, 11, 14 and 18 of the experiment).

	Measured concentration (mg L^{-1})			PAH nominal concentration (mg L^{-1})	% of nominal concentration
	Mean	SD	Min – Max		
Pyrene (mg L^{-1})	705.98	48.66	655.78 – 756.12	750.00	94.130
	217.37	23.69	197.22 – 249.73	187.50	115.931
	52.41	3.86	47.99 – 59.37	46.87	111.810
	14.62	1.61	13.05 – 16.69	11.72	124.769
B[a]P (mg L^{-1})	380.91	14.01	363.36 – 393.63	375.00	101.577
	103.30	3.94	98.07 – 107.67	93.75	110.182
	25.21	1.30	23.55 – 26.65	23.44	107.556
	6.38	0.34	5.74 – 6.94	5.86	108.802

Supplementary Table V-20 – Behavioural anomalies observed in the newborns hatched from the cocoons deposited during the 21-day exposures of *Girardia tigrina* adults to either pyrene or B[a]P. Values represent the average (min – max) percentage of individuals, for each treatment, that exhibited behavioural anomalies.

Type of disorder	Concentration of pyrene (µg L^{-1})					Concentration of B[a]P (µg L^{-1})				
	0.00	**1.17**	**4.69**	**18.75**	**75.00**	**0.00**	**0.59**	**2.34**	**9.38**	**37.50**
Uncoordinated movements[a]	**0.00** (0.00 – 0.00)	**0.16** (0.00 – 0.81)	**0.17** (0.00 – 0.84)	**0.22** (0.00 – 1.05)	**0.71** (0.00 – 3.45)	**0.00** (0.00 – 0.00)	**0.85** (0.00 – 2.67)	**1.46** (0.00 – 3.53)	**8.70** (3.57 – 14.78)	**24.75** (15.79 – 51.02)
Spasms[b]	**0.45** (0.00 – 1.53)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**3.86** (0.00 – 8.33)	**11.37** (0.00 – 40.91)
Vomiting[c]	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.67** (0.00 – 4.08)
Stillness[d]	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.28** (0.00 – 1.26)	**0.24** (0.00 – 1.25)	**0.00** (0.00 – 0.00)	**0.67** (0.00 – 2.78)
Total%	**0.45** (0.00 – 1.53)	**0.16** (0.00 – 0.81)	**0.17** (0.00 – 0.84)	**0.22** (0.00 – 1.05)	**0.71** (0.00 – 3.45)	**0.00** (0.00 – 0.00)	**1.13** (0.00 – 2.67)	**1.70** (0.00 – 3.53)	**12.56** (6.38– 19.32)	**37.46** (20.83– 57.58)

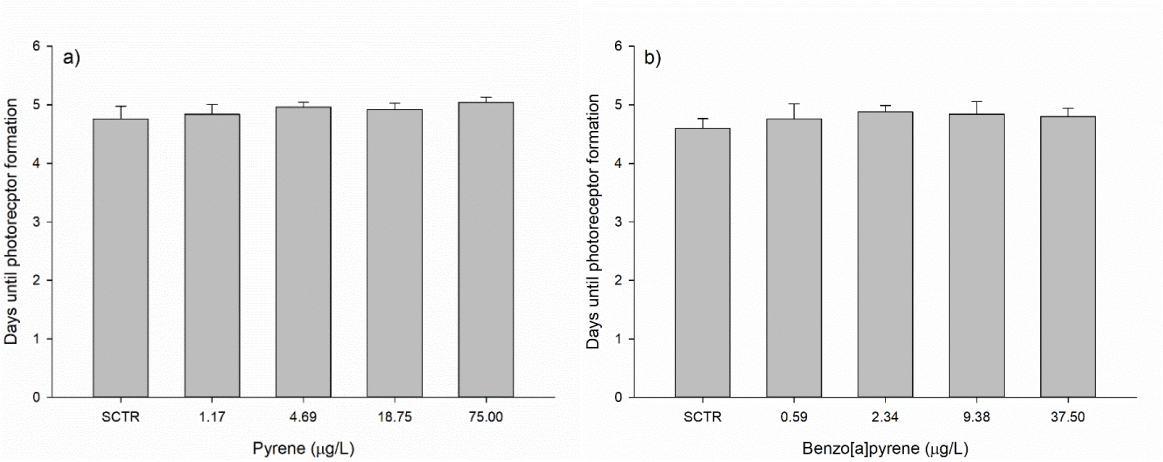
a – Uncoordinated movements include all sudden twists of the body and/or contractions of body parts, but with animals being able to move away from bright lights.

b – Spasm include all events where animals showed contractions of the body, while remaining in the same place and not moving away from bright lights. c – Vomiting include all events where animals showed eversion of pharynx and contents were discharged. d – Stillness includes all animals that were laying on their sides and were unresponsive to stimuli, such as gentle prodding and light.

Supplementary Table V-21 –Morphological anomalies observed in newborns hatched from the cocoons deposited during the 21-day exposure of *Girardia tigrina* adults to either pyrene or B[a]P. Values represent the average, minimum and maximum percentage of individuals, in each treatment, that presented morphological anomalies.

	Concentration of pyrene (µg L^{-1})					Concentration of B[a]P (µg L^{-1})				
Type of disorder	**0.00**	**1.17**	**4.69**	**18.75**	**75.00**	**0.00**	**0.59**	**2.34**	**9.38**	**37.50**
Deformed body parts[a]	**0.60** (0.00 – 1.80)	**0.16** (0.00 – 0.65)	**0.17** (0.00 – 1.12)	**0.22** (0.00 – 1.30)	**0.00** (0.00 – 0.00)	**0.55** (0.00 – 2.41)	**0.28** (0.00 – 1.26)	**0.97** (0.00 – 1.52)	**0.72** (0.00 – 2.13)	**0.33** (0.00 – 2.04)
Altered pigmentation[b]	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.28** (0.00 – 1.26)	**0.00** (0.00 – 0.00)	**0.72** (0.00 – 3.57)	**0.00** (0.00 – 0.00)
Photoreceptor anomalies[c]	**0.15** (0.00 – 0.81)	**0.32** (0.00 – 1.04)	**0.00** (0.00 – 0.00)	**0.22** (0.00 – 1.11)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.28** (0.00 – 1.30)	**0.49** (0.00 – 1.52)	**0.00** (0.00 – 0.00)	**1.34** (0.00 – 4.17)
Extra body parts[d]	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.17** (0.00 – 0.65)	**0.45** (0.00 – 1.05)	**0.71** (0.00 – 4.00)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.24** (0.00 – 0.96)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)
Missing body parts[e]	**0.60** (0.00 – 1.80)	**0.00** (0.00 – 0.00)	**0.17** (0.00 – 0.65)	**0.22** (0.00 – 1.05)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.85** (0.00 – 2.53)	**0.49** (0.00 – 3.03)	**0.48** (0.00 – 1.79)	**0.33** (0.00 – 1.39)
Damages in tegument[f]	**0.15** (0.00 – 0.51)	**0.16** (0.00 – 0.72)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.56** (0.00 – 1.45)	**0.49** (0.00 – 2.5)	**0.48** (0.00 – 2.17)	**0.00** (0.00 – 0.00)
Neoplasia[g]	**0.00** (0.00 – 0.00)	**0.32** (0.00 – 2.08)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)	**0.28** (0.00 – 1.33)	**0.24** (0.00 – 1.52)	**0.00** (0.00 – 0.00)	**0.00** (0.00 – 0.00)
Total%	**1.49** (0.00 – 2.44)	**0.96** (0.00 – 2.61)	**0.52** (0.00 – 1.30)	**1.11** (0.00 – 2.11)	**0.71** (0.00 – 4.00)	**0.55** (0.00 – 2.41)	**2.54** (0.00 – 5.06)	**2.91** (0.00 – 7.58)	**2.42** (1.09– 3.57)	**2.01** (0.00 – 6.94)

a – Includes all animals with deformed body plans and misshaped body parts; b – Any alteration in the pigmented areas, such as larger and darker pigmentation spots than normal; c – Includes all alterations relating to number of photoreceptors, like extra or reduced number of eyespots. Also includes reduced photoreceptors. d – Any structure that is duplicated, such as heads, tails or pharynxes. e – Missing body parts, including tail or head. f – Includes any damages on the skin, such as tears and blisters. g – Includes any abnormal growth.



Supplementary Figure V-24 – Days until regeneration of photoreceptors in *Girardia tigrina* adults after 21-day exposures to PAHs and cephalic regeneration in clean media. Columns represent means with standard deviation. Data is presented as means ± SD.

Chapter VI – Accumulation of PAHs in the head portion of planarians

Accumulation of PAHs in the head portion of planarians

Abstract

It has been previously observed that freshwater planarians can accumulate cadmium in their head portions, with neurotoxicity and head loss accompanying this phenomenon. Since planarians exhibit head loss and symptoms of neurotoxicity in response to PAHs, we investigated the differential accumulation of pyrene and B[a]P in the body and head portions of *Girardia tigrina*, a freshwater planarian. It is evidenced that planarian head fragments present higher amounts of pyrene- and B[a]P-equivalents than body fragments, indicating a differential distribution of these compounds within planarian tissues.

1 Introduction

Planarians are simple animals. Externally, a head and tail portions are recognizable. The head portion of planarians contains the sense organs and the brain, which are fundamental for the acquisition of environmental stimuli, their processing and choice of the appropriate behavioural responses (Ross et al. 2017). In planarians, xenobiotic stress can lead to disintegration of planarian tissues, starting on the head region (e. g. Best et al. 1981a; Villar et al. 1994). Some authors have proposed the hypothesis of a relationship between disintegration of the head region and neurotoxicity, with accumulation of xenobiotics in that part of the planarian body (Wu et al. 2011). So far, it has been observed that the metal cadmium can preferentially accumulate in the head region of planarians (Wu et al. 2011). In previous experiments we have shown that exposure to PAHs can lead to stereotypical behaviours and to the disintegration of the head region in planarians (Chapter III). Hence, we were interested in determining if PAHs also accumulate in the head region of planarians. Our objective was to determine if there is a differential distribution of pyrene and B[a]P-type compounds between the head and body portions of the planarian *Girardia tigrina*.

Keywords: planarians; PAHs; differential accumulation.

2 Methods

2.1 Planarian cultures

G. tigrina cultures were kept in plastic containers with reconstituted hard water (ASTM 2014), at 20 °C. Animals were maintained in near darkness, except when feeding, with room photoperiod set as 16 h light / 8 h dark. Animals were fed once a week with either cow liver or chironomid larvae. Media and containers were changed after feeding and 2 – 3 days after. Prior to experiments, planarians were starved for 7 days to ensure a uniform metabolic state (Oviedo et al. 2008). The length of

the selected planarians was 1.6 ± 0.2 cm. Animals were measured, while fully extended, in a petri dish with a small amount of media and visually inspected with a stereomicroscope.

2.2 Differential accumulation tests

Animals were kept in small crystallizing vials with 20 mL of experimental solution. For each treatment, 10 replicates of 2 animals each were performed. For both PAHs, 3 concentrations were chosen, plus a control (CTR) and a solvent control (SCTR). The chosen concentrations were based on previous experiments that showed behavioural responses in planarians exposed to pyrene and B[a]P, which are consistent with neurotoxicity (sudden contractions / spasm or snake-like movement), but no head loss is observed (Chapter III). The performed treatments were 37.5, 75 and 150 µg L^{-1} for both pyrene (CAS Number 129-00-0; Sigma-Aldrich; purity 98%) and B[a]P (CAS Number 50-32-8; Sigma-Aldrich; purity ≥ 96%). Dimethyl sulfoxide (DMSO) was used as a solvent and kept at 0.01% (v/v) of final solution volume. Solutions were renewed every 2 days. The vials were kept covered with glass lids to avoid evaporation. After the 8 days and for each replicate, one of the animals was dried with paper filter and put in a microtube (whole animals) and the other animal was decapitated and head and tail were dried in filter paper and put in separate microtubes (head and tail fragments). All samples were weighed and kept at -20 ºC until further processing.

2.2.1 Measurement of PAHs

Frozen samples were homogenized with an ultrasonic homogenizer (Branson Ultrasonics™ Sonifier 250) in Milli-Q water (whole animals and tail fragments in 1200 µL and head fragments in 400 µL). From each homogenate, 150 µL were sampled and mixed with methanol (CAS Number: 67-56-1; Fisher Chemical; ≥ 99.8%) at 50%. Mixing of samples was performed using an ultrasonic bath (J. P. Selecta, Ultrasonic Bath) during 1 min and then vortexed. Immediately after, fluorescence readings were performed using 96-well plates (at 20°C). Each sample was performed in quadruplicate with blanks in each plate. PAHs were detected by using the fixed wavelength fluorescence method (FF), similarly as previously described (Aas et al. 2000; Dissanayake and Galloway 2004; Almeida et al. 2012). Calibration curves were performed using curves on known parent PAH concentration. Samples were expressed as ng of PAH-equivalents / mg of tissue. The excitation and emission wavelengths used for pyrene and B[a]P were, respectively, 341 / 383 nm and 380 / 430 nm. Slit widths were set at 2.5 nm. Measurements were performed using a Hitachi F-7000 Fluorescence Spectrophotometer (Hitachi High-Technologies Corporation).

2.3 Statistical analyses

Linear Mixed Models were used to evaluate differences between the different planarian tissues (whole planarian, body fragment, head fragment) within each concentration. For each treatment

(concentration), the type of tissue was included in the model as a fixed effect while planarians ID (nested in Replicate) was used was a random effect, in order to consider the lack of independence between measurements taken in the same animal as well as animals from the same replicate. The validation of all models was done through the analysis of residuals. All analyses were performed with R 4.0.0.

3 Results and Discussion

PAH-type equivalents in controls and solvent controls were below the quantification limit (165.85 and 30.84 µg L^{-1} for pyrene and B[a]P, respectively). The planarian head fragments were observed to contain higher amounts of PAH-type equivalents per weight than tail portions, with PAH-type equivalents increasing in a dose-dependent-way (Figure VI.25). This indicates that PAHs are accumulating in the head region of planarians at the tested concentrations. One possible explanation for the higher PAH-type equivalents in the head portion is related with the lipid content of the brain. For instance, a study by Deb et al. (2000) found that 11 fish species collected in the Hiroshima Bay, accumulated more PAHs in brain and gonad than in other tissues, such as gill, bone, muscle and liver. The authors suggest that this might be related to higher lipid content of these organs. Another study found that 4 fish species had higher PAH loads on brain than in muscle, gill or liver tissues, with the lipid content being a major influencing factor on PAH concentrations in each tissue (Xu et al. 2011). Thus, as brains are generally lipid-rich tissues, the planarian brain might be an accumulation organ for these hydrophobic compounds. An alternative explanation would be an accumulation of these compounds on different structures. For instance, the fungus *Fusarium solani* is able to accumulate pyrene and B[a]P in lipid vesicles and subsequently degrade them (Verdin et al. 2005). Still, no study involving invertebrates found similar evidence, so it is unknown if invertebrates possess similar mechanisms to deal with PAHs. If in fact the distribution of PAHs in planarian bodies is related to the accumulation in the lipid-rich nervous system, then it is possible that this might be associated with the potential neurotoxicity evidenced by the presence of stereotypical behaviours (Chapter III).

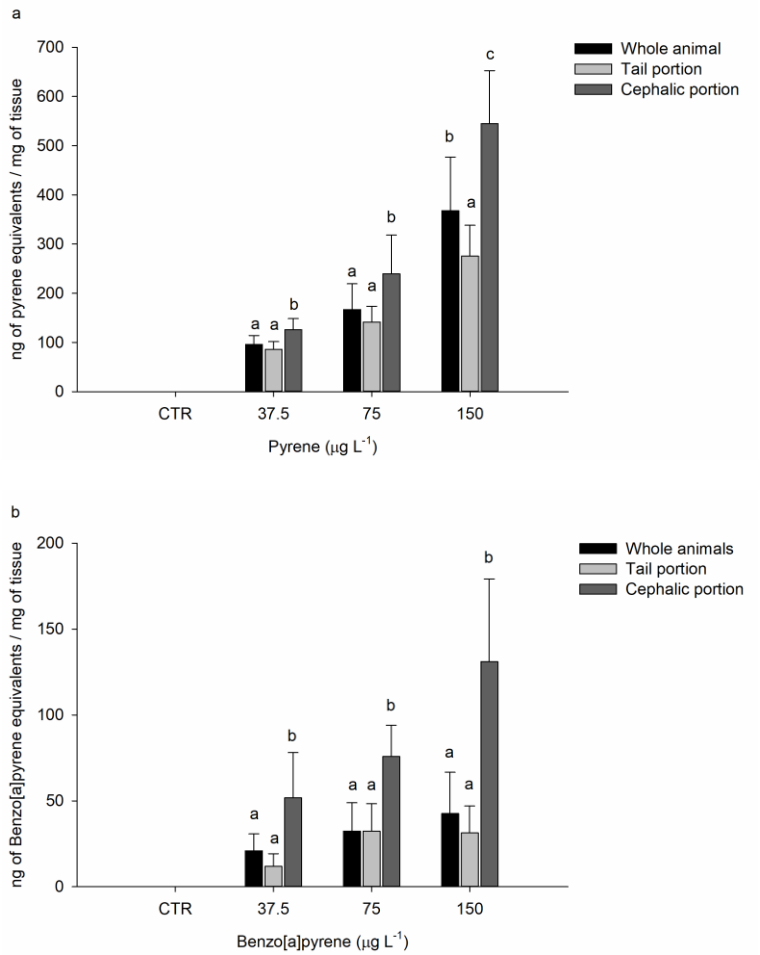


Figure VI.25 – Accumulation of PAHs in intact, tail and head portion of planarians, after 8 days of exposure. Columns represent means with standard deviation. Treatments were CTR (solvent control), 37.5, 75.0 and 150.0 µg L^{-1} of a) pyrene and b) B[a]P. Whole animals and body fragments were compared with Linear Mixed Models. Different letters above columns represent significant differences (within each tested concentration) between whole animals and body fragments.

The only other study investigating the differential accumulation of contaminants in the head versus tail region of planarians, found that cadmium accumulates in the head region, but copper does not (at similar relative toxicity levels - LC_{50} / 2). Moreover, cadmium induced MT levels in the head portion of planarians, with the authors suggesting a connection between cadmium distribution in planarian tissues and MT induction (Wu et al. 2011). It is interesting to note that cadmium, but not copper, accumulated in the head of planarians and greatly induced MTs, which might suggest a difference in detoxification / differential distribution between essential and non-essential metals. Nevertheless, it seems unlikely that MT induction might also be related to accumulation of PAHs in the head portion of planarians, given the specific affinity of MTs for metals (Amiard et al. 2006).

Head loss in planarians occurs as a consequence of exposure to very high levels of PAHs (Chapter III), cadmium (Calevro et al. 1998), methylmercury (Best et al. 1981b), chlordane (Best et al. 1981a) or organophosphorus insecticides (Villar et al. 1993; Villar et al. 1994). At least for cadmium and PAHs, accumulation in the head portion has been shown, while for the other compounds, there

is no information regarding their distribution among planarian tissues. Still, all of these studies report either neurotoxicity or stereotypical and other behavioural responses that may be indicative of neurotoxicity (see Buttarelli et al. 2008 for neuropharmacology studies). If there is a connection between accumulation and head loss in planarians, one might wonder if it is a consequence of the extent of damage or if it could be a protective response to eliminate accumulated contaminants. Invertebrates have strategies to eliminate contaminants, such as accumulation in exoskeletons that are lost during moulting in crustaceans, autotomy of body portions with higher contaminant concentrations in oligochaetes, and it has also been proposed that release of contaminants through eggs might reduce body burdens (Vidal and Horne 2003; McClellan-Green et al. 2007). In planarians, the loss of the head region can also lead to significant costs, since the sense organs are lost as well. Still, since planarians regenerate their head and sense organs within days, animals can recover from this tissue loss, with the advantage of eliminating a great portion of accumulated contaminants and regenerating a new brain. This phenomenon is an acute response elicited before mortality. In natural environments, if planarians are exposed to very high concentrations of PAHs for short periods, head loss could play a role in the survival of populations, since the contaminant load would be reduced in conjunction. Still, further work is needed to determine if differential accumulation in the head region could be related to head loss.

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Chapter VII – Accumulation and trophic transfer of Benzo[a]pyrene in *Chironomus riparius* and *Girardia tigrina*

Accumulation and trophic transfer of Benzo[a]pyrene in *Chironomus riparius* and *Girardia tigrina*

Abstract

Planarians are increasing in relevance for ecotoxicological research. One of the advantages of using planarians as test animals, is the possibility of evaluating a vast range of endpoints in response to chemical stressors. Yet, the possibility of using planarians as predators in studies dealing with the trophic transfer of contaminants has not been explored. Given their epibenthic nature, planarians can be especially useful to evaluate the transfer of pollutants present in sediments, through benthic prey invertebrates. In this work, we exposed *Chironomus riparius* larvae and *Girardia tigrina* to sediments spiked with B[a]P and evaluated contaminant uptake into both species. Moreover, the dietary transfer of B[a]P-equivalents from larvae to unexposed planarians was assessed. Both planarians and chironomids exposed to contaminated sediments had detectable amounts of B[a]P-type compounds in their tissues, indicating that B[a]P was bioavailable. In the dietary experiment, planarians fed on contaminated larvae and trophic transfer of B[a]P-equivalents was observed. These results indicate that planarians have the potential to be used in ecotoxicity studies using contaminated sediments, as well as in trophic transfer studies as invertebrate predator.

1 Introduction

Currently, planarians are model species for areas, such as stem cell, regeneration and neuropharmacological research (Newmark and Sánchez Alvarado 2002; Sánchez Alvarado 2006; Buttarelli et al. 2008). Several features have made them important models, such as the presence of adult stem cells (neoblasts) that allow for the regeneration of missing body parts, and a simple nervous system that shares similarities with that of the vertebrates (Newmark and Sánchez Alvarado 2002; Sánchez Alvarado 2006; Buttarelli et al. 2008). Moreover, they can be sensitive to environmental pollutants and are amenable to the evaluation of a great variety of endpoints in response to chemical stressors, which has led some researchers to propose them as test species for ecotoxicological research (Best and Morita 1991; Ofoegbu et al. 2016; Wu and Li 2018; Ofoegbu et al. 2019). These include the potential to evaluate acute and reproductive toxicity, developmental defects, genotoxicity and neurotoxicity and other biochemical responses, (Best and Morita 1991; Wu and Li 2018). Nevertheless, one feature that seems to be overlooked is planarian's predatory behaviour. Their inclusion in the repertoire of ecotoxicological model species could potentially introduce an invertebrate predator amenable to bioaccumulation and trophic transfer studies. This could be especially relevant for research on persistent hydrophobic contaminants in sediments, considering the (epi)benthic nature of planarians and their invertebrate prey such as gastropods, insect nymphs and larvae, small crustaceans and oligochaetes (Pickavance 1971; Vila-Farré and Rink 2018),

some of which already have representative species in ecotoxicological research. Planarians capture prey by trapping them with mucus and wrapping themselves around them. Once captured, planarians feed by forcing the pharynx inside the prey's body. By muscular and enzymatic action, the prey's tissues are consumed (Jennings 1957; Jennings 1962).

The transfer of contaminants from prey depends on the contaminant and its specific properties. Hydrophobic contaminants, such as benzo[a]pyrene (B[a]P), a 5-ringed polycyclic aromatic hydrocarbon (PAH), have higher affinity for the organic matter present on sediments and can persist in this compartment for long periods of time (Wilcock et al. 1996; Meador 2008; Kainz and Fisk 2009). Sediments can be a source of PAH contamination for aquatic organisms, but the degree of contamination also depends on the preferred habitat or food source of prey and predator organisms (Beasley and Kneale 2002; Logan 2007; Meador 2008; Alegbeleye et al. 2017). Animals that live in close association with sediments are more exposed to PAHs (Logan 2007). Species that feed on particulate matter can ingest PAHs along with sediment particles, while predators depend on their specific feeding strategy and on the level of prey contamination (Meador 2008; Mogren et al. 2013). Invertebrate predators can have distinct feeding strategies that may imply the consumption of all the prey's tissues or of only a part of them (Brooks et al. 2009; Mogren et al. 2013). This latter case is that of the planarians, since they "select" what parts of the prey they feed upon, leaving unwanted particles and structures (such as exoskeletons) behind. It is unknown if the planarian feeding strategy may account for significant intake of PAHs from contaminated prey. So, in this study we focused on 2 invertebrate species, *Chironomus riparius*, whose larval stages (2^{nd} to 4^{th}) are benthic and live buried in the sediments, and *Girardia tigrina*, an epibenthic planarian predator. *C. riparius* was chosen, given that it is a model species in ecotoxicology, whose larval stages are natural prey of planarians and, as benthic organisms, are likely to be affected by contaminants that persist in sediments such as B[a]P. *G. tigrina* was chosen, since it has been shown to be sensitive to PAHs and is a voracious planarian species, making it ideal for studies on trophic transfer of contaminants. We evaluated the uptake of B[a]P for both species, in an experiment with contaminated sediments, and the transfer of B[a]P-equivalents from sediment-exposed *C. riparius* larvae to uncontaminated *G. tigrina* through feeding.

2 Methods

2.1 Planarian and chironomid culture maintenance

Planarians were kept in plastic containers with ASTM hard water (ASTM 2014), at a temperature of 20°C. Animals were kept in containers wrapped with aluminium foil with perforated lids to achieve a dark environment, except when feeding and cleaning. Room photoperiod was kept at 16 h light / 8 h dark. Either cow liver or chironomid larvae were provided as food items weekly. Media and con-

tainers were changed immediately after feeding and 2 – 3 days after. Prior to the experiments, planarians were starved for 7 days to ensure a uniform metabolic state (Oviedo et al. 2008).

Chironomus riparius cultures were maintained in plastic containers with a thin layer of mineral sediment (< 1 mm, and previously burned at 500 °C during 4 h) and ASTM hard water (ASTM 2014). Room temperature was kept at 20°C and aeration was provided to the media. Room photoperiod was set as 16h light / 8h dark. Animals were fed with a suspension of Tetramin® every other day. Culture media, containers and sediments were renewed every two weeks.

2.2 Preparation and spiking of the sediments

Tests were conducted with sediment spiked with Benzo[a]pyrene (B[a]P, purity ≥ 96%; Sigma-Aldrich Portugal, analytical standard). The preparation and spiking of sediments were performed according to the guideline sediment-water chironomid toxicity test using spiked sediment (OECD 2004), with some modifications. Briefly, the artificial sediment was composed by 75% of inorganic sediment, 20% kaolin, 5% α cellulose, 0.1% calcium carbonate ($CaCO_3$), so that each replicate contained 50 g of sediments. The inorganic sediment was washed, sieved (< 1 mm) and burned for 4 – 5 h at 450 ºC. After mixing of all the sediment components, for each replicate, sediments were spiked with 10 mL of B[a]P dissolved in acetone and acetone alone for the solvent control. Three concentrations were prepared: 40, 10 and 2.5 mg of B[a]P kg^{-1} plus the control (SCTR). After spiking, the solvent was evaporated by leaving the vials in a fume hood for at least 72 h in the dark. After this, 10 mL of medium was thoroughly mixed with the rest of the components to form a cohesive mass. Then, 150 mL of medium (ASTM hard water) was gently added to avoid resuspension and left to stabilize at least 48 h in darkness. After this step, gentle aeration was provided to the test vessels and left for 24 h prior to the placement of organisms.

2.3 Uptake of B[a]P from sediments by *C. riparius* and *G. tigrina*

Five replicates of 3 planarians and 10 replicates of 15 8-day larvae were performed. Animals were maintained at 20 °C with a photoperiod of 16h / 8h (light / dark). No food was provided during the exposure period. Total exposure period was 48 h. By the end of the experiment, planarians (Accumulation Planarians – AP) were removed from the vials with a soft brush, washed in clean media, dried with filter paper, put individually in a microtube, weighed and frozen at -20 °C. Each replicate containing larvae was divided in 3 pools of 5 organisms. The 1st pool (Accumulation Chironomids – AC) was dried, put in a microtube, weighed and frozen at -20 °C. The 2nd and 3rd pools were used in the experiments described in the next section (section 2.4).

2.4 Transfer of B[a]P-equivalents from *C. riparius* to *G. tigrina*

The 2 remaining pools of chironomids resulting from the experiment described above, were used in 2 parallel experiments. The first was designed to evaluate the trophic transfer of B[a]P-equivalents from chironomid larvae to unexposed planarians. The second was designed to evaluate if there was transfer of B[a]P-equivalents that leaked from chironomids to the water and then to planarians.

Feeding experiment: five animals (2nd pool from the above experiments) were washed in clean media and fed to a starving planarian in a glass crystallizing vial with 50 mL of clean media, for a total of 10 fed planarians per concentration. Animals were left feeding for 3 to 6 h. After this, each planarian (Feeding planarian – FP) was dried in filter paper, put in a microtube, weighed and kept at -20 °C until further analysis.

Leaking experiment: five larvae were washed, put in a glass crystallizing vial with clean media (50 mL) and left for 3 h. After this period, larvae (Leaking Chironomids – LC) were removed, dried in filter paper, weighed, kept at -20°C for detection of B[a]P-type compounds, and a planarian was placed in the same media. After 3 h, the planarians (Leaking Planarians – LP) were dried in filter paper, weighed and kept at -20°C for detection of B[a]P-type compounds. This step allowed the evaluation of the transfer of chironomid-leaked compounds through the media to the planarians.

2.5 Detection of PAHs in tissues

To detect B[a]P-type compounds in planarian and chironomid tissues, the fixed wavelength fluorescence method (FF) was used, as previously described (Aas et al. 2000; Dissanayake and Galloway 2004; Almeida et al. 2012; Silva et al. 2013). Briefly, planarian and chironomid samples were homogenized in ultra-pure water using a TissueLyser II (Qiagen). 150 µL of the whole homogenate were diluted in 1450 µL of methanol at 50% and well mixed using ultrasonic vibration (1 min) and vortexing. Reading were performed in 96-well plates and each sample analysed in quadruplicates. B[a]P-equivalents were detected by using the following excitation and emission wavelengths pairs: 380 / 430 nm. Slit widths were set at 2.5 nm. Concentrations were estimated by using standard curves of known B[a]P concentrations and used to calculate the limit of quantification (LOQ). Tissue measurements were expressed as ng of B[a]P-equivalents per mg of tissue. Fluorescence reading were performed with a Hitachi F-7000 Fluorescence Spectrophotometer (Hitachi High-Technologies Corporation).

2.6 Statistical analyses

Within each set of tissue samples (AC, AP, FP, LC, LP), treatments were analysed with Kruskal-Wallis, followed by a Dunn's post-hoc test to evaluate differences from the solvent control (SCTR). The number of leftover larvae was compared using the Kruskal-Wallis test. Two-tailed t-tests were

performed between chironomid samples (AC vs LC) exposed to the same concentrations. All analyses were performed with SigmaPlot v. 12.3.

3 Results

In the contaminated sediment experiments, no planarian mortality was recorded. However, by the end of the exposure period, some of the planarians on the 40 mg kg^{-1} treatment exhibited an unusual behaviour, being buried, instead of gliding on top of the sediment layer. Some of the chironomid larvae were not recovered and were presumed dead. All missing larvae were included in the 1st pool (AC), resulting in some pools with only 4 larvae. The vessels containing larvae had a turbid appearance, evidencing higher amounts of suspended particles as a result of bioturbation. Visual inspection of chironomids, revealed that these ingested particulate matter from sediments, since their digestive tracts had discernible white particles inside. Both planarians (AP) and chironomids (AC) had quantifiable amounts of B[a]P-equivalents, after the 48 h exposures (Figure VII-26). B[a]P-equivalents in planarian (AP) tissues followed a dose-dependent increase ($H = 51.219$; 3 d. f; $P < 0.001$), with control replicates being below LOQ. The samples from the chironomids left in clean media for 3 h (LC) presented B[a]P-equivalents that followed a dose-dependent increase ($H = 37.013$; 3 d. f.; $P < 0.001$). LC chironomid samples presented lower amounts of B[a]P-type equivalents in the 40 mg kg^{-1} treatment than AC chironomid samples ($t = -4.322$; 18 d. f.; $P < 0.001$; Figure VII-26).

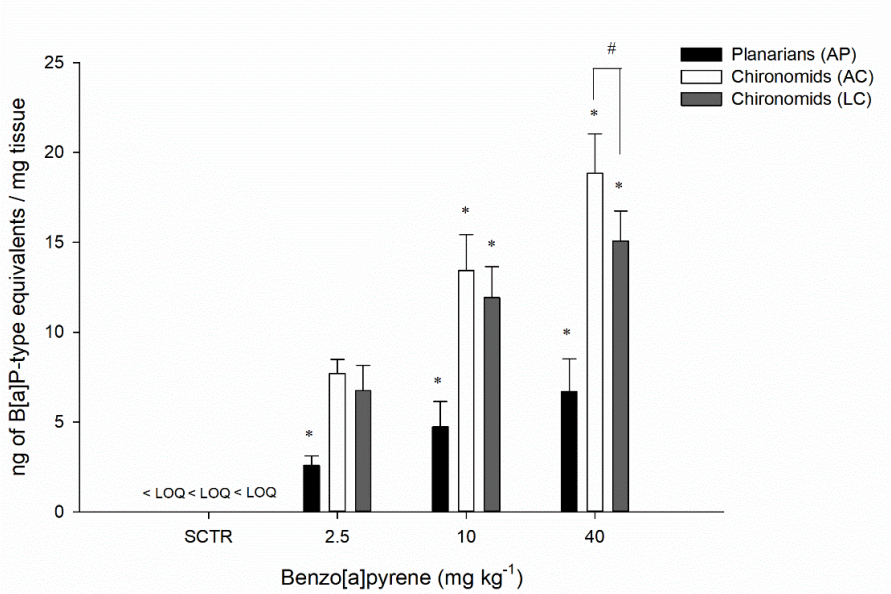

Figure VII-26 – Benzo[a]pyrene-equivalents in tissues of *Girardia tigrina* planarians and *Chrionomus riparius* larvae, exposed to B[a]P-contaminated sediments during 48 h. Chironomids were either immediately frozen (AC) or left in clean media for 3 h (LC). Data is presented as means (±SD). Within each set of tissue samples, treatments (SCTR, 2.5, 10 and 40 mg kg^{-1}) were compared using Kruskal-Wallis one-way analysis of variance followed by Dunn's post-hoc test. Differences from the control group (SCTR) are represented by * ($P \leq 0.05$). Differences between chironomid samples (AC vs LC) within the same concentration were compared with two-tailed t-tests and are represented by # ($P < 0.001$).

In the trophic transfer experiment, planarians were observed to feed on the contaminated larvae. After planarians fed, it was noticeable that the exoskeletons, and sediment particles (that were inside the gut of the larvae) were not ingested by planarians. Most of the planarians did not eat all the provided larvae in the allotted time. Still, unpalatability of contaminated food was not evidenced, since the average leftover larvae was around 2 for all treatments. Tissues of planarians (FP) fed with B[a]P-exposed larvae revealed to have B[a]P-equivalents in a dose-dependent way ($H = 31.265$; 3 d. f.; $P < 0.001$; Figure VII-27). Tissues of planarians kept in the same media where chironomids were left for 3 h (LP), had no quantifiable amounts of B[a]P-equivalents in their tissues (Figure VII-27).

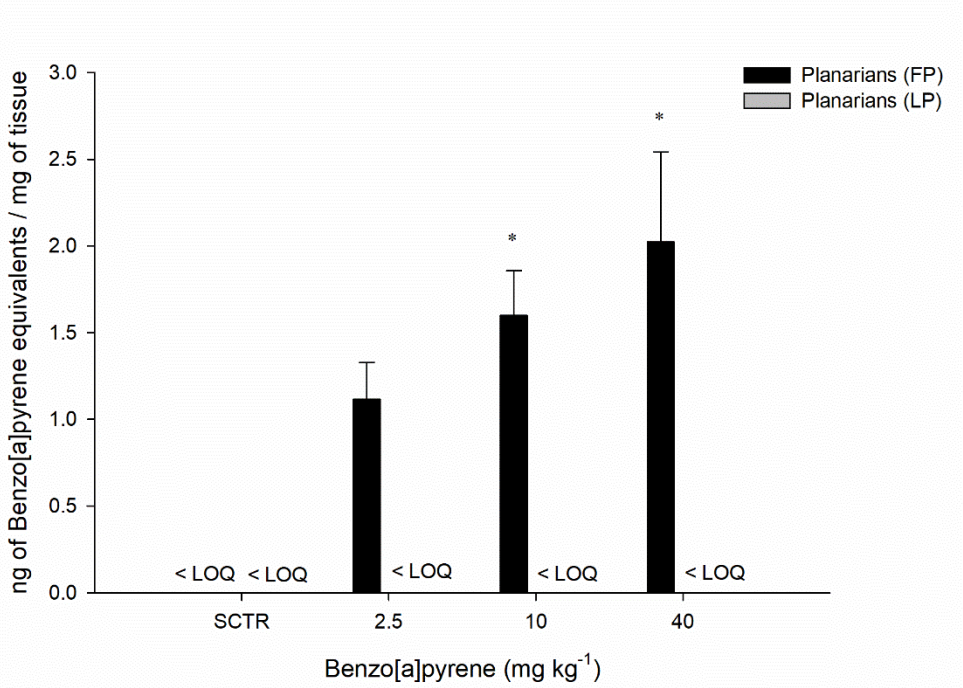

Figure VII-27 – Benzo[a]pyrene-type equivalents in tissues of *Girardia tigrina* fed with benzo[a]pyrene-exposed *Chironomus riparius* larvae (FP) or kept in the same media as chironomids where left during 3 h (LP). Data is presented as means (±SD). Treatments were compared using Kruskal-Wallis one-way analysis of variance followed by Dunn's post-hoc test. Differences from the control group (SCTR) are represented by asterisks. * P ≤ 0.05.

4 Discussion

This work explores the potential transfer of B[a]P present in sediments to benthic *C. riparius* and epibenthic *G. tigrina* and the potential transfer from contaminated prey to predators. The present results show the presence of B[a]P-equivalents in both larvae and planarians, indicating that B[a]P was bioavailable for both species. Moreover, planarians fed on contaminated larvae presented B[a]P-equivalents on their tissues, indicating trophic transfer. These results evidence that both predator and prey might be at risk from B[a]P-contaminated sediments. Moreover, these results show that planarians have the potential to be used in dietary transfer of contaminants by using chironomid larvae as their prey.

The range of concentrations tested in this study is consistent with high and very high levels of PAH contamination that occurs in the environment (Barakat et al. 2011). For instance, in the Gulf of Pozzuoli in the Mediterranean coast, sediments have been polluted for almost a century by several industries in the vicinity, with concentrations of B[a]P ranging from 0.63 to 180 mg kg^{-1} and total PAHs from 7.1 to 2500 mg kg^{-1} (Arienzo et al. 2017). Moreover, the Tianjin rivers in China, highly polluted due to industrial and urban sources, presented B[a]P concentrations in sediments that ranged from 0.03 to 3.99 mg kg^{-1} dry wt, and total PAHs in the range of 0.787 to 1943 mg g^{-1} dry wt (Shi et al. 2005). Hence, such high B[a]P concentrations can be found in areas with elevated human impact posing a threat to aquatic invertebrates.

The uptake of B[a]P by both chironomid larvae and planarians was evidenced by the B[a]P-equivalents found in tissues, which increased with concentrations of B[a]P in sediments. This indicates that B[a]P was bioavailable for both species. However, concentrations of B[a]P-equivalents in chironomid tissues were apparently higher than for planarians. The higher amount of B[a]P-type compounds in chironomid tissues is not surprising, given than *G. tigrina* is epibenthic, gliding on top of the sediment layer and normally not burying in it (Noreña et al. 2014; Vila-Farré and Rink 2018). On the other hand, chironomid larvae (from 2nd to 4th instars) are benthic and remain mostly buried, also ingesting particulate matter present in the sediments (Pinder 1986; Armitage et al. 1995). In our experiment, ingestion of particulate matter was evidenced by the presence of particles in the digestive tracts of larvae. A study by Leppänen and Kukkonen (2000), compared the body burdens of *Lumbriculus variegatus* worms (feeding vs non-feeding) in B[a]P-contaminated sediments and observed that the feeding animals had higher body burdens than non-feeding worms. They concluded that ingestion of particulate matter from sediments was the main source of B[a]P inside organisms, explained by the tight bonds that highly hydrophobic chemicals have with sediment particles. In our experiment, it seems that B[a]P was available for uptake through the body surfaces, given that both of our test species presented B[a]P-equivalents in their bodies, but probably the ingestion of the contaminated organic matter was responsible for the higher body burden of chironomid larvae. The replicates containing chironomids had a turbid appearance, probably indicating reworking of the sediments by the larvae. Clements et al. (1994) observed that *C. riparius* larval density was related to higher turbidity of the overlying and consequent higher B[a]P concentrations in the water. This also resulted in higher amounts of B[a]P in chironomid larvae. In our experiment, this phenomenon could also be (partially) responsible for the higher amounts of B[a]P-equivalents in chironomid larvae.

Interestingly, some planarians in the highest B[a]P treatment were buried in the sediments. This behaviour was not observed in control planarians. In fact, previously, we observed that when planarians were exposed to B[a]P, they would twist and turn with very fast motions, trying to escape bright lights (Chapter III). Possibly, this unusual behaviour, elicited by B[a]P exposure, was an attempt to escape from the illuminated environment to a less irradiated one, seeming to indicate that B[a]P enhanced their negative phototactic response.

The trophic transfer experiment evidenced that B[a]P-equivalents were transferred from chironomids to planarians. This transfer of compounds could be attributed to feeding on contaminated larval tissues and / or uptake of B[a]P-equivalents released by the chironomids. The leaching experiment with chironomids, evidenced a decrease in the B[a]P-equivalents in tissues, pointing to a release of compounds to the media and, the planarians kept in the same media did not present quantifiable amounts of B[a]P-equivalents. These results indicate that the ingestion of contaminated larval tissues was probably the main route of B[a]P-equivalents to the planarian tissues. Also noteworthy, was the consumption of contaminated prey in similar amounts as control ones, indicating that planarians will feed on B[a]P-contaminated invertebrates. Previously, other studies have shown that PAHs can be transferred from organisms exposed to PAH-contaminated sediments to predators feeding on them, such as the dietary transfer of fluoranthene to the grass shrimp *Palaemonetes pugio* feeding on the oligochaete *Monopylephorus rubroniveus* (Filipowicz et al. 2007). However, the feeding strategy of the predator may influence the uptake of contaminants. An experiment evaluating the transfer of arsenic from *Culex tarsalis* to terrestrial and aquatic invertebrate predators over 30 days, found that, of the 2 terrestrial species fed with adults, *Tenodera aridifolia sinensis* (Mantodea: Mantidae) accumulated more arsenic than *Tidarren haemorrhoidale* (Araneae: Theridiidae). Since arsenic was mainly in the exoskeleton of the prey, the authors attribute the observed differences to the feeding modes of the predators: the mantids eat their prey whole, while the spiders inject their prey with enzymes and then suck the resulting fluids. Hence, ingestion of the contaminant was higher for the predator that consumed the entire prey. In our experiment, the planarian feeding strategy implies that hardened structures, such as exoskeletons, are left untouched, and planarians seem to also be able to avoid intake of other prey contents, as was observed by the sediment particles left untouched, previously inside the guts of larvae. Nevertheless, it seems that the contents ingested by planarians contain B[a]P-equivalents, which was not surprising, given that, in chironomids, B[a]P is mostly accumulated in the soft tissues and not in the exoskeleton (Bartell 1982). Interestingly, the amounts of B[a]P-equivalents in tissues of planarians in the trophic transfer experiment (FP - 2.023 ng of B[a]P-equivalents / mg of tissue) fed with larvae from the 40 mg kg^{-1} treatment were not far from the levels in planarians that accumulated B[a]P from sediments in the 2.5 mg kg^{-1} treatment (AC - 2.584 ng of B[a]P-equivalents / mg of tissue) during 48 h. Considering that planarians fed, at most, on 5 larvae, and that planarians are voracious predators, in environments contaminated with B[a]P (and possibly other PAHs) planarians may be especially vulnerable to uptake of these compounds from contaminated prey.

The fixed fluorescence wavelength method was a good first approach to study uptake of B[a]P-equivalents into tissues of chironomids and planarians, since it gives an evaluation of the overall presence of B[a]P and metabolites (Szlinder-Richert et al. 2014). However, more specific techniques for quantification of B[a]P and respective metabolites are needed to uncover several aspects, such as, the metabolizing ability of planarians towards B[a]P and to identify the compounds being transferred from chironomids to planarians. Given that chironomids are known to metabolize

B[a]P (Borchert et al. 1997; Schuler et al. 2003), either the parent compound or related metabolites might be transferred to planarians. It would also be important to evaluate if the B[a]P-equivalents transferred through prey are retained in tissues for significant periods of time and if they can lead to measurable effects in planarians. Some studies have shown that PAH metabolites can be trophically transferred from prey to predators (Carrasco Navarro et al. 2012; Carrasco Navarro et al. 2013), and the transformation products may be more available than the untransformed compound to the predator (Palmqvist et al. 2006), although the ecological implications of this have not yet been elucidated. More studies are needed to elucidate these issues.

5 Conclusion

The present work shows that planarians and chironomids exposed to B[a]P-contaminated sediments uptook B[a]P into their tissues, indicating bioavailability of the compound to both invertebrate species. Chironomids had higher amounts of B[a]P-equivalents in their tissues, probably due to their benthic nature and ingestion of sediment particles. Moreover, B[a]P-equivalents were trophically transferred from chironomids, reared in contaminated sediments, to planarians, indicating that in natural environments, planarians might be at risk from feeding on B[a]P-contaminated prey. This study shows that planarians have the potential to be used in studies with contaminated sediments and to evaluate the trophic transfer of sediment-bound contaminants by feeding on benthic invertebrate species.

6 References

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Chapter VIII – General Discussion

Traditionally, risk assessment procedures use a limited number of model species, which implies that many groups of organisms are not represented. Hence, the addition of alternative model species, to complement the already existing ones, may help improve the risk assessment of chemicals (Breitholtz et al. 2006). Ideally, model species should possess a series of features that allows them to be easily maintained in laboratory conditions, be sensitive to contaminant stress and should already possess a database of background information (Robinson and Thorn 2005). In this context, planarians can be alternative species for ecotoxicological research. In the last decades, information on environmental contaminant toxicity towards planarians has been generated, in addition to the extensive literature from other research areas that have elucidated many processes at cellular and molecular levels (Cebrià 2007; Buttarelli et al. 2008; Wu and Li 2018). In practical terms, planarians are easily obtainable and can also be easily maintained in laboratory conditions, showing a wide tolerance to physicochemical parameters but being sensitive to environmental contaminants (Wu and Li 2018). Moreover, a vast range of effects can be observed in planarians as a response to contaminant stress, such as mortality, behavioural changes, sexual and asexual reproduction, neurotoxicity, teratogenicity or carcinogenicity, all in the same animal model (Wu and Li 2018). The shared features of their brain with the vertebrate nervous system (Buttarelli et al. 2008), make them interesting to study the effects of neuroactive contaminants (Ofoegbu et al. 2019b; Ofoegbu et al. 2019a). Interestingly, planarians belong to the 4th largest animal phylum, are abundant in many freshwaters and can be top predators in some habitats (Riutort et al. 2012; Noreña et al. 2014; Vila-Farré and Rink 2018), yet no species of the Platyhelminthes phylum is a model for ecotoxicological research. Given their unique combination of features, freshwater planarians would be an interesting addition to the repertoire of animal models used in ecotoxicological research.

The present work intended to explore the potential of freshwater planarians for ecotoxicology by using polycyclic aromatic hydrocarbons (PAHs) as model contaminants. As PAHs are ubiquitous, persistent, and occur in complex mixtures, they have been the focus of many works. Nevertheless, the evaluation of PAH effects has proven challenging, given the variety of potential effects, toxicity of complex mixtures or differences in metabolization among organisms (Logan 2007; Meador 2008). Although the metabolization of these compounds in vertebrate species has been extensively studied, for many invertebrates (such as Platyhelminthes) it is still unknown if, how and to what extent these compounds can be metabolized (Logan 2007; Meador 2008; Ball and Truskewycz 2013), raising questions on their potential effects upon these organisms and on organisms feeding on them. In the following paragraphs, major findings on the toxicity of PAHs towards planarians are highlighted.

A crucial step towards testing the suitability of planarians for ecotoxicological research was the development and adaptation of protocols to evaluate the effects of contaminants on *Girardia tigrina*. As planarians are not model species for ecotoxicology, no standard protocols exist in this context. Therefore, in the present work, a preliminary task implied choosing the testing conditions, experimental design and endpoints. Whenever possible, standardized methods and conditions for

ecotoxicological model invertebrates were chosen. This was the case for the culture media (reconstituted hard water, ASTM 2004) and maintenance temperature (20°C) commonly used in ecotoxicity testing and culturing of invertebrate species (such as daphnids or chironomids) of temperate freshwaters. Likewise, the duration of the tests was a compromise between the duration of standardized tests using invertebrate species, the existing planarian studies and adequacy in evaluating the chosen endpoint. Hence, a scale of days for mortality, behavioural, regeneration and biochemical endpoints was chosen, as well as weeks for reproduction-related endpoints. Moreover, since PAHs were chosen as test substances and since they have a low solubility in water, a solvent was needed to perform test solutions. We chose dimethyl sulfoxide (DMSO), given the high solubility of PAHs in it, but in accordance with general recommendations of using a low solvent concentration (ASTM 2004) and specific recommendations of keeping DMSO concentration below 500 µl L^{-1} (0.05%) in planarians studies (Stevens et al. 2014). The performed endpoints were selected in order to provide a robust characterization of PAH (and other contaminant) effects, and spanned key endpoints such as mortality, cephalic regeneration, behaviour, biochemical biomarkers and reproduction. Within the behavioural endpoints, we chose to evaluate planarian feeding rate and locomotion. Locomotion is a commonly used endpoint in ecotoxicity studies using planarians and a simple protocol, developed in the context of neuropharmacology (Raffa et al. 2001), is almost universally used. Although very easily employed and sensitive, it is not without its drawbacks, mainly the experimenter bias (e.g. different ways to count line crosses and re-crosses) and the time consumption of the observations (10-min for each planarian). Rodrigues et al. (2016) evaluated the locomotor activity of planarians using video-tracking system and compared it to the manual protocol by Raffa et al. (2001), and found that the automated method was able to detect effects of chlorantraniliprole at lower concentrations than the manual method, showing its sensitivity. Hence, as a sensitive, robust, cost and time effective alternative, we chose to use an automated method to evaluate the locomotor activity of planarians. Moreover, we can highlight the **development of a protocol to evaluate feeding rate of *G. tigrina* as an ecotoxicological endpoint, in accordance with the predatory behaviour of this species** (Chapter II). The developed protocol, that uses live chironomid larvae as prey, is simple, but its sensitivity to detect negative effects of different contaminant classes was demonstrated (metals, salinity, PAHs; Chapters II and III), showcasing the potential of planarian behavioural assays for ecotoxicological research.

By evaluating the effects of PAHs on multiple endpoints, the present work is not only relevant in exploring the potential of freshwater planarians for ecotoxicity testing but also allowed the generation of new information regarding PAH effects upon freshwater invertebrates. **PAH exposure elicited a variety of effects in *G. tigrina* including cephalic disintegration, locomotor and feeding impairments, changes in biotransformation enzymes and decreases in reproductive output.** Interestingly, while phenanthrene (3-ringed PAH) appeared to be more toxic than pyrene (4-ringed PAH) and benzo[a]pyrene (B[a]P – 5-ringed PAH) based on the mortality results, the inverse was observed for the sub-lethal endpoints (Chapters III and IV). It is not uncommon that smaller PAHs

cause more acute toxicity in animals than larger PAHs (Manzetti 2013), although this may not always be the case (e.g. *Daphnia*: 48 h LC_{50}s – 730.67, 135.8 and 250 µg L^{-1} for phenanthrene, pyrene and B[a]P, respectively; Atienzar et al., 1999; Brausch and Smith, 2009; Verrhiest et al., 2001). The differences in acute toxicity are possibly related with the bioaccumulation and metabolization of the compounds (Meador 2008). For instance, a study observed that the PAH uptake rate in copepods is faster for smaller when compared to larger PAHs (phenanthrene vs benzo[a]pyrene) (Jensen et al. 2012). In our case, a faster uptake rate of phenanthrene could explain the occurrence of mortality in a short timeframe, due to the inability of animals to cope with the very high internal concentrations. Conversely, the slower uptake rate of the larger compounds could allow for the upregulation of cellular defences or to elimination of compounds, thus allowing the survival of organisms, at least at short timeframes. Added to this, we also observed that glutathione-S transferase (GST) activity increased with increasing phenanthrene concentrations (Chapter IV), which suggests that this compound might be metabolized (see Hayes et al. 2005) by planarians. Hence, it is also possible that this compound is more actively metabolized (in comparison with pyrene or B[a]P) by planarians, as suggested for low compared to high molecular PAHs in other invertebrates (Jensen et al. 2012), thus leading to a fast production of reactive compounds that, at high concentrations, might lead to death. Conversely, at lower concentrations this metabolization might in turn favour the survival of planarians by promoting the elimination of the more water-soluble metabolites. Still, a better understanding of PAH metabolism, uptake, accumulation, elimination and specific cellular interactions in planarians is needed to uncover these issues. Nevertheless, one important conclusion can be drawn regarding the mortality endpoint in planarians: it may underestimate the toxicity of compounds that do not lead to death in a short time period. As we have shown in this work, even if mortality does not occur in a short time period, both pyrene and B[a]P can severely affect feeding and locomotion (Chapter III). **At longer exposure periods, these compounds lead to decreased reproductive output and size of reproducing planarians** (Chapter V). Hence, in ecotoxicological studies using planarians, it is advisable that sublethal endpoints should be used in combination with the evaluation of mortality, since the latter may be a poorly informative endpoint.

Another interesting result stems from the preferential accumulation of PAHs in the head portion (at least of pyrene and B[a]P) (Chapter VI), which might be related to the severity of the observed effects in the sub-lethal exposures (Chapter III). Considering planarian behaviours, they depend on the correct functioning of the nervous system (see Buttarelli et al. 2008) and the preferential accumulation of compounds in the head portion may lead to a disruption of neurochemical pathways. Furthermore, considering the post-exposure regeneration endpoint, the decapitation of planarians after exposure and regeneration in clean media, implies that a large portion of the PAHs are being removed from the regenerant individual. Thus, the animal will possess a much lower body burden than before, which might explain only slight (pyrene) or no delays (B[a]P) observed for the cephalic regeneration endpoint. On the other hand, the evaluation of regeneration while ani-

mals remain in contaminated media, implies that at the beginning of the exposure, PAHs cannot accumulate in the head portion, since this is missing. Cadmium has also been observed to accumulate preferentially in the head portion of planarians (Wu et al. 2011), and, when in cadmium-exposed media, planarians were able to regenerate without visible malformations or delays (Calevro et al. 1998), similarly to our observations (Chapter III). Interestingly, a study has observed that berberine-regenerating planarians possessed lower body residues than intact ones (Balestrini et al. 2014), which offers a potential explanation for our observations. If regenerating planarians simply uptake fewer amounts of PAHs, than the lower body burdens could translate in fewer negative effects. The regeneration endpoint also seemed to indicate that none of the tested PAHs was teratogenic to planarians, since no malformations were observed (Chapter III), an observation further evidenced by the (almost) absence of malformations in newborns (Chapter V). Still, additional information on the regeneration of the nervous system and reappearance of normal behaviours would be interesting to evaluate, since regenerated planarians might exhibit an apparently normal morphology, but be otherwise affected. For instance, Best and Morita (1991) observed that planarians regenerated in methylmercury chloride (at 0.02 and 0.04 mg L^{-1}) presented no visible morphological abnormalities. However, synaptic density and type (simple versus complex synapses), as well as righting time, motility and prey capture were found to be altered in mercury exposed animals. Interestingly, we observed stereotypical behaviours not only in intact but also in the exposed regenerants and in newborns of B[a]P-exposed progenitors (Chapters III and V), which is a potential indication of neurotoxicity (Buttarelli et al. 2008). Interestingly, the neurotoxicity biomarker evaluated, the activity of acetylcholinesterase (ChE), was unaffected by PAH exposure (Chapter IV). Still, it is unknown if other neurotransmitter-related enzymes or neurotransmitters are being affected. Hence, this preferential accumulation of PAHs in the head region of planarians is an extremely relevant result for future ecotoxicity studies using planarians, since other contaminants might exhibit a similar accumulation pattern and lead to similar behavioural effects. This phenomenon merits further investigation on its underlying mechanisms, and future studies should take the potential differential accumulation of contaminants within head / tail sections of planarians in consideration.

In the present work, it was evidenced that planarians also have the potential to be used in studies evaluating effects of contaminated sediments (Chapter VII). As animals that live in close contact with the sediment layer in freshwaters (Vila-Farré and Rink 2018), planarians will likely be exposed to contaminants present in this compartment (Alegbeleye et al. 2017). Planarians can uptake contaminants, such as B[a]P, through direct contact, but also through the ingestion of contaminated prey. Their feeding mode, by forcing the pharynx into the prey's body and sucking contents but leaving hardened structures untouched (see Jennings 1957), does not preclude the transfer of B[a]P (Chapter VII), an observation probably explained by the accumulation of B[a]P mainly in the chironomid's soft tissues (Bartell 1982). Possibly, in the case of contaminants that accumulate mainly in the hardened structures of prey (such as exoskeletons or shells), this transfer might be lower. Nevertheless, planarians have the potential to be used in studies dealing with the

trophic transfer of chemicals, especially considering their position on the trophic web, consuming other invertebrates and being preyed upon by larger invertebrate and vertebrate predators (Vila-Farré and Rink 2018). **Their features of small size, voracious and epibenthic nature, make planarians interesting organisms to be used in multi-species studies as invertebrate predators.**

Regarding all the evaluated planarian endpoints in this work, it was evidenced that some were much more sensitive than others. At short-term exposures, the biochemical biomarkers were some of the most sensitive endpoints, although different biochemical effects were observed for each PAH. Interestingly, energy-related parameters were responsive for phenanthrene and pyrene exposures, pointing to increased metabolic costs elicited by these compounds (Chapter IV). Nevertheless, at whole-organism level, the behavioural endpoints were very sensitive, especially the feeding endpoint, which was able to detect deleterious effects for all PAHs, Hg and NaCl. Nevertheless, the locomotion endpoint was as sensitive as the feeding rate in the pyrene experiment, and more sensitive in the case of Hg exposure (Chapters II and III). With longer exposure periods, the fecundity was shown to be a sensitive endpoint, but, as evidenced by the B[a]P experiments, it is advisable that the progeny should also be inspected, since effects might be carried over to the next generation (Chapter V). It is, however, noteworthy that the most sensitive endpoints in the present study might not be the same for other xenobiotics, since the observed effects depend on the mode of action of the tested substance. Hence, the best course of action is, undoubtedly, to evaluate a combination of these (and potentially other) endpoints, in order to take advantage of the full potential of using planarians for ecotoxicity testing.

The method of fixed wavelength fluorescence (FF) used in the present work enabled us to detect the presence of PAH-type compounds in planarian tissues, and hence confirm that all tested PAHs were uptook by planarians from experimental solutions in a concentration-dependent manner (Chapters III and V), with pyrene and benzo[a]pyrene partitioning more to the head than to the tail portion (Chapter VI). **The FF method also allowed for the detection of trophic transfer of PAH-type compounds** from contaminated chironomid larvae to planarians (Chapter VII). However, since this method is unable to discriminate closely related compounds, such as parent PAHs and respective metabolites (Beyer et al. 2010), it is not possible to exactly know if the planarian tissues contained the parent PAH, metabolites or a combination of both. It is also not possible to pinpoint the specific compounds that were acquired through the contaminated food items (Chapter VII). To uncover these issues, a more sensitive method is required to identify and quantify the compounds present in planarian tissues. This will allow for a better understanding on the metabolic abilities of planarians towards PAHs, and, if so, identify the metabolites being formed. This knowledge will potentially help explain the differences in the toxicity and the different effects elicited by each of the different PAHs.

Future research in planarian ecotoxicity testing

With this work, we have shown that planarians have great potential as alternative model species for ecotoxicological research, but some issues merit further examination. Because planarians can regenerate from injuries such as decapitation, it is possible to evaluate lethal effects of contaminants on regenerating animals. Some works have compared the mortality endpoint between intact and regenerating animals and, although some have observed that regenerants may be more sensitive (Kustov et al. 2014), they can have lower (Hagstrom et al. 2015) or similar (Calevro et al. 1998) sensitivity toward contaminants. It is argued by Calevro et al. (1998) that a similar sensitivity towards contaminants is not surprising, since regeneration serves the purposes of organismal repair as well as reproduction in the asexual strains. Moreover, differences in uptake rates of xenobiotics between regenerating and intact animals (Balestrini et al. 2014) may further contribute to the observed differences. It seems that planarian physiological stage's sensitivity might depend on the tested chemical, its mode of action and uptake dynamics. Therefore, it may be useful to evaluate the sensitivity of both developmental stages towards contaminants, since regeneration is a fundamental process for individual homeostasis and population maintenance. Another noteworthy issue is that, for sexually reproducing planarians, the lethality of contaminants can also be evaluated in planarian newborns. Some studies on this subject indicate that newborns can be more sensitive to contaminants than adult planarians (Knakievicz and Ferreira 2008; Zhang et al. 2010; Ribeiro and Umbuzeiro 2014). Nevertheless, newborns have been shown to be less amenable to the evaluation of other endpoints such as locomotion, since they are frequently rendered immobile by contaminants (Knakievicz and Ferreira 2008; Zhang et al. 2010). Probably other endpoints would be difficult to evaluate in newborns, but, presently, there is no information available on this subject. Finally, although planarians are long-lived and seem to avoid metabolic ageing (Mouton et al. 2011), it is unknown how / if differences in the age of animals could influence the sensitivity to contaminants. It is possible that metabolic changes occur throughout the lifetime of planarians, especially considering sexually reproducing animals, but further research is needed.

One of the most pressing challenges regarding the usage of planarians for ecotoxicological research is the lack of uniformity in species and testing conditions used by the several laboratories working with planarians. Ideally, a few species should be chosen, to allow for a better standardization and interlaboratory comparisons. From the existing literature, it can be seen that some planarian species seem to be more sensitive to chemical stressors than others. For instance, some evidence points to a lower uptake of metals (cadmium, copper and zinc) by *Dugesia japonica* when compared to *G. tigrina*, and is apparently related to the higher tolerance in metal-contaminated media by the former species (Wu and Li 2017). Moreover, chemical carcinogenicity has been demonstrated in a few species (*G. dorotocephala* and *G. tigrina*), but not for others (see Wu and Li 2018). Also, although many planarian species can be easily maintained in laboratory conditions, they may have distinct requirements, for instance needing more space per animal to be properly

maintained (Rivera and Perich 1994; Knakievicz et al. 2006). These issues must be taken in consideration when choosing the most adequate test species. This should involve a comparison of the most commonly used planarian species on their maintenance effort, tolerance to physicochemical changes, testing amenability, sensitivity to chemical stress, and number of endpoints that can be easily tested. Besides the choice of planarian species, the food provided to cultures is also of paramount importance. This may constitute a source of variation regarding nutritional status that might affect growth, development or synthesis of important reproduction-related molecules (Klein Breteler et al. 1999; Li et al. 2016) and may ultimately affect the sensitivity towards chemical stressors (e.g. Leung and Furness 2001). The usual food provided to planarian cultures varies from chicken or cow liver, egg yolk or live invertebrates such as chironomid larvae or oligochaetes (Knakievicz et al. 2006; Medvedev 2008; Zhang et al. 2010). In the present work we observed that, although cow liver allowed for planarian growth, development and cocoon laying, the resulting cocoons were mostly infertile (Chapter V). We observed that the best diet was a mixed one, alternating chironomid larvae with liver, and this diet allowed for a good development of planarians, as well as high fertility rates. Possibly the liver alone was insufficient in providing the needed nutrients for proper embryo development. Likewise, overfeeding planarians may lead to “obesity” and to a lower reproductive rate (Dunkel et al. 2011). Ideally, the food supply and feeding frequency should be optimized for each planarian species and standardized among laboratories in order to overcome such issues.

The testing conditions can be quite different between studies, which, makes it very difficult to compare results. The exposure media varies considerably throughout the literature (see Wu and Li 2018 for detailed list), with potential implications for molecular interaction, speciation or realized toxicity of contaminants (Sunda et al. 1978; Klaine et al. 2002). A sound strategy for interlaboratory standardization would be the adoption of reconstituted media recipes already used in ecotoxicological studies, to ensure a similar chemical composition of the media between laboratories and allow comparisons with other test species. The differences in exposure time further hinder comparisons, although, the type of endpoint to be evaluated typically dictates the temporal scale. For instance, effects that are more immediately observed are evaluated in a period of days (such as mortality or regeneration), while endpoints that take more time to be observed (such as on reproductive parameters or chemical carcinogenesis) are evaluated in a period of several weeks or months (see Wu and Li 2018). Nevertheless, this is a crucial test parameter that needs to be well defined. In order to optimize the exposure periods, the planarian species should be chosen first, since the timing of experiments should take into account specific features, such as the time needed to regenerate (e.g. 6 days for photoreceptor formation in *D. etrusca* versus 4 days in *G. tigrina*, Calevro et al., 1998; present work), or the time until reproduction or emergence from cocoons (e. g. Knakievicz et al. 2006), among other features.

Temperature is another factor that should be standardized, but that will also undoubtedly depend on the chosen species. For instance, while *D. japonica, G. tigrina* or *Schmidtea mediterranea* tests

are usually performed at *circa* 20 °C (Knakievicz and Ferreira 2008; e. g. Plusquin et al. 2012; Zhang et al. 2014; present work), *Polycelis felina* tests are usually performed at 15 °C or less (Horvat et al. 2005; e. g. Alonso and Camargo 2015). Ideally, the chosen planarian species should allow the testing in the context of different biogeographical regions. Species such as *G. tigrina* would be ideal for this purpose, since they are eurythermic and already inhabit different biogeographical regions (Vila-Farré et al. 2011; Vila-Farré and Rink 2018).

Finally, the photoperiod may be a somewhat challenging factor to optimize. Planarians are photonegative animals (Vila-Farré and Rink 2018) and are usually kept in a dark environment (e. g. Oviedo et al. 2008). In some experimental setups, planarians have been kept in a dark environment (e. g. Hagstrom et al. 2015), while others have maintained them in 16 h / 8 h or 12 h / 12 h light / dark cycles (Calevro et al. 1998; Zhang et al. 2011). It is advised by Wu and Li (2018) that planarian tests should be performed in a 16 h / 8 h or 12 h / 12 h light / dark cycle, in order to maintain their normal physiological condition. They base this advice in a study by Itoh and Igarashi (2000), that observed reductions in the amplitude of serotonin fluctuation along the day in planarians maintained in complete darkness. Still, these issues should be further studied, since it is unclear if keeping planarians in a light protected, but not completely dark environment (similarly as our culturing setup), influences the circadian rhythm of planarians in the same way. Moreover, it is also unclear how exposure to light for such long periods of time (12 or 16 h) might affect planarians, given their photophobic nature.

As demonstrated in the present work and throughout the literature, planarians are amenable to the development of simple protocols that take advantage of their features and allow for the evaluation of xenobiotic effects. Yet, it seems that some planarian aspects that would be important to characterize in terms of contaminant stress have been overlooked. For instance, there is virtually no information on the effects of contaminants upon cocoons, except for one study indicating that incubation in ethanol-containing media can affect the viability and size of newborns (Best and Morita 1991). If cocoons can be susceptible to contaminants, it is crucial to determine the extent of this susceptibility, since these structures harbour the developing embryos and are vital for the maintenance of sexually reproducing planarian populations. Moreover, the effects of contaminants on the sexual reproduction process of planarians is almost unknown and should be further elucidated. Although planarians may take several months to reach sexual maturity after hatching (personal observation), it is possible to prompt sexually mature planarians to degenerate and reform their sexual organs in a matter of weeks by cutting them (Miyashita et al. 2011). In this way, it is possible to evaluate the effects of contaminants on the reappearance of sexual organs and ability to reproduce in a relatively short time. Another interesting issue is the sexual versus asexual reproduction in planarians and how this can affect the sensitivity to contaminants. As opposed to sexual individuals, asexual planarians do not possess sexual organs (Newmark and Sánchez Alvarado 2002), and it has never been investigated if / how these physiological differences can reflect on the effects elicited by contaminants. Potentially, sexual individuals might be affected by contaminants that

target the male and female reproductive system, and species such as *G. tigrina*, that possess sexual and asexual populations, might be useful to investigate such questions.

Another interesting possibility might be the usage of planarians for *in situ* and biomonitoring studies, since they are slow moving animals and have low dispersal abilities (Vila-Farré and Rink 2018), rendering them vulnerable to local contamination events. Added to this, planarians can be very easily collected and are abundant in many natural environments, being possible to obtain a great number of individuals with little effort. Still, more information on uptake, bioaccumulation and metabolism of contaminants would be important to understand the potential of planarians for biomonitoring studies. In fact, one of the current limitations of using planarians in ecotoxicology is the lack of information on uptake and metabolism of contaminants. This is obviously a very important matter and is paramount for a better understanding on the observed effects of different compounds. Still, the usage of biochemical biomarkers presents a simple approach (and planarians are very easily homogenized since they do not possess hardened structures) that can provide insights on the toxicity mechanisms of contaminants and some researchers have begun to investigate the effects of contaminants upon planarians at the biochemical level (e.g. Plusquin et al. 2012; Wu et al. 2015). Moreover, genome sequencing has been performed for some planarian species, such as *S. mediterranea* and *D. japonica* (Robb et al. 2008; An et al. 2018), with transcriptomic (Wheeler et al. 2015; Almazan et al. 2018), and proteomic (Fernández-Taboada et al. 2011; Bocchinfuso et al. 2012) data and approaches also being available, providing a useful resource for future ecotoxicological studies.

Finally, and besides feeding and locomotor behaviour, very few studies have evaluated other behavioural alterations in response to contaminant stress, although Zhang et al (2018) have proposed a planarian screening platform that evaluates other behavioural endpoints such as phototaxis, thermotaxis and scrunching. For instance, considering the work of Inoue et al. (2015), it is evidenced that simple but sensitive assays can be developed for a series of planarian features, such as chemotactic, phototactic, thigmotactic / kinetic and thermotactic behaviours. These or similar protocols could be used to better characterize behavioural effects of different contaminants planarians.

Overall, it is clear that planarians allow for a comprehensive evaluation of contaminant effects (including teratogenicity, carcinogenicity, behavioural toxicity, asexual and sexual reproductive toxicity or neurotoxicity). Although more research is needed, as soon as even more researchers acknowledge the potential of freshwater planarians, then ecotoxicology will gain a powerful model organism.

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