Universidade de Aveiro
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Departamento de Biologia

Pearl Ugochi Ofoegbu

AVALIAÇÃO ECOTOXICOLÓGICA DE DROGAS PSIQUIÁTRICAS USANDO PLANÁRIAS DE ÁGUA DOCE

ECOTOXICOLOGICAL ASSESSMENT OF PSYCHIATRIC DRUGS USING FRESHWATER PLANARIANS

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, especialização em Ecotoxicologia e Biologia Ambiental, realizada sob a orientação científica do Professor Doutor Amadeu Mortágua Velho da Maia Soares, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro, e do Doutor João Luís Teixeira Pestana, Investigador auxiliar do Departamento de Biologia da Universidade de Aveiro.

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This work is dedicated to my family.

o júri

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palavras-chave

Drogas psiquiátricas, Fluoxetina, Carbamazepina, Avaliação Ecotoxicológica, Planárias de água doce, *Schmidtea mediterranea.*

resumo

Os fármacos psiquiátricos são atualmente considerados compostos emergentes nos ecossistemas aquáticos. Estes compostos são bioativos e com diferentes modos de ação, são desenhados para exercer efeitos a baixas concentrações exibindo toxicidade e efeitos secundários a concentrações mais altas. Para a avaliação do risco ecológico destes compostos é necessária uma escolha cuidada das espécies usadas em ensaios ecotoxicológicos. O presente trabalho teve como principal objetivo estudar o uso de planárias de água doce como organismos modelo para a avaliação dos efeitos ecotoxicológicos de compostos como a fluoxetina e carbamazepina. As planárias de água doce são organismos-modelo em estudos neuro-farmacológicos sendo que o seu sistema nervoso se assemelha ao de vertebrados. *Schmidtea mediterranea* é uma das espécies mais bem caracterizadas e usadas para estudos de regeneração e biologia do desenvolvimento. Para além disso, é uma espécie fácil de manter em culturas laboratoriais e usar em ensaios ecotoxicológicos. Este estudo envolveu assim a exposição de organismos, em condições laboratoriais, a baixas concentrações de cada um destes compostos com um enfoque em parâmetros organismais e populacionais. Para além disso e dado que estes compostos ocorrem em misturas complexas nos ecossistemas naturais, foram estudados também os efeitos em cenários relevantes de exposição incluindo a exposição simultânea a outros contaminantes. Em primeiro lugar, a adequabilidade e sensibilidade de diferentes parâmetros ecotoxicológicos em *S. mediterranea* foram validados em ensaios laboratoriais sendo avaliados os efeitos de tributylestanho (TBT) na locomoção, regeneração e danos do DNA. Desenvolveu-se ainda um ensaio de alimentação que pode ser usado como parâmetro comportamental sensível. Todos os parâmetros mostraram sensibilidade às concentrações testadas e *S. mediterranea* mostrou ser uma das espécies mais sensíveis ao TBT. Relativamente aos efeitos dos compostos psiquiátricos, os ensaios laboratoriais mostraram que a fluoxetina foi mais tóxica para *S. mediterranea* que a carbamazepina. A exposição a concentrações baixas de fluoxetina causou genotoxicidade, decréscimos nas taxas alimentares e na reprodução das planárias e também um aumento da atividade locomotora. Por outro lado, e apesar de aumentos na locomoção e dano no DNA observados nas concentrações intermédias, não foram observados efeitos claros da exposição à carbamazepina. Estes resultados realçam a *S. mediterranea* como um organismo modelo adequado para avaliação de efeitos ecológicos de drogas psiquiátricas em águas doces e também a relevância dos parâmetros usados nestas avaliações. Os ensaios laboratoriais para avaliação de efeitos combinados dos dois compostos psiquiátricos não revelaram interações significativas relativamente aos parâmetros comportamentais nem na reprodução e reforçaram a maior toxicidade da fluoxetina. Relativamente à exposição combinada da fluoxetina com o TBT, usado como modelo para contaminantes neurotóxicos, os resultados revelaram interações significativas e complexas quer para o dano no DNA quer para locomoção das planárias, sugerindo que os efeitos da fluoxetina podem ser alterados pela exposição simultânea ao TBT. No entanto a magnitude dos efeitos causados pelas concentrações usadas quer de TBT quer de fluoxetina convidam a uma investigação mais detalhada acerca dos efeitos combinados de concentrações mais baixas de ambos os compostos. Por último os efeitos de stressores naturais tais como a salinidade na toxicidade da fluoxetina foram investigados. Os níveis de salinidade nos ecossistemas dulçaquícolas têm aumentado devido em parte às alterações climáticas e pressão antropogénica e os nossos resultados mostram que níveis de salinidade baixos (0.75 – 3.0 g NaCl L^{-1}) afetam negativamente as planárias diminuindo as suas taxas alimentares, locomoção e reprodução, atrasando também a regeneração. A exposição combinada a salinidade e fluoxetina revelou efeitos opostos e interações significativas no que respeita à atividade locomotora, sendo que não foram observadas interações significativas para a reprodução. Em conclusão, este estudo sugere que as planárias podem e devem ser usadas como organismos teste para avaliação de risco ecológico dos fármacos psiquiátricos em águas doces, utilizado para isso parâmetros comportamentais, genotoxicidade e reprodutivos.

keywords

Psychiatric Drugs, Fluoxetine, Carbamazepine, Ecotoxicological Assessment, Freshwater Planarians, *Schmidtea mediterranea*.

abstract

Psychiatric pharmaceutical substances are emergent chemical contaminants of the freshwater environment. They are bioactive with many different modes of action and side effects, and are meant to elicit beneficial effects at low concentrations but eliciting toxicity at higher concentrations. One of the major challenges to risk assessment of psychiatric substances in freshwaters is the choice of test organisms. The main objective of the present study was to investigate if the freshwater planarian *Schmidtea mediterranea* could be used as test organisms for the assessment of ecotoxicological effects of psychiatric pharmaceuticals namely fluoxetine and carbamazepine. Freshwater planarians are used as model in neuro-pharmacology and their nervous system resemble those of vertebrates, especially humans. *S. mediterranea* is among the best characterised animal models for regeneration and developmental biology studies. Moreover, this species is amenable to laboratory culturing and ecotoxicological testing. The study involved exposure of freshwater planarians to varying low concentrations of each of the psychiatric substances with a focus on organismal and population level endpoints. Moreover, since these compounds may occur together or concomitantly with other stressors within freshwaters, their effects when in mixtures were also evaluated. First the suitability of different *S mediterranea* responses as ecotoxicological endpoints were validated using laboratory assays and effects of tributyltin (TBT) exposure on locomotion, head regeneration and DNA damage (genotoxicity). Moreover, a feeding bioassay was developed as an additional sensitive behavioural endpoint. All parameters showed sensitivity to the tested concentrations with *S. mediterranea* showing to be one of the most sensitive invertebrates to TBT exposure. Concerning the effects of psychiatry drugs, laboratory assays showed that fluoxetine was more toxic than carbamazepine to *S. mediterranea*. Exposure to low concentrations of fluoxetine elicited decreases in feeding rates and reproduction (fissioning) and also an increase in locomotor activity and DNA damage. On the other hand, and despite some increases in locomotion observed in intermediate concentrations, no clear effects were observed for carbamazepine exposures. These responses show *S. mediterranea* as a putative good model organism to evaluate the ecological effects of psychiatric drugs in freshwaters and the relevance of these endpoints for these assessments. Laboratory assays testing for effects of the concomitant exposure to the two drugs revealed no interactions on planarian behavioural endpoints and reproduction, reinforcing at the same time the higher toxicity of fluoxetine. Concerning combined exposures of fluoxetine together with TBT here used as a model neurotoxic contaminant, results revealed complex interactions on DNA damage and locomotion, suggesting that effects of fluoxetine can be mediated by the simultaneous exposure to TBT. However, strong effects of both TBT and fluoxetine in all parameters call for additional research directed at the effects of this mixture using even lower concentrations. Lastly, effects of natural stressors like salinity on fluoxetine toxicity were investigated. Salinity level in freshwater environments recently has increased due to climate change and human activities and our results show that salinity levels as low as 0.75 – 3.0 g NaCl L^{-1} can adversely affect planarians causing decreases in locomotion, feeding and reproduction and also delaying head regeneration with some developmental abnormalities. Combined exposure showed significant interaction on planarian locomotor activity since both stressors elicited contrasting responses and no significant interactions were observed for reproduction. In conclusion, the results obtained in this thesis suggest that freshwater planarians can and should be used as alternative non-model invertebrates, for the risk assessment of psychiatric pharmaceuticals in freshwater environments and that behavioural, genotoxicity and reproduction endpoints should be included in this evaluation.

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Chapter 1

General introduction

Chapter 1

General introduction

The freshwater habitat consists of about 0.8% of earth's surface but contains about 0.01% of World's water and is rich in species diversity, supporting almost 6% of all described species (Dudgeon et al., 2006). Freshwater species diversity is an important economic, educational, cultural, aesthetic, natural and scientific resource (Dudgeon et al., 2006, Strayer and Dudgeon, 2010). Freshwater habitats also serve domestic, agricultural, economic, recreational and waste disposal purposes. The rate of these activities over the years in developed and developing countries has increased due to increased population and industrialization, thereby subjecting freshwaters to a lot of stress due to pollution among other factors.

Pollution of freshwaters has led to exposure of organisms and ecosystems to numerous chemical compounds on a daily basis, which may have the potential of eliciting detrimental effects and drastic alterations of the mass and species richness, and ecological status of the ecosystem. Pollution of freshwaters may also result to some economic loss with respect to associated human morbidity and mortality, cost of water treatment and pollution control.

A number of chemical pollutants have been detected in the freshwater environment in the past years. However, in recent years with the help of higher quality chemical analytical techniques, other groups of chemical contaminants have been detected in the freshwater environment. It is anticipated that the presence of emergent chemical contaminants such as pharmaceuticals and other personal care products may affect freshwater organisms and the functioning of freshwater ecosystems. As a result, it is crucial to monitor and protect the freshwater environment from their deleterious effects.

Protection of freshwaters from possible negative effects due to pollution involves ecotoxicological studies among other measures. Ecotoxicology is a branch of science with a focus on the interaction of environmental chemicals with living organisms, as well as their toxic effects and effects on functioning of ecosystems (Fent, 1996) so that the environment can be shielded from the deleterious effects of these contaminants. It deals with fate of environmental chemical contaminants, their exposure and effects on biota (Fent, 1996), utilizing bioassays as one of the means for a better understanding of the impacts of these contaminant on the environment.

1.1. Freshwater planarians as invertebrate model for ecotoxicology

Pollution of the aquatic environment is often caused by a complex mixture of chemical compounds, and according to Gray (1989) the responses of different organisms and at different life stages to these stressors vary. Thus, adequate evaluation of the detrimental effects of pollutants on the environment requires the use of a range of sensitive species (Gray, 1989) and endpoints of toxicity (Alonso and Camargo, 2011). In addition, it is important to understand the dose-effect relationship, the mechanisms of action, the targets of the toxin and its resultant effects. To this end, a number of invertebrates and vertebrates have been used in tests with environmental contaminants. However, invertebrates with wide natural distribution, high reproductive potential, short life cycle, cheap and easy to maintain under laboratory conditions and with minimal or no ethical concern have been suggested as better biological models (Choi, 2004). Among these invertebrates, a number of organisms ranging from annelids, nematodes, insects, crustaceans to molluscs have been used as models in environmental risk assessment and bio-monitoring. However, it is apparent that test guidelines used in these tests may not reveal all unfavourable conditions posed by environmental stressors and few sensitive species and life stages used do not cover all organisms at risk of exposure in their habitat (Cardone et al., 2008). As such, there is need to include more vulnerable sensitive species from other phyla in addition to these established model species, to provide reasonable information about the impact of environmental contaminants on the aquatic environment.

Freshwater planarians like these established test species possess attributes that make them suitable for ecotoxicological studies. A lot is known about their morphology, biochemistry and physiology at different stages of life. Also, information about their ecology and distribution in natural and artificial freshwater bodies abound. Several published articles where they were used to evaluate the effects of different environmental stressors are available. Even previous reviews suggesting freshwater planarians use as test models in teratogenesis (Best and Morita, 1982), tumorigenesis (Schaeffer, 1993), neurotoxicology (Hagstrom et al., 2016) studies and bio-indicator for freshwater system (Knakievicz, 2014) are available. The aim here is to give an overview of enviable qualities that make freshwater planarians eligible model test organisms in ecotoxicity bioassasys and of some bioassays where they were used to evaluate toxic effects of some environmental contaminants. Furthermore, their potentials as useful test species for pharmaceuticals and other emerging contaminants will be emphasized. Finally, the need for development of a standard protocol for ecotoxicity tests with freshwater planarians while

advocating for their inclusion as test species in environmental risk assessments and bio-monitoring, will be discussed.

1.1.1. Phylogenetic relationship of freshwater planarians

Freshwater planarians are free living triclad flatworms of the phylum Platyhelminthes. Phylum Platyhelminthes are Metazoans with triploblastic (3 embryonic layers - ectoderm, mesoderm and endoderm) and bilaterally symmetrical body. They comprise more than 20,000 described species, and are fourth in abundance following phyla Arthropoda, Mollusca and Chordata (Ruppert et al., 2003, Riutort et al., 2012). Platyhelminthes belong to the Lophotrochozoa clade, sister to Ecdysozoa clade to which *Caenorhabditis* species, *Drosophila* species, *Daphnia* species and *Anopheles* species belong (Egger et al., 2009).

Ambiguities had existed in the classification of members of the phylum Platyhelminthes, but recent advances in molecular biology and morphological studies have been used to place triclad members inhabiting freshwater bodies into 3 families – Planariidae, Dendrocoelidae and Dugesiidae (Riutort et al, 2012). Generally, these freshwater flatworms comprise about 40 genera and over 300 species (Baguna et al., 1990) and make up about a fifth of up to 6500 described species of the world flatworms (Tyler et al., 2006, Schockaert et al., 2008). Family Planariidae consists of gen*us Polycelis* and 11 others, while Family Dendrocoelidae comprises of genera *Dendrocoelum, Dendrocoelopsis* and 20 others (Riutort et al., 2012). Members of the family Dugesiidae have been studied more than the other freshwater planarians and consist of 11 genera which include genera *Dugesia, Schmidtea, Cura* and 8 others (Riutort et al., 2012). Genus *Dugesia* is made up of 75 species (including *D. subtentaculata, D. tigrina, D. japonica, D. dorotocephala*) while genus *Schmidtea* consist of 4 described species (*S. polychroa, S. mediterranea, S. lugubris, S. nova*) among which are 7 biotypes distinguished by chromosome morphology and ploidy level (Riutort et al., 2012).

Commonly used genera in scientific research are *Dugesia* species, *Schmidtea* species (fig. 1.1), *Polycelis* species and *Dendrocelium* species (Baguna et al., 1990, Newmark and Sánchez-Alvarado, 2002).

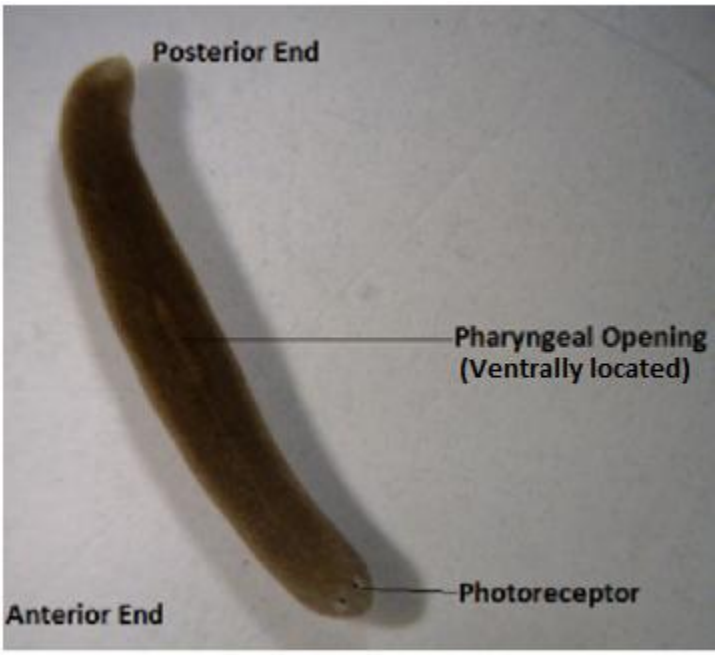


Fig. 1.1: *Schmidtea mediterranea* (Intact worm)

1.1.2. Distribution, ecology and biology of freshwater Planarians

Freshwater planarians are widely distributed in many parts of the world – Europe, Asia, Africa, Australia and the Americas (Reynoldson, 1958, Kawakatsu, 1964, Hay and Ball, 1979, de Vries, 1986, Gee and Young, 1993, Young and Reynoldson, 1999, Young and Sutcliffe, 2001, Charni et al, 2004, Temelkov, 2004, Harrath et al 2004, 2012a, b, Grant et al., 2006, Sluys et al., 2007, Zhang et al., 2008, Riutort et al., 2012, Stocchino et al 2009, 2014, Manenti and Bianchi, 2014). These studies have provided information on the localization and isolation of different populations, divergence in sensitivity among different populations, and their abundance and seasonal variations in their natural aquatic habitats. Such information will be beneficial in the use of indigenous species in the assessment of contamination of aquatic systems in different areas.

Planarians are key members of benthic communities, present in different freshwaters like streams, rivers, ponds, springs and lakes (Knakievicz, 2014, Manenti and Bianchi, 2014) where they are usually found under stones and among leaves and roots of aquatic plants. Macro-benthic organisms are renowned vital biological indicators of water quality and community structure of freshwaters (Manenti and Bianchi, 2014) and field and laboratory studies have shown that freshwater planarians are very sensitive to changes in water quality parameters such as salinity, temperature, conductivity, pH, metals, dissolved oxygen and organic components (Kapu and

Schaeffer, 1991, Rivera and Perich, 1994, Harrath et al., 2004). As a result of their susceptibility to these factors and abundance in unpolluted freshwater systems, they may be good candidates for monitoring changes in water quality (Kapu and Schaeffer, 1991, Rivera and Perich, 1994, Manenti and Bianchi, 2014). The sensitivity of organisms to chemical contaminants under laboratory conditions has been evaluated using acute toxicity tests. Acute toxicity tests are deemed helpful to complement chemical analysis in the determination of the impacts of pollutants on the environment. Since freshwater planarians are known to be sensitive to changes in their environment, and tests with the established models have been shown not to cover all vulnerable species to chemical contaminants, acute toxicity tests with planarians will be helpful. Such tests may be compared with tests using established models to provide more information on ecological effects of chemicals towards aquatic biodiversity.

Besides wide distribution in various lotic and lentic water systems, freshwater planarians can also be easily maintained under laboratory conditions. Standard techniques for culturing and maintaining freshwater planarians in the laboratory which can be modified for all species are available (Cebria and Newmark, 2005, Oviedo et al., 2008; Sanchez Alvarado, 2008). Laboratory cultures of planarians are cheap to maintain as they can be fed with fresh chicken or bovine liver, mosquito larvae or daphnids and do not involve the use of sophisticated equipment. Freshwater planarians are small, with size ranging from 4 mm or less to about 40 mm (for Dendrocoelids) (Baguna et al., 1990). Considering this, they may not occupy much space in the laboratory and there are no specific requirements for animal housing with the exception of temperature and photo-period controlled rooms. Additionally, exposures for ecotoxicity tests may be done in microplates with fewer amounts of reagents and utilizing less space (Hagstrom et al., 2015, 2016). Moreover, some species are commercially available (Kenk, 1937, Oviedo et al., 2008, Pagan et al., 2009), and thus can be a fast and easy source of animals for bioassays. Ecotoxicity tests using planarians will not be delayed because of ethical concerns as there is no need for ethical clearance for the tests and in the disposal of used animals.

Importantly, seeing that it is possible to get freshwater planarians from contaminated and uncontaminated water habitats and raise laboratory populations, comparative studies with these different populations using various sensitive endpoints can be undertaken with ease.

Freshwater planarians have soft, flattened, broad and leaf-shaped body which is covered by mucous secreted by sub-epithelial glands. The mucous is rich in proteins and is involved in locomotion (crawling and gliding), adhesion to substrate, protection (innate immunity) and

predatory behaviour to trap prey (Pearl, 1903, Pedersen, 1963, Talbot and Schötz, 2011, Bocchinfuso et al., 2012). Some studies have shown that exposure to environmental contaminants can alter secretion of mucous by the gland cells (Calevro et al., 1998, Sabourin et al., 1985). Consequently, monitoring rate of mucous secretion in planarians can serve as a toxicity endpoint in ecotoxicological studies. Alteration in mucous secretion due to exposure to contaminants may affect some of the activities mentioned above which are associated to surface mucous. Also, since planarians lack exoskeleton, their surface membrane is open to interaction with toxins in their environment which may result to some morphological alterations. Some studies have shown that exposure to toxins resulted to formation of visible lesions and ulcerations on planarians bodies (Best et al., 1981a, Best and Morita, 1982, Villar et al., 1993, Calevro et al., 1998).

Freshwater planarians move mainly by gliding and crawling (Stringer, 1917) but may exhibit specific behavioural responses to certain stimuli (Pearl, 1903, Inoue et al., 2014). Locomotion by gliding involves cilia located ventrally on the body while crawling is performed by muscular contractions, and both are aided by mucous secretion (Pearl, 1903, Stringer, 1917, Martin, 1978, Nishimura et al., 2007a, Talbot and Schötz, 2011). In addition to crawling and gliding, planarians are known to display some other behavioural responses such as depression, C-like position, screw-like movement, etc. (Pearl, 1903, Grebe and Schaeffer, 1991, Wu et al., 2012). These behavioural responses are controlled by the central nervous system in addition to the muscular system (Stringer, 1917, Talbot and Schötz, 2011).

Planarian central nervous system is complex and consists of a bi-lobed primitive brain anteriorly, and a pair of ventral nerve cords (Buttarelli et al., 2008). Their brain and neurotransmitter systems (dopaminergic, serotonergic, cholinergic, etc.) are structurally and functionally more similar to those of vertebrates than invertebrates (Buttarelli et al., 2008, Cebrià, 2007, Nishimura et al., 2010), and about 95% of genes linked to *Dugesia japonica* nervous system have homologs in humans (Mineta et al., 2003).

Based on the above mentioned features, planarians have been useful test organisms in toxicology (Best and Morita, 1991), neuropharmacology (Buttarelli et al., 2008) and neurotoxicology (Hagstrom et al., 2016), where they have displayed a variety of quantifiable behavioural responses to different chemicals and levels of toxicity. Moreover, they have proved useful in studying mechanisms of drugs used in the treatment of Parkinson disease (Raffa et al., 2013a). Some behavioural responses scored using planarians in these studies include

resting/depression/withdrawal/contraction, glidding, thermotaxism, phototaxism, chemotaxism, thigmotaxism, crawling, righting response, prey catching ability, scrunching, head wiggles, frequent position changes/sharp turns, protrusion of pharynx/vomiting, screw-like and snake-like movement, walnut, C-like and bridge-like positions etc. (Best and Morita, 1991, Grebe and Schaeffer, 1991, Buttarelli et al., 2000, Raffa and Desai, 2005, Raffa et al., 2001, 2003, Inoue et al., 2004, 2015, Ramakrishnan and DaSaer, 2011, Wu et al., 2014, Hagstrom et al., 2016). Moreover, some neurotransmitter systems and related genes associated to these behavioural responses and their distribution in planarians have been identified (Raffa et al., 2001, Nishimura et al., 2007a, b, 2008, 2010, Inoue et al., 2004, 2015, Umesono et al., 2011, Hagstrom et al., 2016). Furthermore, several studies have shown the application of these responses in various ecotoxicity tests (Best et al., 1981a, b, Best and Morita, 1991, Grebe and Schaeffer, 1991, Kapu and Schaeffer, 1991, Villar et al., 1993, Calevro et al., 1998, Medvedev et al., 2006, Zhang et al., 2010, Plusquin et al., 2012a, Paskin et al., 2014, Hagstrom et al., 2015, Wu et al., 2012, 2014, 2015, Inoue et al., 2004, 2015, Rodrigues et al., 2015, Ofoegbu et al., 2016). Recognition of freshwater planarians as model test organism in ecotoxicology will aid in widening our knowledge on impacts of neurotoxic compounds and especially neuro-pharmaceuticals in the aquatic environment on development of the nervous system, behaviour, and neural activity. Planarian behaviour may be a sensitive endpoint to test effects of psychoactive/neuroactive pharmaceuticals in the aquatic environment as it has been utilized successfully in neuropharmacology to study the mechanism of action of some of these drugs. Besides, planarians may be simple but useful animal models for getting insight into effects of contaminants on non-target vertebrates or protected laboratory animals with which researches are ethically restricted. Notably, behavioural responses in planarians may not only serve as a sub-lethal endpoint but a lethal endpoint as well. For instance, alterations in planarian behaviour such as laboured movement, depression or no response to stimuli and unconsciousness have been used in addition to mortality to assess lethal/acute toxicity responses (Grebe and Schaeffer, 1991, Wu et al., 2012).

In their natural aquatic environment, freshwater planarians are important predators feeding mainly on small invertebrates like crustaceans, insects (mosquito larvae), molluscs (egg mass and juveniles), annelids, and also on protozoans, diatoms, plant materials, bacteria, fungi, decaying organisms and other organic materials present (Seaby et al., 1995, Manenti and Bianchi, 2014). Planarians are known to display positive chemotactism and possess advanced

sensory system which aids them to perceive and locate their food (Ash et al., 1973, Ogren, 1995, Inoue et al., 2015). They trap their prey with the slimy mucous released from their membrane while crawling and capturing it with their protractible pharynx (Ash et al., 1973, Ogren, 1995). Since it is evident that environmental toxins can alter sub-epithelial mucous secretion and/or behavioural activities involved during feeding, potential exist for feeding behaviour of planarians to be affected by these toxins. Planarian prey capture ability or feeding rate can be sensitive endpoints and has been used in some studies (Best and Morita, 1991, Rodrigues et al., 2015, Ofoegbu et al., 2016) to assess effects of environmental stressors. Besides, planarians may be exposed to environmental contaminants through contaminated food and may be useful in studying indirect effects of contaminants (Medvedev and Komov, 2005, Medvedev et al., 2006). Planarians also serve as prey to some freshwater mollusc (adults), hirudinea, Pisces, amphibians, odonatans, trichopterans and plecopterans (Davies, 1969, Davies and Reynoldson, 1969, 1971; Wright, 1975). Through these predator and prey interactions, they may be seen as a link between organisms in the lower and higher trophic levels of the freshwater food chain. Thus, planarians may be potential test model to evaluate effects of chemical contaminants along food chains and in bioaccumulation studies where they may serve as prey or predator.

Various modes of reproduction exhibited by different species of freshwater planarians are of ecotoxicological relevance. For instance *Schmidtea mediterranea* has 2 different strains, each specific for either sexual or asexual reproduction (Chong et al., 2011), *Dugesia* has 4 forms - sexual, asexual, parthenogenetic and physiological (one that can alternate between asexual and sexual mode of reproduction depending on season due to degeneration and regeneration of sexual organs) forms (Kenk, 1937, Jenkins, 1967, Vowinckel, 1970, Gee et al., 1998, Vreys et al., 2002, Knakievicz et al., 2006), while *S. polychroa* has sexual race and parthenogenetic forms (Beukeboom et al., 1996, Weinzierl et al., 1999). Freshwater planarians reproduction types may provide a platform to study the impact of environmental stressors on different modes of reproduction.

Sexual strains and parthenogenetic forms are hermaphrodites but reproduce by cross fertilization. However, the sperms released during such crossing in parthenegenetic forms only help to trigger egg development without contributing genetic material to the offspring (pseudogamous parthenogenesis) (Storhas et al., 1999). Mating behaviour of planarians has been described by Vreys et al. (2002). During mating the heads of the mating partners facing opposite direction are firmly attached to substrate or wall of culture container while their tail ends are

raised to allow contact between their ventrally located gonopores (Vreys et al., 2002). After corpulation the raised tail ends are lowered and the animals move away (Vreys et al., 2002). Eggs are deposited in cocoons with about 1 to 20 embryos according to the species (Newmark and Sánchez-Alvarado, 2002). The eggs are ectolecithal as yolk cells reside outside the embryo (Newmark and Sánchez-Alvarado, 2002, Cardona et al., 2006), and hatch into juveniles after about 3 weeks (Salo, 2006), with no larval stage. Embryonic development in some freshwater species has been extensively studied (Cardona et al., 2005, 2006, Vara et al., 2008, Martín-Durán et al., 2010). Parthenogenetic planarians in addition retained their male functions by producing haploid sperms which enables them to fertilize sexual ones (Storhas et al., 1999).

Asexual reproduction on the other hand is achieved by transverse fission of the tail posterior end involving neuromuscular actions (Morita and Best, 1984). During asexual reproduction, a body constriction first forms at the fissioning site on the body of the planarian (fig. 1.2), resulting to the formation of posterior/tail/caudal and anterior/rostral portions (Morita and Best, 1984). Following this, the anterior portion pulls away from the posterior portion which adheres

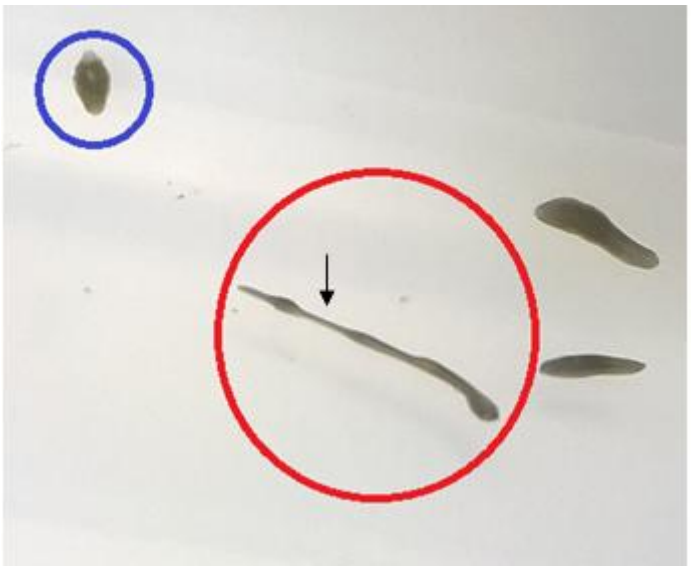


Fig. 1.2: Fissioning process in *S. mediterranea* (encircled in red colour is an adult about to fission; black small arrow points to the constriction site; encircled in blue colour-regenerated fissioned piece)

to a substrate or surface of culture container and the constricted area stretches becoming thinner until it ruptures (Morita and Best, 1984). Rupturing of the constriction area terminates asexual

reproduction, and the resulting pieces regenerate lost parts and grow into normal worms within a week.

Fissiparous reproduction, production of many offspring from one cocoon and short life cycle will support high rates of increase, making enough organisms available for high through-put chemical screening. Also, fissiparity and parthenogenesis will encourage production of clones, thus reducing variability in responses to environmental stressors that may arise due to sexual reproduction.

Concerning planarians' physiological race, considerable efforts have been made to study their switch from one reproduction type to another. These studies revealed that alternation between sexual and asexual mode of reproduction is triggered mainly by environmental factors especially changes in temperature (Kenk, 1937, Jenkins, 1967, Vowinckel, 1970, Gee et al., 1998, Kobayashi et al., 2009). Furthermore, reproduction types in physiological forms are not controlled by chromosomal alterations such as polyploidy and mutation, and inheritance of genetic factors did not follow Mendellian pattern (Kobayashi et al., 2009). In addition, switch from asexual to sexual type in *Dugesia* species has been achieved successfully under laboratory condition (Kobayashi and Hoshi, 2002, Hoshi et al., 2003, Kobayashi et al., 2009). Since switches between asexual and sexual reproduction modes is common among metazoans (Kobayashi and Hoshi, 2002, Hoshi et al., 2003, Kobayashi et al., 2009) and considering current alterations in environmental conditions due to climate change, *Dugesia* physiological forms may be useful in monitoring temperature modifications due to climate change and its impact on reproductive strategies in organisms. Possibly, the physiological form may be resistant to some toxicants which the other races are sensitive to, and may be used to determine exact developmental or detoxification pathway(s) involved in the toxic response(s). Moreover, fissioning, reproductive organ development, embryonic development, rate of cocoon production and hatchlings of sexual and parthenogenetic types have been used as endpoints in ecotoxicity studies with freshwater planarians (Kenk, 1937, Legner et al., 1976, Best and Morita, 1991, Sheiman et al., 2003, Knakievicz et al., 2006, Miyashita et al., 2011, Ribeiro and Umbuzeiro, 2014).

Freshwater planarians also have long life span (Kenk, 1937, Salo, 2006)). Long life span is beneficial as these animals may conveniently be used for long term tests. On the basis of this feature, freshwater planarians have been used to evaluate chronic effects of some environmental

stressors (Best and Morita, 1991, Medvedev and Komov, 2005, Knakievicz et al., 2006, Alonso and Camargo, 2011, Ribeiro and Umbuzeiro, 2014).

1.1.3 Developmental plasticity and regeneration in planarians

Freshwater planarians also have unique developmental plasticity which has been attributed to the presence of the totipotent stem cell; neoblasts. Neoblasts are involved in cell renewal or replacement during physiological processes and regeneration after injury (Newmark and Sanchez-Alvarado, 2000). A small fragment of planarian can regenerate into a whole organism (Eisenhoffer et al., 2008). In addition, planarians are known to survive without food for a long time (Kenk, 1937, Baguna et al., 1990) and also grow and shrink or de-grow depending on nutritional conditions. Studies showed that an adult of about 16 or 20 mm in length can shrink to about 1.0 mm after long period of starvation but grow back to adult size when fed (Baguna et al., 1990, Newmark and Sánchez-Alvarado, 2002). The process of growth or de-growth has been associated to differences in cell turnover or differences in the ratio of cell loss (by cell death or apoptosis) to cell proliferation (new cells by cell division) (Baguna et al., 1990, Newmark and Sánchez-Alvarado, 2001). Planarians can thus be used to evaluate post-exposure recovery rate to a stressor or to investigate the effects of environmental stressors such as carcinogens on cell turnover in aquatic organisms. Additionally, their ability to survive without food will reduce interference with food on outcome of short term ecotoxicity tests as they may be carried out without feeding the animals. Except for long term tests, planarians used for ecotoxicity tests were usually starved before commencement of experiments (3 days and up to 7 days) and during the tests to ensure animals used are of the same metabolic state and avoid influence of food on their responses to the stressor(s) (Oviedo et al., 2008, Wu and Persinger, 2011).

Similarly, freshwater planarians are known for their exceptional regenerative ability such that when a planarian is cut, the pieces re-grow the missing portions in a week or less than 14 days depending on species (fig. 1.3). Infact, it has been reported that a small piece 1/279 of a planarian can regenerate a complete worm (Newmark and Sánchez Alvarado, 2002). This matchless regenerative ability is prevalent in both juvenile and adults as neoblasts responsible for regeneration are distributed throughout the body of both juveniles and adults (Reddien and Sánchez Alvarado, 2004). Neoblasts make up about 30% of all planarian cells (Beane et al., 2011), and are highly undifferentiated cells (stem cells) and the only cells in adult planarians with mitotic activity able to differentiate into all cell types – somatic and germ cells (Newmark

and Sánchez Alvarado, 2000; Salo, 2006). Planarians, as a result, can serve as a model to evaluate teratogenic effects of some environmental chemical contaminants, and effects of toxicants on wound healing and growth, and hence on mitotic activity of cells.

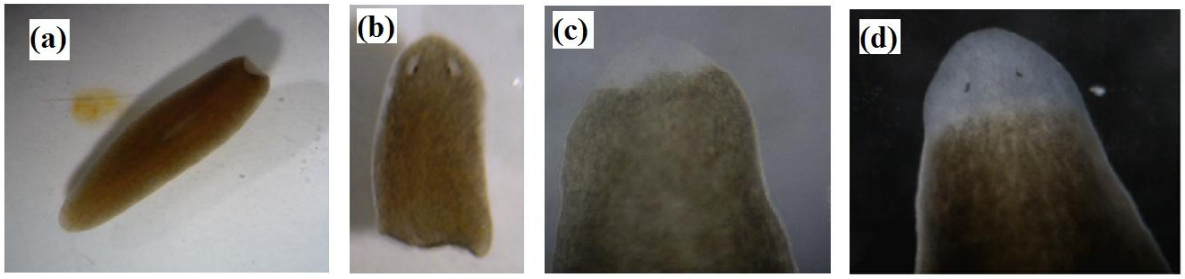


Fig. 1.3: Regeneration in planarian **(***S. mediterranea***)**: (a) newly decapitated worm (Day 0), (b) head piece from decapitated worm, (c) Newly formed blastema on prior decapitated worm (Day 3), and (d) Newly formed head with photoreceptor (Day 7)

Regenerative ability in addition to sexual or asexual reproduction can also aid in the production of organisms to be used in bioassays. This high regenerative ability together with asexual reproduction makes it possible for clonal lines to be obtained easily for studies, avoiding complexities and variability in responses to stressors that may arise due to sexual reproduction. It also provides identical genetic setting, reduces irregularities in experiments and sequence polymorphism normally seen in wild populations (Newmark and Sanchez Alvarado, 2002). Additionally, regeneration has been used as a sensitive endpoint to test teratogenic, neurotoxic and cytotoxic effects of natural and chemical environmental stressors (Best and Morita, 1982, 1991, Jenrow et al., 1996, Calevro et al., 1998, Medvedev and Komov, 2005, Medvedev et al., 2006, Kalafatić et al., 2006, Hagstrom et al., 2015, Inoue et al., 2015, Ofoegbu et al., 2016). Besides, planarian neoblasts can serve as a future potential source for cell line studies in ecotoxicity tests for effects of contaminants on stem cell differentiation. It can also be used to evaluate effects of contaminants on cells in-vivo. Regeneration can also be evaluated with behaviour to determine effects of environmental contaminants on the restoration of normal behaviour in regenerates.

1.1.4 Adaptability to molecular, cellular, automated tracking and high resolution imaging techniques

Freshwater planarians are easily compliant to research techniques such as high resolution imaging systems, automated tracking systems, techniques for expression based approaches like polymerase chain reaction based techniques (genomic and transcriptomic techniques, microprofiling technique, RNA interference technique) and techniques for structural studies (in situ hybridization and immunohistochemistry), colorimetric techniques and others. These, if applied in ecotoxicologal studies will result to higher throughput chemical screening.

Head regeneration traditionally is scored by microscopically monitoring blastema development and photoreceptor formation. However, head regeneration can be monitored immediately after decapitation measuring the time of photoreceptor or auricle formation using higher resolution light microscope with automated imaging systems (Balestrini et al., 2014, Kustov et al., 2014, Hagstrom et al., 2015). This technique can easily be used to quantify blastema growth rate in addition to monitoring morphological changes during head regeneration and the effects of contaminants on regeneration of auricles and photoreceptors. The method is better than the former method of scoring as it gives a more reliable result considering that some stressors may not affect or delay photoreceptor or auricle formation but blastema development and growth rates which may be ignored or go unnoticed or inaccurately scored. Also, the method makes it easier to measure size of blastema which may not be easy to measure with regular light microscopes.

Automated tracking systems (instead of the commonly used visual scoring) has enabled the quantification of planarian behavioural responses like locomotor velocity, gliding, wiggling, phototaxism, chemotaxism, thigmotaxism, thermotaxism and scrunching (Talbot and Schötz, 2011, Li, 2012a, Balestrini et al., 2014, Inoue et al., 2004, 2015, Hagstrom et al., 2015, Cochet-Escartin et al., 2016, Rodrigues et al., 2016). These methods help for faster and more precise screening and measurement of more replicates and concentrations, and various behavioural responses. Additionally, automated tracking systems make it easier for behavioural defects which may be associated to neurodevelopmental alterations due to exposure to contaminants to be determined. Moreover, it will be possible to trace neurotransmitter system affected by the contaminant since the relationship between these behavioural responses and specific neurotransmitters are known. Furthermore, locomotory behaviour of RNAi treated planarians can

be tracked (Talbot et al., 2014) making it easier for altered behaviour to be linked to specific gene in exposed planarians.

Freshwater planarians are widely used animal models in regeneration, developmental and neuropharmacology because of their compliance to genomic and transcriptomic techniques. A number of laboratories in the US (Alejandro-Sanchez's lab, Peter Reddien's lab, Phil Newmark's lab and Oviedo lab), Spain (Francesc Cebrià's lab, Emili Saló's group, Rafael Romero's lab), Japan (Kiyokazu Agata's lab) and other places are involved in molecular studies using freshwater planarians to unravel complex molecular interactions in regeneration and developmental biology. Studies in these laboratories have provided information on gene expression during planarian regeneration (Callaerts et al., 1999, Sanchez-Alvarado et al., 2002, Oviedo et al., 2010, Qin et al., 2011, Wagner et al., 2012), homeostasis (Molina et al., 2009), embryonic development (Martín-Durán et al., 2010), of mitochondrial genome (Solà et al., 2015), planarian proteome and mucous subproteome (Bocchinfuso et al., 2012) and planarian transcriptome datasets are available (Abril et al., 2010). More importantly, the genome of some species has been characterized (Robb et al., 2008). These techniques may be relevant in ecotoxicological studies like in ecotoxicogenomics. For instance, some stable genes under metal toxicity and varying salinity stress conditions useful as reference genes for quantitative real time PCR (qPCR) experiments have been identified in *S. mediterranea* and *M. Lignano* (Plusquin et al., 2012b). Similarly, gene expression associated to signal pathway activation in *S. mediterranea* under $CdCl_2$ stress (Plusquin et al., 2012a), yolk gland in regenerating sexual *D. ryukyuensis* under steroid and estrogenic endocrine disrupter exposures (Miyashita et al., 2011); and to certain tissues and extracellular matrix remodelling in regenerating *D. japonica* under Berberine exposure (Balestrini et al., 2014) have been reported.

In addition, Gene knockdown using RNA Interference (RNAi) techniques have been extensively used to characterize developmental regulatory genes during planarian regeneration and maintenance (Agata, 2003, Cebria and Newmark, 2005, Cebrià et al., 2007, Forsthoefel and Newmark, 2009, Fraguas et al., 2011, Forsthoefel et al., 2011, Tu et al., 2012, Wagner et al., 2012), regulatory genes associated to some neurotransmitter systems such as serotonergic, dopaminergic and gabaergic systems (Curie and Pearson, 2013, Nishimura et al., 2007a, b 2008) and genes involved in eye function (Agata, 2003), trophic factor (heterogenous group of molecules which support cell growth and survival) which are involved in planarian central body region neuron survival and asexual reproduction (Bueno et al., 2002). This technique has been applied to characterize genes associated to planarian visual system and their expression in

association to planarian phototaxism (Inoue et al., 2004), determine the importance of neural activity in the brain for planarian chemotaxism, thigmotaxism and thermotaxism (Inoue et al., 2014, 2015) and neurotransmitter pathway involved in planarian thermotaxism (Inoue et al., 2014), and also genes involved in locomotory behaviour, neoblast activities and epithelial integrity (Talbot et al., 2014). This technique has also been applied in toxicity studies to identify specific genes implicated in behavioural, developmental, neural and regeneration defects (Balesrini et al., 2014). Determining neurotransmitters and pathways linked to behaviour in an organism and characterizing behavioural and neuro-developmental defects due to exposure to stressor may assist for in-depth analysis of the molecular mechanisms involved (Hagstrom et al., 2016).

Freshwater planarians also have capabilities to serve as good models for epigenetic studies because of the presence of the stem cell neoblast which is maintained in the whole body of the adult worm, identified molecular markers for stem cells and their progeny and numerous bioinformatic tools (genome assembly, annotations and browser) (Robb and Sánchez-Alvarado, 2014). Numerous key histones, histone clusters, histone variants and many post-translational modifications are present in planarians (Robb and Sánchez-Alvarado, 2014). Thus, it is possible to carry out epigenetic studies in vivo, study progression of cell lineage from undifferentiated stem cells, and customize bioinformatic tools (Robb and Sánchez-Alvarado, 2014). Because epigenetic studies can reveal interactions of environmental and genetic factors leading to visible phenotypic traits, with regeneration and homeostasis as some of the targets of environmental factors (Robb and Sánchez-Alvarado, 2014), planarian regenerative and developmental plasticity may be useful tools for epigenetic studies with respect to effects of environmental contaminants. Availability of large number of neoblasts in planarians will increase the perception of the significance and functions of environmental and genetic factors, and modifying enzymes in neoblast activities. In addition, RNAi techniques can further be used to identify specific genes affected or responsible for the displayed phenotypic traits due to exposure to contaminants.

Also, High Performance Liquid Chromatography HPLC has been used to quantify amino acid neurotransmitters implicated in planarian behavioural responses (Eriksson and Panula, 1994, Itoh and Igarashi, 2000, Umeda et al., 2005, Rawls et al., 2006, 2007). This technique has been used to evaluate levels of neurotransmitters and their metabolites involved in toxicity response to Cadmium exposure (Wu et al., 2015). Planarians adaptability to this technique may offer

opportunity for changes of measurable organismal endpoints to be linked to levels of neurotransmitters involved.

Further, because planarians are known to absorb fluids through their surface epithelium and pharynx, they have the potential of absorbing and accumulating contaminants in solution into their body. The accumulation of these chemicals in the tissues may be linked to the level of toxicity on such tissues. Bioaccumulation of chemical contaminants in planarians body have been characterized using spectrophotometric techniques (Plusquin et al., 2012a, Wu et al., 2012, Balestrini et al., 2014). In addition, bioaccumulation studies may help to understand planarians ability to retain or metabolize toxic chemicals and specific tissues/organs prone to toxic effects of particular chemical contaminants.

Moreover, improved and more advanced histochemical techniques such as in-situ hybridization, whole mount immunohistochemistry, immunofluorescence studies and fluorescence dye tracing used for cellular and anatomical structural effects characterization (Hagstrom et al., 2016), have been used for in depth characterization and visualization of the central nervous system and neural network in planarians (Cebria, 2008, Nishimura et al., 2007a, b, 2008). Freshwater planarians (intact and regenerating worms) adaptability to in-situ hybridization techniques has been used to show an array of expression patterns by neural genes in their central nervous system, giving insight into more specific molecular regions of neural genes (Cebria et al., 2002). Also, tissue specific expression pattern of genes essential for nervous system functions and behaviour have been characterized using in-situ hybridization (Talbot et al., 2014). These techniques have been used to determine effects of contaminant exposure on regeneration of planarian brain, visual system, sexual organs and neoblast activity (Miyashita et al., 2011, Plusquin et al., 2012a, Balestrini et al., 2014, Hagstrom et al., 2015). Further application of these techniques in ecotoxicological studies will provide more information for better understanding of morphological/structural defects and location of genes whose expressions were altered in association to these defects due to chemical toxicity.

Furthermore, DNA microarrays and expressed sequencing tags (EST) have been used to isolate/characterize expression of neural specific genes in intact and regenerating planarians (Cebria et al., 2002). In another study by Nakazawa et al (2003), planarian brain was characterized with DNA microarray showing the compliance of these organisms to the technique and possibilities of their application in ecotoxicity assays. Ecotoxicological evaluation involving

DNA microarrays utilizing intact and regenerating planarians will help to detect specific genes altered by exposure to environmental contaminants.

In addition, freshwater planarians can be subjected to colorimetric and spectrophotometric techniques to determine the interaction between contaminant exposure and activities of enzymes and antioxidants. Colorimetric assays using homogenates of planarians have been used to analyze changes in biochemical enzymes (acetylcholinesterase, monoamine oxidase, ATPase, catalase, superoxide dismutase, glutathione peroxidase) due to exposure to environmental stressors (Guecheva et al., 2003, Li, 2008, 2012a, Plusquin et al., 2012a, Wu et al., 2012, Yuan et al., 2012, Garcia-Medina et al., 2013, Zhang et al., 2014).

Other techniques such as micronucleus assay for micronuclei formation due to mutagenicity (Knakievicz and Ferreira, 2008, Knakievicz et al., 2008), DNA strand break by comet assay for genotoxicity (Guecheva et al., 2001, Horvat et al., 2005, Frenzilli et al., 2009, Garcia-Medina et al., 2013, Ofoegbu et al., 2016) have also been applied in ecotoxicity assessments with planarians. In addition, pollution-sensitive genes such as heat shock protein 90 (hsp90) and metallothionein genes, and glioma pathogenesis related proteins have been analysed in *Dugesia japonica* (Qu et al., 2008) paving way for more detailed molecular studies on toxicity and a better understanding of interaction between DNA and environmental contaminants.

1.2 **Freshwater planarians in ecotoxicology**

Freshwater planarians are known to be sensitive to changes in their environment and are able to display various responses in respect to these changes. Based on some of the above mentioned features, they have been used to assess impact of several environmental stressors utilizing a wide range of sensitive endpoints. Freshwater planarians responses to some of these environmental pollutants are herein discussed as well as their sensitivity in comparison with other established model organisms.

1.2.1 Metals

Metals present in freshwaters are associated to natural sources and anthropogenic activities. Although, some metals may be essential for some biological processes like gene regulation or enzymatic functioning, they become toxic when their concentration in water and/or food is more than what an organism's homeostatic control mechanism can handle (Prosi, 1981) or due to

displacement from binding sites resulting from interaction with other metals (Mason and Jerkins, 1996). Pollution of freshwater system by heavy metals may be due to natural processes like weathering or combustion of fossil fuel, wastes and releases from industries, agriculture, homes and sewage treatment plants (Yadav and Trivedi, 2009, Dhanakumar et al., 2015). These metals in addition to being toxic to aquatic organisms are persistent in the environment, bio-accumulate and bio-magnify in the food chain.

Effects of various metals such as cadmium, mercury, copper, aluminium, chromium, silver, lead and zinc on freshwater planarians have been investigated. Studies with some freshwater planarians showed that they are sensitive to heavy metals in the aquatic environment like other freshwater organisms used in ecotoxicity assays (table 1.1).

The above LC_{50}s of metals may show planarians to be less sensitive to metals than *Daphnia magna* however, since planarians are known to display various morbidity responses in addition to mortality (Grebe and Schaeffer, 1991, Wu et al., 2014), assessing their sensitivity based on death alone may not be appropriate. Exposure of planarians to Cd for instance caused lethal or near lethal effects such as increased secretion of mucous from the surface membrane, body lesions or ulcerations and head loss or dissolution, before death of exposed planarians (Calevro et al., 1998, Sabourin et al., 1985). Apart from lethal responses, planarians may display other responses to metal exposure.

Table 1.1: Lethal concentrations (LC_{50}, mg L^{-1}) of some metals for freshwater planarians and *Daphnia magna*

Contaminants	**Planarian species**	**48 hrs LC_{50}**	**96 hrs LC_{50}**	***D. magna* 48 hrs LC_{50}**
Cd^{2+} ($CdCl_2.2.5H_2O$)	*D. dorotocephala*	-	0.69[A]	0.065[B]
Zn^{2+} ($ZnSO_4$)	*D. tigrina*	1.16[A]	-	29.4[C]
Cr^{6+} ($K_2Cr_2O_7$)	*D. tigrina*-newborn	9.27[D]	4.56[D]	0.38[D]
Al^{3+} ($Al_2(SO_4)_3.18H_2O$)	*P. felina*-adult	-	1100 (5 days LC_{50})[E]	38.2[F]
Cu^{2+} ($CuSO_4.5H_2O$)	*D. tigrina*-newborn	-	12[G]	0.0826[H]

Note: [A-]Garcia-Medina et al., 2013, [B-]Biesenger and Christensen, 1972, [C-]Gale et al., 1992, [D-]Preza and Smith, 2011, [E-]Kovačević et al 2009, [F-]Kimball, 1978, [G-]Knakievicz and Ferreira, 2008, [H-]Guilhermino et al., 2000

Studies have shown that Cd accumulates in the body of planarians even when there is no clear toxic effects, with higher concentrations in the head region, and the concentration increasing with increasing exposure concentration and time (Plusquin et al., 2012a, Wu et al., 2011, 2015). Accumulation of more Cd in the head indicates the involvement of planarian brain in its toxicity and neurotoxicity (Best and Morita, 1982, Wu et al., 2015), and these may be associated to loss of head as well as head resorption, suppression of fissioning and disruption of neurotransmission system in exposed planarians (Best and Morita, 1982, Calevro et al., 1998, Wu et al., 2011, 2014, 2015). Interruption of the activity of the neural system in planarians due to exposure to Cd may as well be related to decrease in serotonin and dopamine levels and alterations of monoamine oxidase activities (Wu et al., 2011, 2014, 2015). Also, associated to Cd neurotoxicity were various behavioural alterations reported for exposed planarians. Locomotor activity of adult *D. japonica* (Zhang et al., 2010, Wu et al., 2014) and *S. mediterranea* (Plusquin et al., 2012a) exposed to Cd was decreased while it was severely impaired or inhibited in juveniles (Zhang et al., 2010). Other behavioural alterations like irregular body shape, abnormal body elongation, screw-like hyperkinesias and bridge-like position were also elicited by Cd in planarians (Wu et al., 2014). Cd toxicity to planarians may also result to induction of DNA damage and chromosomal aberration, induction of oxidative stress and damage (activities of catalase and superoxide dismutase, and changes in gene expression patterns of heat shock proteins) (Kalafatić et al. 2004, Plusquin et al., 2012a, Garcia-Medina et al., 2013). Furthermore, Cd caused reduction of planarian body size (reduction in growth) (Plusquin et al., 2012a) but had no affect on head regeneration (Sabourin et al., 1985, Calevro et al., 1999). In regenerating *P. felina* Cd exposure caused inhibition of mitototic activity after 6 hrs exposure but the effect was temporary (Kalafatić et al. 2004). In intact *S. mediterranea* increased neoblast activity (cell proliferation) occurred during 3 weeks exposure to Cd (Plusquin et al., 2012a). It was observed that while Cd inhibition of neoblast mitotic activity and reduction of locomotion and planarian body size were transient as planarians recovered after exposure, neoblast cell proliferation continued post-exposure (Kalafatić et al. 2004, Plusquin et al., 2012a). Increased neoblast activity may be a defence mechanism which planarians use to cope with Cd stress or display of planarian developmental plasticity and ability to recover from Cd stress, or linked to the cadmium's carcinogenic potential (Plusquin et al., 2012a). Moreover, Hall et al (1986) observed development of type I tumor (benign neoplasia) especially at post-pharyngeal region in *D. dorotocephala* during post exposure in clean culture medium after long term exposure in Cd.

Planarian mortality due to Cd toxicity may be seen as insensitive endpoint considering high LC_{50} (Plusquin et al., 2012a). However, endpoints such as behavioural alterations which occurred at low level exposure, accumulation in body and increased neoblast mitotic activity are more sensitive to to measure sublethal effects of Cd (Plusquin et al., 2012a).

Methyl mercury exposure to freshwater planarians elicited many neurotoxic and teratogenic responses in intact and regenerating worms. Methyl Hg exposure caused abnormal head regeneration with rudimentary auricles, re-sorption of newly regenerated head and head of intact worms, decreased locomotion and prey-capture response in intact worms and regenerates, increased time for righting response, non-coordinated and difficulty in righting response, paralysis of part of caudal region and suppression of fissioning in *D. dorotocephala* (Best and Morita, 1982, 1991, Best et al., 1981b). Discrepancies in righting response, prey capture response and locomotion displayed by regenerates exposed to Hg may be linked to their less developed head or brain as electron microscopy studies revealed impairment in development of their brain synapses (Best and Morita, 1991, Best et al., 1981b). Also, studies on the activity of biochemical enzymes showed that exposure of *D. japonica* to methyl Hg led to inhibition of alkaline phosphatase activity, an enzyme implicated in cell proliferation and differentiation during planarian regeneration (Chen et al., 2013). In addition, the relevance of planarians in bio-accumulation studies and indirect effects of contaminants through food chain was evident when mercury-exposed chironomid and oligochaete larvae were used to feed *D. tigrina*, *D. lugubris* and *Polycelis tenius*. Hg dietary toxicity caused abnormal head regeneration, delay or in severe cases suppression of head regeneration as well as accumulation of Hg in planarians (Medvedev and Komov, 2005, Medvedev et al., 2006). Although developmental malformation and suppression of fissioning are sensitive endpoints and occurred at sublethal concentrations, behavioural alterations due to methyl Hg exposure appeared more sensitive as it occurred even at much lower concentrations (Best and Morita, 1982).

Copper exposure to freshwater planarians *D. schurbarti* and *D. tigrina* resulted to induction of DNA damage, increase in micronucleus frequency especially in newborn and regenerates, increase in catalase activity, cytotoxicity at higher concentrations, but no effect on heat shock protein (hsp) 60 (Guecheva *et al*., 2001, 2003, Knakievicz and Ferreira, 2008). Knakievicz and Ferreira (2008) observed that in *D. tigrina* Cu concentrations as low as 0.05 mg L^{-1} caused prominent reduction in fecundity and hatching while 0.1 mg L^{-1} caused increase in micronucleus frequency in regenerating worms, reduction of locomotion in intact and regenerating worms, and

delay in time for head regeneration. In addition, bioaccumulation of Cu in the body of planarians was observed (Knakievicz and Ferreira, 2008). Following the outcome of Knakievicz and Ferreira's study, Cu concentrations causing toxicity in this freshwater planarian was within reported Cu concentration range 0.0005 to 1.0 mg L^{-1} in surface waters (WHO, 2004) and below safe water Cu concentrations in some areas (Knakievicz and Ferreira, 2008). This supports the need to involve wider range of organisms and sensitive endpoints in biomonitoring studies. Moreover, consequent to freshwater planarians sensitivity, they were suggested as a model for the detection and assessment of Cu pollution of freshwater environment and its effects on aquatic organisms (Guecheva et al., 2001, 2003, Knakievicz and Ferreira, 2008).

Aluminium exposure increased mucous secretion from epidermal layer, accumulation of Al in the body and caused different behavioural alterations like severe body twisting, uncoordinated and lack of response to stimuli, writhing response, reduced and disordered locomotion and motionlessness in *D. estruca* and *P. felina* (Calevro et al 1998, 1999, Kovačević et al 2009). Morphological changes also linked to Al toxicity include depigmentation, ulcerations and lesions on the body of the worms, visible oedemas and body contraction (Calevro et al., 1998, 1999, Kovačević et al 2009). In addition, intact planarian experienced reduction of auricle and acephaly (Kovačević et al 2009). Similarly, inhibited regeneration followed by death of regenerates in severe cases, and altered blastema, auricle and photoreceptor development was also observed in regenerationg planarians exosed to Al (Calevro et al., 1998, 1999), showing its teratogenic effects. Histological changes such as damage to epithelial and muscular layers resulting in decreases of their thickness, increase in the number of neoblast in the parenchyma and changes in the number of reticular cell occurred in planarians exposed to Al (Kovačević et al 2009). Increase in the number of neoblast may be due to increase in cell proliferation to replace damaged ones due to Al toxicity while changes in number of reticular cell may be linked to their phagocytic role, which provided protection at lower concentration but at high concentration failed under high Al load (Kovačević et al 2009). These responses show that Al targets planarian nervous system. Again, Freshwater planarian mortality may not be a sensitive endpoint considering high 48 and 96 hrs LC_{50} for Al, but sublethal responses like behavioural, histological and morphological alterations may be relevant for bioassays involving Al in the aquatic environment.

Exposure of freshwater planarians to magnesium chloride resulted to inhibition of head regeneration in decapitated *Dugesia polychroa* worms (Schürmann and Peter, 1998). Organisms

exposed to this metallic salt showed altered muscular contraction at the cut site and suppressed differentiation of neoblasts which accumulated at the wound site resulting in delay in wound healing and developmental malformation. Mg salt may have impaired interaction between epithelial and parenchymal cells which would have initiated differentiation of neoblasts to regenerate lost head parts (Schürmann and Peter, 1998). Freshwater planarian regeneration may thus provide information on how environmental contaminants interfere with epithelial and parenchymal cellular interactions needed for the well being of organisms.

Other metals like chromium (Cr), zinc (Zn), silver (Ag) and lead (Pb) were shown to induce various toxic responses in planarians. Cr exposure increased secretion of mucous from surface membrane, inhibited head regeneration (Calevro et al 1998, 1999), suppressed neoblast mitotic activity and induced chromosomal aberration in planarians (Kalafatić and Taborŝak, 1998). Exposed regenerating planarians developed abnormal and reduced blastema which failed to develop photoreceptors and auricles (Calevro et al 1998). Lead acetate exposure has been shown to cause head resorption in intact *D. dorotocephala* planarians in addition to visible body lesions and altered appearance (Best and Morita, 1982). Zn exposure induced DNA damage, and oxidative stress resulting in increased level of lipid peroxidation, superoxide dismutase and catalase activities in planarians (Garcia-Medina et al., 2013). Ag nanoparticle and $AgNO^3$ suppressed head regeneration and mortality in intact and regenerating *D. tigrina* (Kustov et al., 2014).

These studies have shown how individual metals may affect freshwater planarians survival, physiology and population growth. However, in the natural habitat, these metals may not occur singly but as mixtures which may have more profound effects than individual exposures. Kapu and Schaeffer (1991) exposed asexual *D. dorotocephala* to zinc, iron, boron, selenium, chromium IV, lead, copper and nickel individually and as mixtures based on Illinois water quality standards concentration and criteria levels. They observed that exposure of planarians to metal mixtures at their water quality criteria levels for 60 minutes resulted in several toxic responses such as restlessness, hyperkinesias, spiralling, head twist, shape change and laboured movement. This shows the need to consider effects of metals mixtures when setting metal water quality standards and the relevance of freshwater planarians for such studies (Kapu and Schaeffer, 1991).

Metals generally were toxic to freshwater planarians, eliciting various responses ranging from mortality to mutagenicity, teratogenicity, cytotoxicity, genotoxicty and neurotoxicity. Increased

secretion of mucous from the epithelial layer experienced by planarians exposed to metals may be an early response to exposure and a protective response to reduce diffusion of the metals into their body. More importantly behavioural and possibly neoblast mitotic activity and regeneration endpoints appear to be sensitive parameters to be used in metal biomonitoring studies involving freshwater planarians.

1.2.2 Pesticides

Freshwater planarians have been shown to be sensitive to exposure to various pesticides (table 1.2).

Table 1.2: Lethal concentrations (LC_{50}, mg L^{-1}) of some pesticides for freshwater planarians and *Daphnia magna*

Contaminants	**Planarian species**	**LC_{50}**	***D. magna* 48 hrs LC_{50}**
Chlorpyrifos	*D. dorotocephala*-dark strain	7 days LC_{50}-0.88[A]	0.344[B]
Methyl parathion	*D. dorotocephala*-dark strain	7 days LC_{50}-0.76[A]	0.81-0.97[C]
Malathion	*D. dorotocephala*-dark strain	7 days LC_{50}-2.84[A]	0.354[B]
p-Nitrophenol	*D. dorotocephala*-light strain	7 days LC_{50}-1.42[A]	11[B]
Permethrin	*D. japonica*-intact worm	8 days LC_{50} -150.25[D]	0.022[E]
Dichlorvos	*D. japonica*-intact worm	8 days LC_{50}-0.42[D]	0.159[F]
Chlordane	*D. dorotocephala*	10 days LC_{50}-1-5[G]	0.0113-0.0158[H]
Tributyltin (TBT)	*S. mediterranea*	48 hrs LC_{50}-0.00187[I]	21 days LC_{60}-0.0025[J]

Note: [A-]Villar et al., 1993, [B-]Guilhermino et al., 2000, [C-]Keppeler et al., 2015, [D-]Hagstrom et al., 2015, [E-]Simanov et al., 1984, [F-]Saler and Sağlam, 2005b, [G-]Best et al., 1981a, [H-]Manar et al., 2009, [I-]Ofoegbu et al., 2016, [J-]Oberdörster et al., 1998.

Based on the LC_{50} data, *D. magna* appeared more sensitive to majority of the pesticides than planarians. Nevertheless, planarians exposed to some of these pesticides exhibited general toxic effects subsequent to mortality. Malathion for instance caused invagination at the neck followed by head loss and growth of abnormal tissue at wound surface before death of exposed planarians (Villar et al., 1993). Despite mortality, these pesticides also induced other toxic effects in exposed planarians.

Organophosphorus herbicide norflurazon caused behavioural alterations such as twisting, curling, disordered and reduced locomotion, and sometimes stillness in exposed *Polycelis felina* (Horvat et al., 2005). Planarians also experienced morphological alterations including body contraction, crenellation and invagination of body edge, de-pigmentation, loss or altered auricle or acephaly in some cases. Other toxic effects like damages to the mucous layer, epidermis and parenchyma, loss of rhabdites, induction of DNA damage and apoptosis after longer exposure were observed. Behavioural alterations in exposed planarians were shown to be reversible when planarians were transferred to clean medium except those from the highest concentration which was an indication of severe damage to the nervous system (Horvat et al., 2005). However, some morphological alterations were evident in planarians after the exposure period (Horvat et al., 2005).

D. dorotocephala exposed to 4 pesticides chlorpyrifos, malathion, p-nitrophenol, methyl parathion and chlordane exhibited various structural and behavioural alterations. Chlorpyrifos caused depigmentation, head contraction, depression, head waving/wiggles, increased frequency of sharp turns, suppression of thermotactism, crenellation of edge and coiling of exposed animals (Villar et al., 1993). Malathion caused abnormal body elongation, head contraction, head waving, restlessness, and frequent changes in directions of movement (Villar et al 1993). Histological study of regenerating *D. tigrina* exposed to malathion showed alteration on the ventral epidermal layer due to over dilation of pores, loss of ventral cilia, changes in many tissues, necrosis, malformation and underdevelopment of blastema (Butturi-Gomes et al., 2016). Also, exposure to methyl-parathion caused formation of depigmentation line from eye to auricle, head contraction, abnormal movement and frequent changes in direction while p-nitrophenol caused head contraction, head swelling, supernumerary eye in intact worms, ulceration of epidermal origin, abnormal body elongation and movement (Villar et al 1993). Generally, chlorpyrifos, malathion, p-nitrophenol and methyl parathion exposure caused delayed head regeneration and resulted in teratogenicity expressed as development of abnormal auricle and/or abnormal eyes in *D. dorotocephala* (Villar et al 1993). Delayed head regeneration due to malathion exposure is proabably linked to underdevelopment of blastema and tissue changes in exposed regenerating planarians (Butturi-Gomes et al., 2016). Exposure to chlordane resulted to abnormally extended body, protrusion of pharynx, head resorption, lesion on head, increased fissioning at lower concentration and a decrease at higher concentrations, hyperactivity, convulsion and disorganized writhing which are signs of neurotoxicity (Best et al., 1981a, Best and Morita,

1982). In addition, Amaya-Chavez et al. (2009) observed that planarians have the ability to influence the degradation of methyl parathion in their culture medium. They reported that presence of planarian *D. dorotocephala* in 1.25 µg methyl parathion mL^{-1} solution enhanced its degradation with up to 69% efficiency compared to the chemical degradation in solution without planarians.

Similarly, Hagstrom et al. (2015) in another study with *D. japonica* exposed to chlorpyrifos, dichlorvos and permethrin observed various responses associated to toxicity due to these compounds. *D. japonica* like *D. dorotocephala* exposed to chlorpyrifos experienced increased frequency of sharp turns, head wiggles, suppression of thermatactic response but reduced glidding speed in only regenerating worms (Hagstrom et al 2015). Also, whereas Villar et al. (1993) reported that chlorpyrifos exposure delayed head regeneration in *D. dorotocephala*, Hagstrom et al (2015) only observed reduction of brain size as the impact of chlorpyrifos on *D. japonica* head regeneration. On the other hand, dichlorvos and permethrin reduced gliding speed and movement of intact and regenerating *D. japonica* but regenerating worms seem to be more sensitive than whole worms (Hagstrom et al 2015). Athough neither permethrin nor dichlovos affected blastema development, permethrin exposure caused delay in photoreceptor formation and reduction in brain size while dichlorvos exposure affected only brain density (Hagstrom et al., 2015).

Other pesticides whose toxic effects have been tested on freshwater planarians are chlorotolurone on *Polycelis felina* (Kalafatić et al., 2001), chlorantraniliprole on *D. subtentaculata* (Rodrigues et al., 2016) and the biocide tributyltin chloride (TBT) on *Schmidtea mediterranea* (Ofoegbu et al 2016). Chlorotolurone exposure to *P. felina* caused reduction in locomotion, body contraction, increased secretion of mucous from surface membrane, disruption of mitotic activity, reduction in number of neoblasts and inhibition of head regeneration (Kalafatić et al., 2001). *D. subtentaculata* exposed to chlorantraniliprole experienced reduction in behaviour with a LOEC of 131.7 µg L^{-1} for feeding and locomotion scored visually while the LOEC of locomotion scored by automated system was 26.7 µg L^{-1} (Rodrigues et al 2016). The difference between the LOEC for planarian locomotion measured by visual scoring and automated system shows the reliability of automated system of scoring of planarian locomotion an endpoint in ecotoxicology. TBT exposure induced reduction in locomotion and motionlessness/stillness, reduction in feeding, induction of DNA damage and delay or inhibition of head regeneration in planarians (Ofoegbu et al., 2016, Chapter 2).

Pesticides generally are formulated to induce toxic effects. Studies have shown their ability to induce various toxic effects on different planarian species with behavioural alterations showing to be responsive endpoints to all compounds. Freshwater planarian behaviour in addition to structural/morphological alterations, genetoxicity and teratogenecity may be sensitive endpoints for the assessment of pesticide effects. Teratogenic effects will include among others their effects on neoblast activity and regeneration with consequences for tissue regeneration and developmental malformations.

1.2.3 Pharmaceuticals

Several studies have shown that freshwater planarians are sensitive to exposure to pharmaceutical compounds with responses ranging from mortality, altered and suppressed behaviour, and altered gene expression to genotoxicity. Mortality due to exposure of planarians to different groups of pharmaceutical substances such as analgesics, psychoactives, beta blockers, antibiotics, antihypertensives, endocrine disruptors and antihistamines have been reported (table 1.3). Before death, exposed planarians exhibited morbidity responses like different behavioural and morphological changes (body coiling, crenelation of edges, restlessness, and altered locomotion) (Li, 2013).

Freshwater planarians have also been used extensively in place of vertebrate models in pharmacology to determine mechanisms of action in addition to molecular markers associated to several drugs, especially drugs of behaviour and abuse. They are used as animal models for drug screening due to close similarity of their neurotransmitter systems to those of vertebrates (Buttarelli et al, 2002). Specific behavioural responses to psychoactive substances like cannabinoids, amphetamines and opiates, caffeine and nicotine have been observed in exposed planarians (Carolei et al., 1975, Palladini et al., 1996, Kusayama and Watanabe, 2000, Buttarelli et al, 2002, Raffa and Desai, 2005, Pagan et al., 2008, Ramakrishnan and DeSaer, 2011). For instance, amphetamine, cocaine or nicotine were shown to induce screw-like and C-like hyperkinesias, body contraction resulting to walnut positions in planarians (Carolei et al., 1975, Ramakrishnan and DeSaer, 2011, Rawls et al., 2011). Also, withdrawal response following chronic exposure to caffeine, methamphetamine and cocaine (Sacavage et al., 2008), and discontinuation of amphetamines, benzolediazepines, cannabinoids, nicotine, cocaine and opioids exposure was observed in planarians (Rawls et al., 2007, 2011). In addition, some neurotransmitters and receptors associated to some of these behavioural responses induced by

these pharmaceutical compounds have been identified (Palladini et al., 1996, Passarelli et al., 1999, Buttarelli et al., 2000, Raffa et al., 2001, 2013b, Rawls et al., 2007). Further, Ramakrishnan and DaSaer (2011) demonstrated potential of carbamazepine, an antiepileptic, to

Table 1.3: Lethal concentrations (mg L^{-1}) of some pharmaceuticals for freshwater planarian *D. japonica* and *Daphnia magna* (Li, 2013)

Pharmaceutical	**48 hrs LC_{50} D. japonica**	**48 hrs LC_{50} *D. magna***
Naproxen	8.6	166.3
Acetaminophen	371.5	20.1
Acetylsalicylic acid	95.9	88.1
Diclofenac	5.3	68
Ibuprofen	39.1	31, 101.2
Mefenamic acid	3.3	10
Carbamazepine	163.8	55, 111
Caffeine	633.2	182
Lincomycin HCl	>1000	>3651
Ciprofloxacin	>1000	65.3
Trimethoprim	271.6	167.4
Chlortetracycline HCl	141.4	137.6
Tetracycline	760.6	617.2
Erythromycin	>100	210.6
Ofloxacin	195.2	17.4
Sulfamethoxazole	282.8	189.2
Acebutolol HCl	>1000	51
Nadolol	621.4	>100
Metoprolol	290.2	438
Propranolol	7.2	0.5, 7.7
Atenolol	>500	180, 313
Pindolol	67.5	30
Diltiazem HCl	14.1	28
Diphenhydramine HCl	9.8	0.4

restrain drug-induced epileptic seizure-like condition in *D. tigrina*. In addition to behavioural alterations and mortality, nicotine exposure caused suppression of motility and fissioning in *D. dorotocephala* (Best and Morita, 1991, Rawls et al., 2011). As well, sertraline (3.06 mg L^{-1} or 10µM) exposure to *D. tigrina* resulted to reductions in locomotion, motionlessness, tissue damage and death but lower concentrations which are within therapeutic concentration caused reduction in locomotion and altered behaviour (Thumé and Frizzo, 2017).

Other compounds such as methyl methanesulfonate (MMS) and cyclophosphamide (CP) used for the treatment of cancer caused chromosomal aberration in exposed *D. tirgrina* and *D. schubarti* (Lau et al., 2007). The steroid 17 β-estradiol caused mortality at concentrations above 1.0 mg L^{-1} after 5 days in regenerating *D. ryukyuensis* (Miyashita et al., 2011). Also, exposure to this steroid caused a reduction in the expression of *Dryg* gene involved in development of yolk gland resulting to suppression of development of female yolk gland in regenerating *D. ryukyuensis* (Miyashita et al., 2011).

Berberine useful in the treatment of diabetes and an alkaloid contained in eye drops, caused regeneration of abnormal eye/photoreceptor and inhibition at higher concentrations in exposed *D. japonica*, though without any effect on cell proliferation (Balestrini et al., 2014). Other toxic effects associated to berberine exposure were altered movement/reduction in locomotion, alteration in the expression of the genes linked to different tissues but cytotoxic effects in the intestine only (Balestrini et al., 2014).

Pharmaceuticals have been shown to cause acute toxicity, but at very high concentrations. Thus, mortality may not be a very sensitive endpoint for bioassays. However, behavioural, genotoxicity, asexual reproduction by fissioning, gene expression and cytotoxicity endpoints may be useful in ecotoxicological studies using freshwater planarians.

1.2.4 Personal care products

As a result of wide scale and persistent uses of personal care products such as body cream and lotion, soaps (including shampoos and detergents), toothpaste, insect repellents, sunscreen and fragrances, their active ingredients are detected in aquatic environment (Brausch and Rand, 2011, Blair et al., 2013). This group of chemical compounds have been recognised as one of the emerging aquatic pollutants, though it has received little attention compared to other aquatic chemical pollutants (Brausch and Rand, 2011). These products are used externally and might not

undergo metabolic modification resulting to their release in unchanged form into aquatic systems (Ternes et al., 2004, Brausch and Rand, 2011). Personal care products are released into the aquatic environment directly through bathing, washing, runoff from surroundings of aquatic systems like beaches, and indirectly through wastes from production facilities and effluents from sewage treatment plants. Some are known to be bioactive, persistent in the environment, bioaccumulate and adsorb to water sediment (Peck, 2006, Brausch and Rand, 2011). Little is known about their toxic effects to non-target aquatic organisms because research on their effects is still scarce (Brausch and Rand, 2011). Information on their effects on freshwater planarians are limited to few studies on organic UV filters, preservatives and some surfactants present in personal care products and other household products (Li, 2008, 2012a, 2012b, Hagstrom et al., 2015). Acute toxicity studies with freshwater planarian *D. japonica* showed that planarians might be as sensitive as *Daphnia magna* to these chemical compounds (table 1.4).

Table 1.4: Lethal concentrations (mg L^{-1}) of UV filters and paraben preservatives for *Dugesia japonica* and *Daphnia magna* (Li, 2012b)

Common name	**Chemical name**	**48 hrs LC_{50} for *Dugesia japonica***	**48 hrs LC_{50} for *Daphnia magna***
UV filters			
Benzophenone	BP	5.0	0.3 (24 hrs)
2,4-Dihydroxybenzophenone	BP-1	2.8	7.9
Oxybenzone	BP-3	0.9	1.9
Sulisobenzone	BP-4	146	50
4,4′-Dihydroxybenzophenone	DHBP	12.3	12
Preservatives			
Methyl 4-hydroxybenzoate	Methyl Paraben	77	62
Ethyl 4-hydroxybenzoate	Ethyl Paraben	31	32
Propyl 4-hydroxybenzoate	Propyl Paraben	12.3	23
Butyl 4-hydroxybenzoate	Butyl Paraben	7.8	9.2

1.2.5 Abiotic natural stressors

Studies have shown that water quality parameters prevalent in the freshwater environment may influence the survival and population of freshwater planarians (Rivera and Perich, 1994, Harrath et al., 2004, Manenti and Bianchi, 2014). The effects of changes in water quality parameters such as salinity, temperature, hardness, pH, dissolved oxygen and dissolved organic matter on freshwater planarians was evaluated using *D. tigrina*, *D. dorotocephala*, *Dendrocelopsis vaginatus* and *Cura foremanii* (Rivera and Perich, 1994). These authors observed that increased salinity in addition to causing mortality may as well alter asexual reproduction and feeding in freshwater planarians (Legner et al., 1976, Rivera and Perich, 1994). *D. dorotocephala* feeding was significantly impaired below 0.25 g L^{-1} and above 1.0 g L^{-1} salinity (Cl^-) (Legner et al., 1976). *C. foremanii* is the most sensitive species to salinity (NaCl) tolerating up to 0.25 g NaCl L^{-1} and with 100% mortality at 3.0 g NaCl L^{-1}, *D. dorotocephala* and *Dendrocelopsis vaginatus* survived up to 3.0 NaCl g L^{-1} while *D. tigrina* is the most tolerant showing no deleterious effects on asexual reproduction and survival at 3.0 g NaCl L^{-1} (Rivera and Perich, 1994).

Increased temperature on the other hand, affected survival and reproduction of the 4 planarian species. Temperature increase up to 30°C induced asexual reproduction in *D. dorotocephala* but caused significant mortality to *C. foremanii* and *D. vaginatus* (Rivera and Perich, 1994). Similarly, Legner et al. (1976) reported that *D. dorotocephala* is intolerant to temperatures above 26°C but that gradual temperature increases above adverse limits could lead to adaptation and induce asexual reproduction. Also, a field study on a *S. mediterranea* population showed that salinity concentrations up to 2.9 g L^{-1} and temperature between 25 and 27.5°C had negative effects on sexual reproduction and survival with the few survivors showing tears/lesions on their body and poor general condition (Harrath et al., 2004).

Increased hardness (160 to 320 mg L^{-1} $CaCO_3$) enhanced asexual reproduction in *D. tigrina* but had no significant influence on the survival or asexual reproduction of the other 3 species (Rivera and Perich, 1994). Studies on effects of pH showed the four planarians species used by Rivera and Perich can tolerate pH ranges between 4 and 9, while pH 3 caused 100% mortality in all species. Similarly, Oviedo et al. (2008) suggested pH ranges between 7.5 and 9.5 for *D. tigrina* and up to 7.5 for *D. japonica* laboratory cultures. High dissolved organic matter affected asexual reproduction and survival of *D. dorotocephala* and *D. tigrina* but had no effect on those of *C. foremanii* and *D. vaginatus* (Rivera and Perich, 1994). Decrease in planarians' asexual

reproduction could be associated to insufficient nutrition due to reduction in planarians feeding/prey capture ability at high dissolved carbon concentration (Rivera and Perich, 1994). Impairment in planarian prey capture ability could be linked to interaction between planarian mucous substance and carbon in the medium resulting to loss of mucous substance adhesiveness needed by planarians to trap prey (Rivera and Perich, 1994).

Dissolved oxygen affected survival and reproduction of all 4 species causing significant mortality at 6 mg L^{-1} oxygen (Rivera and Perich, 1994). Additionally, these studies support previous findings that freshwater planarians are found in the wild in unpolluted freshwaters with dissolved oxygen levels close to saturation (Kapu and Schaeffer, 1991, Rivera and Perich, 1994).

Freshwater planarians are known to inhabit sheltered freshwater habitats (Manenti and Bianchi, 2014), which may be associated to their photophobic behaviour as the sheltered streams may have reduced light illumination. A number of studies have assessed the effects of light radiation of different wavelengths on planarians (Spencer and Klein, 1979, Kalafatić et al., 2006, Paskin et al., 2014), and showed that planarians exhibit wide range of behavioural responses to light radiations consisting of general and wavelength specific responses (Paskin et al., 2014). Exposure of *S. mediterranea* to Infra red, red, UV, blue and green radiations induced escape responses but UV, blue and green radiations suppressed exploratory behaviour (Paskin et al., 2014). However, red radiation did not affect exploratory behaviour and planarians exposed to infra red radiation were attracted to the light source (Paskin et al., 2014). Kalafatić et al. (2006) observed that exposure of *D. tigrina* and *P. felina* to UV light resulted to mortality, folding or wrinkling of the epidermis of lateral region, reduced response to stimulus, damage to auricles, epidermal and mucous layers, contraction of body, reduction of the intestine lumen, increased secretion of mucous in the intestine and accumulation of pigments on the body. In addition, they observed accumulation of neoblasts at the wound area and that planarians recover from toxic effects some days after exposure. Similarly, in another study, exposure to broad band light near UV radiation affected survival and regeneration of planarians while exposure to blue, green, red and far red radiations did not have any effect (Spencer and Klein, 1979). Broad band near UV radiation of the UV-B range suppressed regeneration, caused injury and death of intact while sublethal intensities caused melanogenesis (deep pigmentation) in *D. dorotocephala* and *D. tigrina* (Spencer and Klein, 1979). Altered regeneration may be associated to near UV radiation interaction with nucleic acids and proteins in exposed planarians (Spencer and Klein, 1979). The severity of toxic effect due to UV exposure is a function of exposure time as planarians have the

potential to repair damages (Spencer and Klein, 1979, Kalafatić et al., 2006). Also, *D. tigrina* appeared more sensitive to UV radiation than *P. felina* and *D. dorotocephala* due to presence of lesser endogenous pigment, melanin (Spencer and Klein, 1979, Kalafatić et al., 2006).

These abiotic factors had deleterious effects on freshwater planarians with significant impacts on their survival and population growth. Planarians survival, reproduction and behaviour may be sensitive endpoints for bioassays considering these factors. Regeneration, mucous secretion, morphological, histological and cytological alterations can be used as complementary endpoints to evaluate effects of light radiations. Moreover, planarians deep pigmentation may be used as a marker for photoactivated melanin synthesis (Spencer and Klein, 1979) and a measure for sublethal effects of UV-B. Freshwater planarians may thus be useful in studying effects of climate change on the freshwater environment.

1.2.6 Other stressors of freshwater habitat

Freshwater planarians are also sensitive to other freshwater environmental stressors in addition to metals, pesticides, pharmaceuticals and abiotic factors. Environmental stressors such as unionized ammonia, bisphenol A, polyaromatic compounds, synthetic dyes and surfactants have been shown to be toxic on planarians.

Planarians have been shown to exhibit high sensitivity to unionized ammonia ($N\text{-}NH_3$) with significant mortality occurring at very low concentrations 96 hrs LC_{50} of 0.39 mg L^{-1} and more than 60% mortality at 0.05 mg L^{-1} after 30 days of exposure (Alonso and Camargo, 2006, 2011). Also, 30 days chronic exposure of *P. felina* caused reduction in locomotor activity in planarians from 0.02 mg L^{-1} $N\text{-}NH_3$ (Alonso and Camargo, 2006, 2011). Results from these studies show that in the natural habitat where limits for safe concentrations have been set at 0.01 and 0.10 mg $N\text{-}NH_3$ L^{-1}, planarian survival may be threatened (Alonso and Camargo, 2011). Behaviour and mortality endpoints may be used in the environmental impact assessment of unionized ammonia using freshwater planarians.

Further, exposure of *D. dorotocephala* to polyaromatic compounds dimethylbenzanthracene (DMBA), benzopyrene (BP) and benzanthracene (BA), and phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) were shown to be toxic to planarians. DMBA was teratogenic causing supernumerary eyes and heads, and tumors, BA was weakly teratogenic while BP was lethal (Best and Morita, 1991). Similarly, 12-O-tetradecanoylphorbol-13-acetate caused 100% at 0.05 mg L^{-1} after 5 days exposure but at 0.01 mg L^{-1} suppressed asexual

reproduction by fissioning and caused malignant type II tumor in *D. dorotocephala* (Hall et al., 1986). Mortality, reproduction as well as morphological and developmental alterations may be used to evaluate the effects of these polyaromatic compounds in the freshwater environment.

Bisphenol A (BPA) used in making plastics and an endocrine disruptor caused mortality at concentrations above 1.0 mg L^{-1} after 5 days in regenerating *D. ryukyuensis* (Miyashita et al., 2011). Also, exposure to the endocrine disruptor caused a reduction in the expression of *Dryg* gene involved in development of yolk gland resulting to suppression of development of female yolk gland in regenerating *D. ryukyuensis* (Miyashita et al., 2011).

Synthetic dyes are another group of emerging contaminants in the aquatic systems. They are known to induce genotoxic and other toxic effects in addition to mortality on non-target exposed aquatic organisms. Among these synthetic dyes is azo dye used in the production of textiles, foodstuffs, cosmetics, paints, inks and household products (Ribeiro and Umbuzeiro, 2014). Azo dye is released into the aquatic environment through wastes from factories and effluents from waste treatment plants (Umbuzeiro et al., 2004, 2005). The effects of disperse red 1, an azo dye which have been detected in some places known for dyeing activities was evaluated using planarian *D. tigrina* (Ribeiro and Umbuzeiro, 2014). Apart from mortality, exposure to disperse red caused increase in secretion of mucous from the epidermal layer, altered movement and red colouration of surface membrane in adults and regenerates (Ribeiro and Umbuzeiro, 2014). Additionally, disperse red exposure delayed or suppressed head regeneration, caused irregular twisting and contraction of body, retractile pharynx, reduction of feeding and reproduction in planarians (Ribeiro and Umbuzeiro, 2014).

Other studies evaluated acute toxicity and sub-acute responses of freshwater planarians to surfactants: linear alkylbenzene sulfonate (LAS), sodium dodecyl sulfonate (SDS), benzethonium chloride (Hyamine 1622), cetyl trimethyl ammonium bromide (CTAB), 4-nonylphenol (NP), dodecyl trimethyl ammonium bromide (DTAB), benzalkonium chloride (BKC). Also, planarian sensitivity to these chemicals is comparable to that of other sensitive organisms like *Daphnia magna* (table 1.5).

Additionally, CTAB, hyamine 1622, NP and SDS caused a decrease/inhibition of cholinesterase activities; triton X-100 caused an increase in cholinesterase activities while LAS increased catalase activities (Li, 2008). DTAB decreased planarian movement (Li, 2012a) but triton X-100 and SDS decreased movement and gliding speed in intact and regenerating

Table 1.5: Lethal concentrations (LC_{50}, mg L^{-1}) of water quality parameters and surfactants for freshwater planarians and *Daphnia magna*

Contaminants	Planarian species	LC_{50}	*D. magna* 48 hrs LC_{50}
Salinity	*Cura foremanii*	14 days-3000[A,B]	2999[C]
Oxygen	*D. dorotocephala*	14 days-2[A,B]	0.7[D]
DTAB	*D. japonica*	48 hrs-126.79[E]	0.25[E]
BKC	*D. japonica*	48 hrs-2.27[E]	0.16[E]
CTAB	*D. japonica*	48 hrs-2.83[F]	0.05[F]
LAS	*D. japonica*	48 hrs-1.79[F]	9.55[F]
NP	*D. japonica*	48 hrs-0.87[F]	0.19[F]
SDS	*D. japonica*	48 hrs-0.36[F]	19.13[G]

Note: [A-]100% mortality, [B-]Rivera and Perich, 1994, [C-]Ghazy et al., 2009, [D-]Nebeker et al., 1992, [E-]Li, 2012a, [F-]Li, 2008, [G-]Guilhermino et al., 2000

worms (Hagstrom et al., 2015). Also, SDS exposure caused defect in brain morphology of regenerates and altered thermotactic response while triton X-100 caused delay in blastema development, photoreceptor formation, defect in brain size and suppression of thermotactic responses (Hagstrom et al., 2015).

1.3 Research needs for the use of freshwater planarians in ecotoxicology

All these evidences of effects of different contaminants show that freshwater planarians display lethal and sub-lethal responses to most stressors of the freshwater environment, and have provided more information on diverse ways these stressors can affect non-target aquatic organisms. It is obvious that freshwater planarians offer great potential as invertebrate models to study environmental contaminants. However, studies with planarians face many constraints such as – (a) varying exposure and observation time used by different laboratories for a contaminant; (b) absence of environmentally relevant or low concentrations in some studies; (c) varying culturing conditions and size of planarians used in the studies; (d) varying and inadequate number of replicates and test animals per replicate; (e) focusing research efforts on few species of freshwater planarians and few endpoints, and (f) varying method of classification/scoring of responses.

To overcome these limitations, future research involving freshwater planarians should consider the standardization of culturing and testing procedures and the continuous evaluation of ecological effects of different contaminants. In response to this need, a simple protocol for ecotoxicity assays using freshwater planarians was drafted in the course of this work (Annex 1).

To ensure uniformity in laboratory culturing of freshwater planarians to be used for ecotoxicity tests and for comparison with established ecotoxicity test models, laboratory populations of planarians should be maintained in standard mediums such as ASTM medium (ASTM, 2004). Otherwise, planarians may be cultured with media as suggested in Oviedo et al., (2008). Standardizing culture medium used to maintain freshwater planarians is important as culture medium may influence their sensitivity to contaminant and also allows for direct comparison with toxicity data acquired for other species cultured and tested under similar conditions.

Moreover, since sexual, asexual and physiological types of freshwater planarians have high regenerative ability it will be appropriate for laboratories to maintain a clonal line for such studies reducing variability of responses.

Studies with freshwater planarians like those with established test models like *Daphnia magna*, or chironomids, should involve similar test protocols for all species/genera. More studies with more genera and species of freshwater planarians should also be encouraged as well as studies including sexual and asexual strains, and physiological forms.

Standardization may as well involve both manual and automated scoring protocols so that small and high budget research can be undertaken effectively. Importantly, Grebe and Schaefer (1991), Wu et al. (2012) and Hagstrom et al. (2016) have developed methods of scoring neurotoxicity in planarians. However, there is need for a generally accepted method of scoring and one that will consider all endpoints. Also, sizes/age of planarians used in tests should be normalised, as well as exposure and observation periods. Standardizing testing protocols including dilution medium and solvents and minimum number of replicates and organisms will encourage reliable, repeatable and reproducible results and enable comparison between different species and laboratories.

Additionally, more studies are needed to evaluate chronic effects of stressors on planarians in addition to short term studies. Long term bioassays with planarians are possible since they have long life span and fertility, and fecundity is easily measured. Multigenerational studies will also

identify possible carry over and/or epigenetic effects of contaminants and should not be restricted to sexual or parthenogenetic forms but also include the asexual strains.

Further, ecotoxicological studies with freshwater planarians have rarely considered effects of contaminants on planarian biochemical parameters, respiration and energetic biomarkers. Given the available protocols for measureing these responses in other invertebrates, adjusting protocols should be a simple task allowing their routine assessment in planarians. Moreover, the wealth of information available for molecular studies with planarians and their amenability to expression based PCR techniques should encourage more studies assessing impacts of contaminants on gene expression and epigenetic modifications associated to exposure that can also be linked to other endpoints. Furthermore, microarray studies with exposed planarians will provide more information on impact of interaction between genetic and environmental stressors on planarians. Even RNAi techniques can be used to identify specific genes responsible for various developmental alterations and behavioural responses induced by exposure to contaminants. In addition, histological studies involving immunohistochemistry and in-situ hybridization may be encouraged for in depth characterization of possible anatomical and cellular alterations caused by exposure to contaminants. Moreover, more studies on exposed planarians evaluating DNA damage with Comet assay and mutagenecity with micronuclei assay are required, as exposure to contaminants may affect the DNA in various ways which may be linked to some toxic responses.

Most reported studies with planarians considered toxicity of the chemicals with very high concentrations which are not relevant to the aquatic environment while others evaluated the toxicity of chemical compounds with more or less no consideration of their ecotoxicological implications. As such, studies aiming at evaluating the effects of environementally relevant concentrations of different contaminants such as pharmaceuticals, personal care products and pesticides (especially recently formulated pesticides) and of their mixtures should be encouraged. Mediating effects of natural stressors such as increasing salinity, temperature and Ultra violet radiation as well as the consideration of possible indirect effects of contaminants on trophic chains where planarian have a pivotal role should also be taken into consideration.

1.4. Psychiatric Pharmaceuticals as emerging contaminants of the aquatic environment

Pharmaceuticals are one of the most important classes of emerging chemical contaminants in the aquatic environment. Pharmaceuticals are a group of chemical compounds designed to have

specific biological effects in their target organisms, many of which may be long lasting, and with some side effects (Halling-Sørrenen et al., 1998, Fent et al., 2006). These include pharmaceutical products used in human medicine, growth promoters and therapeutic substances in veterinary medicine, and feed additives in aquaculture (Halling-Sørrenen et al., 1998). They are made such that they can pass through membranes, and be persistent, bioactive, and exert the expected effect(s) in the organism (Halling-Sørrenen et al., 1998, Calisto and Esteves, 2009). Reports on the presence of pharmaceuticals in the aquatic environment dates back to the 1970s and 1980s in Germany and the UK, respectively (Kümerer, 2001). Following this, in the 1990s and subsequent years and due to improved analytical techniques, several authors have reported and reviewed numerous field studies in Europe, America, Asia, Australia and Arctic region on the presence of different pharmaceutical products in different aquatic systems (Halling-Sørrenen et al., 1998, Ternes, 1998, Jones et al., 2001, Kümerer, 2001, 2009a, Heberer, 2002a, 2002b, Fent et al., 2006, Calisto and Esteves, 2009, Blair et al., 2013, Yuan et al., 2013, Fedorova et al., 2014, Huber et al., 2016). Pharmaceutical substances detected in these freshwater environments include psychiatric pharmaceuticals, analgesics, beta blockers, blood lipid regulators, antibiotics, antineoplastics, cancer therapeutics, contraceptives, anti-inflammatory and antitumor agents.

Several routes through which these chemical compounds enter the aquatic environment have been identified. It has been shown that after ingestion and having an effect on the organism, these pharmaceuticals are released from the organism's body through urine and feaces as parent compound, metabolites or conjugates (Halling-Sørrenen et al., 1998). Sometimes some unused pharmaceuticals are discarded with domestic waste or flushed down the drain (Jones et al., 2001). In addition, wastes from pharmaceutical production facilities and hospitals have been reported to contain high concentrations of pharmaceutical compounds (Kümmerer, 2001, Larsson et al., 2007, Philips et al., 2010). Also, some of the feed additives used in fish farming are not utilized by the fish but settle on the water beds (Halling-Sørrenen et al., 1998). Moreover, sewage sludge, manure or wastes from animals or animal husbandry used as fertilizers in agriculture have been shown to contain pharmaceuticals which can contaminate the soil and through run-off from farms or leaching through the soil reach surface waters and ground waters (Boxall et al., 2003, Chen et al., 2006, Zuccato et al., 2006). Consequently, these chemical products may enter into the aquatic environment through human and animal wastes, fish feed additives, disposal of unused drugs within domestic wastes, effluents from hospitals,

pharmaceutical production plants and sewage treatment plants (Heberer, 2002b, Ternes, 1998, Kümmerer, 2001, Lindqvist et al., 2005, Roberts and Thomas, 2006, Gross et al., 2007, Philips et al., 2010, Sim et al., 2011, Metcalfe 2014). The sewage treatment plants have been shown to be inefficient in the complete removal of pharmaceuticals because while some can degrade or may become inactive others do not, resulting in the release and presence of the parent compounds, their metabolites and conjugates in the sewage effluents, surface and ground waters (Halling-Sørrenen et al., 1998, Ternes, 1998, Kümmerer, 2001, Heberer, 2002b, Heberer et al., 2002, Jones et al., 2005, Roberts and Thomas, 2006). The removal effectiveness of pharmaceuticals by sewage treatment plants may vary between more than 80% to less than 20% (Larsson et al., 2007). Overall, major factors responsible for the presence of pharmaceuticals substances in the environment are population density and consumption rate, fate of the drug in human body, efficiency of sewage treatment plants and volume and flow rate of the receiving waters (Heberer, 2002b).

Concentrations of pharmaceuticals in the aquatic environment usually range from ng L^{-1} to µg L^{-1} (Jones et al., 1998, 2001, Heberer et al., 2002, Kümmerer, 2009b, Blair et al., 2013) and may sometimes increase to mg L^{-1} in aquatic ecosystems near pharmaceutical production facilities (Larsson et al., 2007, Philips et al., 2010). The environmental concentrations of individual pharmaceuticals may not likely have any acute toxicity effects on aquatic organisms, since most of the acute toxicity effects are usually above environmental concentrations (Pascoe et al. 2003, Fent et al., 2006). However, since these pharmaceutical substances are designed to elicit effects in their human and veterinary animal targets at low concentrations, effects may likely occur in exposed non-target organisms at these low environmental concentrations (Escher et al., 2005). Moreover, since pharmaceutical products are used and released into the aquatic system on a regular basis (Daughton and Ternes, 1999), exposure of aquatic organisms may occur throughout their entire life-cycle involving all life stages and many generations too (Daughton and Ternes, 1999, Fent et al., 2006, Brausch et al., 2012). Thus, long term exposure to low environmental concentrations of pharmaceuticals may increase the chances of detrimental effects on aquatic organisms.

Studies have shown that environmental concentrations of these pharmaceuticals can affect some physiological systems of non-target aquatic organisms (Halling-Sørrenen et al., 1998, Fent et al., 2006, Rivetti et al., 2016, Ford and Fong, 2016). Among pharmaceuticals whose

effects at environmental concentration on non-target aquatic organisms have been evaluated are psychiatric pharmaceuticals.

Psychiatric pharmaceuticals include drugs which modulate behaviour by acting on the central nervous system to interrupt some neuro-endocrine signaling (Calisto and Esteves, 2009). They are grouped into 6 classes namely antidepressants, antipsychotics, anxiolytics, antiepileptics, mood stabilizers (Lithium and anti-convulsants), and others like methadone, stimulants and non-stimulants (Martin-Vazquez, 2012). Psychiatric drugs are among the commonly used pharmaceutical products worldwide (Calisto et al., 2011, Chiffre et al., 2014). Several field studies have reported various psychiatric pharmaceuticals in many aquatic environments (Ternes, 1998, Heberer, 2002b, Kolpin et al., 2002, Philips et al., 2010, Gracia-Lor et al., 2011, Schultz et al., 2010, Sui et al., 2011, Yuan et al., 2013, Fedorova et al., 2014). Like other pharmaceuticals, different ways through which psychiatric may be released into the environment have also been identified (fig.1.4).

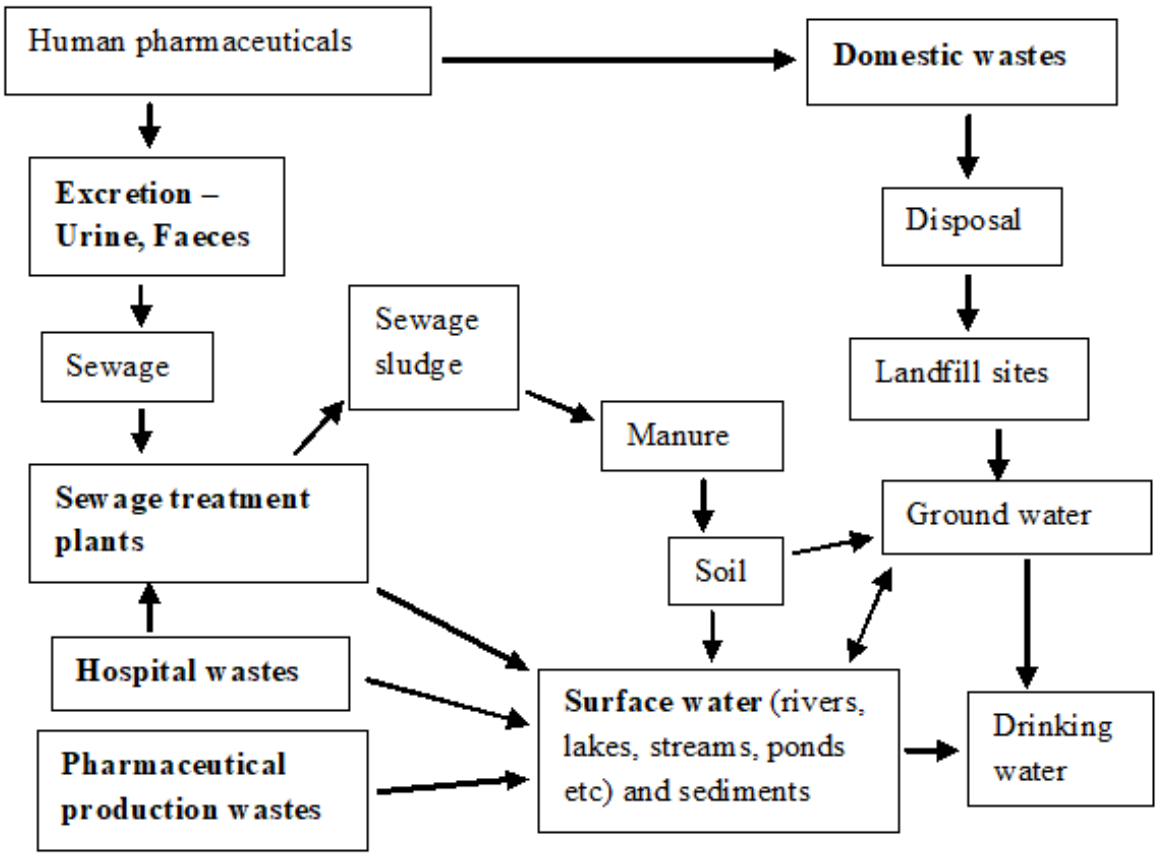
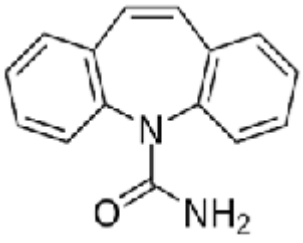
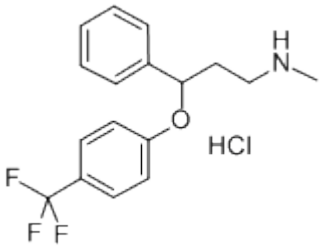


Fig. 1.1: Chart showing possible route through which psychiatric pharmaceuticals reach aquatic systems (Adapted from Ternes, 2001)

Amongst psychiatric pharmceuticals most commonly detected within freshwater ecosystems are carbamazepine and fluoxetine (table 1.6).

Table 1.6: Pharmacological information about carbamazepine and fluoxetine

Information	**Carbamazepine**	**Fluoxetine**
Common names	Tegretol, Epitol, Carbatrol etc	Prozac, Sarafem etc
CAS No	298-46-4	562960-78-7
IUPAC name	5*H*-dibenz[*b*,*f*]azepine-5-carboxamide	Benzenepropanamine N-methyl-gamma-[4-(trifluoromethyl) phenoxy]-hydrochloride
Producer	Sigma Aldrich	Tokyo Chemical Industry (TCI)
Molecular weight	236.27 g mol^{-1}	345.79 mg mol^{-1}
Chemical formula	$C_{15}H_{12}N_2O$	$C_{17}H_{18}F_3NO.HCl$
Water solubility	17.7 mg L^{-1}	14 mg mL^{-1}
Log K_{OW}	2.45	4.05
Drug class	Antiepileptic, anxiolytic, anticonvulsant, mood stabilizer[A]	Anti-depressant[A]
Target ailment	Epilepsy, convulsion, manic symptoms, bipolar disorder, anxiety disorder[A]	Depression, obsessive-compulsive disorder, panic disorder, bulimia nervosa, obesity, premenstrual syndrome, post traumatic stress disorder, social phobia, alcoholism[A, B]
Major target	Voltage-gated Sodium ion channel[C]	Serotonin uptake site and receptor[B]
Other targets	Calcium ion and Potassium ion channels, serotonergic, dopaminergic and glutamergic systems, adenosine and peripheral-type benzodiazepine receptors and cyclic adenosine monophosphate (cAMP)[C]	Dopaminergic, adrenergic, histaminergic, GABAergic, melatonergic,, benzodiazepine, opiate etc receptors and uptake sites[B, D]
Structural formular		

CAS No-Chemical Abstract number, [A]- Martín-Vázquez, 2012, [B]- Wong et al., 1995, [C]- Ambrósio et al., 2002, [D]-Reierson et al., 2009

The main target of carbamazepine is the voltage-gated sodium (Na) ion channels where it blocks the release of neurotransmitters and alters neuronal excitability (Post et al., 1983, Ambrósio et al., 2002, Datar, 2015). Carbamazepine reduces synaptic transmission by blocking Na^+ channels and firing action potential at the pre-synapse, and reduces high frequency firing action by enhancing Na^+ channel inactivation at the post-synapse (Datar, 2015).

Carbamazepine is known to act shortly after administration (Post, 1988, Ramakrishnan and DeSaer, 2011, Rivetti et al., 2016). After ingestion, 72% of carbamazepine oral dose is absorbed while 28% is excreted in unchanged form through faeces (Zhang et al., 2008). The absorbed portion is subjected to metabolic processes in the liver after which 1 to 3% are excreted as parent compound through urine while the remaining are released through urine as metabolites and conjugates (Ternes, 1998, Ambrósio et al., 2002, Clara et al., 2004, Zhang et al., 2008, Petrie et al., 2015). Consequently, carbamazepine from faeces and urine is carried in influent water to the sewage treatment plants.

Studies on sewage influents and effluents showed that the removal rate of carbamazepine in sewage treatment plant is low, less than 1% or about 8% (Heberer et al., 2002, Clara et al., 2004). This may be associated to carbamazepine resistance to microbial degradation and adsorbtion to sewage sludge, and negligible photo-transformation in sewage treatment plants (Heberer, 2002b, Clara et al., 2004 Zhang et al., 2008). In addition, carbamazepine does not adsorb to sediments, and is resistant to biodegradation and adsorption during groundwater or underground water passage (Heberer, 2002b, Clara et al., 2004). As a result, carbamazepine is frequently detected in sewage treatment plant effluents (Ferrari et al., 2003) and freshwater systems (Ternes, 1998), particularly where waste waters from sewage treatment plants are discharged (Heberer, 2002a, Yang et al., 2015). Ordinarily, carbamazepine is regarded as a marker of human interference in the aquatic ecosystem (Clara et al., 2004) and sewage contamination of surface waters (Heberer, 2002b). Studies in many countries have reported carbamazepine in influent and effluent water, water sediments, surface, ground, well and drinking water (table 1.7). Carbamazepine has been detected in sewage sludge reaching concentrations of up to 291.1 ng Kg^{-1}, 2900 ng Kg^{-1} and 3.4 ng g^{-1} (Zuccato et al., 2006, Gao et al., 2012, Muhapatra et al., 2012) and in biosolids at 0.9, 2.9 and 5.6 µg Kg^{-1} (Spongberg and Witter, 2008, McClellan and Halden, 2010, Ding et al., 2011). Moreover, carbamazepine is persistent in aqueous solution and its half-life under semi field condition has been estimated to be 82 ± 11 days (Lam et al., 2004, Sandersen et al., 2004).

Table 1.7: Environmental concentrations of carbamazepine

Influent wastewater (ng L^{-1})	Effluent (ng L^{-1})	Sediment (ng g^{-1})	Surface water (ng L^{-1})	Ground water (Drinking/well water) (ng L^{-1})	References
950-2593	826-3117	-	610	-	Petrie et al., 2015
19-161	13-184	-	-	-	Yuan et al., 2013
300-1170	250-1200	-	-	-	Verlicchi et al., 2012
-	-	-	23.1-175.3	-	Zuccato et al., 2006
-	-	4.81	-	-	Beretta et al., 2014
-	-	-	250-12000	-	Loos et al., 2009
-	-	-	6-128	-	Ramaswamy et al., 2001
-	-	-	80-3090	-	Ginebreda et al., 2010
-	-	-	43	-	Zhao et al., 2010
2200	1630-2000	-	1300	1100 (10)	Ternes, 2001
21600	21000	-	-	-	Sim et al., 2011
-	6400	-	-	-	Ternes, 1998
-	226	-	25	-	Kim et al., 2007
1780	1630	-	25-1075	160-360 (20)	Heberer et al., 2002
-	-	41.6	60	-	Thacker, 2005
-	-	-	9	-	Skadsen et al., 2004
-	219-344	-	-	-	Hua et al., 2006
100	65	-	-	-	Benotti and Brownwell 2007
-	-	-	-	13.9-43.2	Rabiet et al., 2006
-	155-6300	-	-	(155-610)	Drewes et al., 2002
-	-	-	-	83	Osenbrück et al., 2007
-	1094	-	-	560	Arye et al., 2011
-	-	-	-	420	Fram and Belitz, 2011
-	-	0.1-32.89	-	-	Yang et al 2015
-	-	6.6	-	-	Vulliet et al., 2014
-	7-126	-	2-650	-	Metcalfe et al., 2003
-	--	-	310	-	Rocco et al., 2011
-	-	-	20-7100	-	Wiegel et al., 2004

The antidepressant fluoxetine (table 1.6) is another psychiatric substance detected in the freshwater environment. Fluoxetine is chiral and distributed as racemic mixtures of R- and S-fluoxetine hydrochloride (Stanley et al., 2007). Fluoxetine has as its major function selective serotonin uptake inhibition (Wong et al., 1995). Generally, fluoxetine is known to have very high affinity to serotonin uptake carriers where it binds to the uptake site to block the uptake of serotonin in the synaptosome back into the neuron (Wong et al., 1995). This results to an increase in the level/concentration of serotonin in the synapse (Wong et al., 1995). In addition, fluoxetine may also increase extracellular dopamine and norepinephrine levels in the prefrontal cortex and inhibit uptake of the catecholamines (Pozzi et al., 1999, Bymaster et al., 2002), and increase synthesis and level of melatonin (Reierson et al., 2009, Kirecci et al., 2014).

The inhibition of serotonin uptake occurs within minutes in animals and up to hours in humans after fluoxetine administration though the therapeutic effects may be delayed for some weeks (Wong et al., 1995).

Studies showed that about 95% (consisting of 80% in urine and 15% in faeces) of administered oral dose is excreted after metabolic processes in the liver (Bergstrom et al., 1988). Also, 11% out of 80% released through urine is excreted as parent compound in unchanged form while the rest consists of the metabolite and conjugate forms (Bergstrom et al., 1988, Petrie et al., 2015). The prescription and use of fluoxetine in recent times has increased because of its efficiency in mood-lifting and increased number of depression cases (Stanley et al., 2007, Nentwig, 2008, Petrie et al., 2015, Silva et al., 2015). This has resulted in the increase in the rate at which it is released to the environment.

The removal rate of fluoxetine in sewage treatment plant has been estimated as 22.55% (Johnson et al., 2007). Johnson et al. (2007) also observed that it is resistant to biodegradation, hydrolysis, persistent in water medium and may possibly adsorb to sludge. Also in other studies fluoxetine was shown to be resistant to biodegradation, hydrolysis and photolysis but readily adsorbed to sediments where it is persistent (Lam et al., 2005, Kwon and Armbrust, 2006). Fluoxetine displays stabilty with negligible degradation (2 to 3%) in buffer solution (pH 5, 7, 9), synthetic humic water and lake water (Kwon and Armbrust, 2006). As such, fluoxetine has been detected in sewage treatment plant influents and effluents (Petrie et al., 2015) from where it may be released into the aquatic environment. Concentrations of fluoxetine in sewage influent and effluents, surface water and water sediment have been reported by many studies in various countries (table 1.8). Similarly, fluoxetine was also detected in sewage sludge in concentration

reaching 170 ng Kg^{-1} and in biosolids at concentrations of 4700 and 37.4 µg Kg^{-1} (Furlong et al., 2004, Kinney et al., 2006, Petrie et al., 2015). In addition, the half life of fluoxetine in lake water is 63 days (Tixier et al., 2003) and 38 days in natural water under the influence of sunlight (Andreozzi et al., 2002) while Kwon and Armbrust (2006) reported half-life ranges from 102 to

Table 1.8: Environmental concentrations of Fluoxetine

Influent waste water (ng L^{-1})	**Effluent waste water (ng L^{-1})**	**Sediment (ng g^{-1})**	**Surface water (ng L^{-1})**	**Drinking water (ng L^{-1})**	**References**
14-86	16-29	-	5.8-14	-	Petrie et al., 2015
17	10-21	-	-	-	Yuan et al., 2013
-	99	-	-	-	Monteiro and Boxall, 2010
55-190	10-69	-	-	-	Verlicchi et al., 2012
-	64	-	-	-	Hua et al., 2006
-	-	0.39-19.37	0.8-43.2	-	Schultz et al., 2010
-	38-99	-	13-46	-	Metcalfe et al., 2003
-	540	-	320	-	Weston et al., 2001
-	590	-	-	-	Chen et al., 2006
-	-	-	12	-	Kolpin et al., 2002
600	560	-	-	-	Benotti and Brownawell, 2007
-	-	-	3	-	Pait et al., 2006
-	-	17.4	104-119	-	Bringolf et al., 2010
-	99	-	-	-	Brooks et al., 2005
-	-	-	10-12	-	Schultz and Furlong, 2008
-	-	-	0.42	-	Lajeunesse et al., 2008
-	-	-	3.9-10.1	-	Conley et al., 2008
-	29.3	-	9	-	Baker and Kasprzyk-Hordem, 2011
-	-	-	5.5	-	Vanderford et al., 2003
-	-	-	-	0.5	Synder et al., 2008
-	-	1.84	-	-	Furlong et al., 2004

385 days in buffer solutions and 112 to 133 days in lake water.

A number of studies have been carried out to establish environmental quality standard values for pharmaceutical substances. Through these studies the derived water quality guidelines for carbamazepine protective for 90% and 95% of species were 58 and 9.2 µg L^{-1} respectively, and carbamazepine environmental quality standard value in Germany and Switzerland is 0.5 µg L^{-1} Kumar et al., 2016). Similarly, derived water quality guidelines for fluoxetine protective for (90% and 95% of species were 3.8 and 1.6 µg L^{-1} respectively while predicted no effect concentrations in different studies were 0.004, 0.012, 0.031 and 0.05 µg L^{-1} (Kumar et al., 2016). Nevertheless, the presence of these pharmaceutical substances in the aquatic environment may pose threat to biota since responses of non-target organisms may vary due to individual species differences and pharmaceutical substances various mechanisms of actions (Escher et al., 2005, Fent et al., 2006). Moreover, some targets of carbamazepine and fluoxetine in humans are preserved among species and have been identified in many non-target vertebrates and invertebrates (Welsh and Moorhead, 1960, Fong, 1998, Fent et al., 2006). Orthologs to 86% and 61% of drug targets has been detected in zebrafish (*Danio rerio*) and the crustacean *Daphnia pulex* respectively (Gunnarsson et al., 2008). Moreover, neurotransmitter systems such as serotonergic system in crustaceans (*D. pulex*), molluscs (*Mytilus edulis*), hydrozoans (*Hydra*), zebrafish (*D. rerio*), rainbow trout (*Oncorynchus mykiss*), Japanese medaka (*Oryzias latipes*), frogs (*Xenopus laevis, X. tropicalis*) (Welsh and Moorhead, 1960, Gunnarsson et al., 2008, Mennigen et al., 2011), GABAergic (γ-aminobutyric acid) system in blackfish (*Orthodon microlepidotus*) (Cole et al., 1984) and voltage-gated sodium ion channel in *D. pulex*, *D. rerio*, *X. laevis, X. tropicalis* (Gunnarsson et al., 2008) have been identified. Thus, there is a possibility these pharmaceuticals may interact with their targets in these aquatic organisms. Furthermore, a neurotransmitter system may be involved in many biological processes some of which may not have been considered during the derivation of these water quality guidelines. Serotonin for instance, is known to influence physiological activities like behaviour, reproduction, metabolism, immune responses and moulting in animals (Fong, 1998, Bossus et al., 2014), and exposure to pharmaceutical substance which interacts with serotonin may perhaps affect these activities in organisms.

Ecotoxicolgical assessments of the effects of fluoxetine and carbamazepine on non-target aquatic organisms have shown that their environmental concentrations may alter some of their biological processes (Fent et al., 2006, Rivetti et al., 2016). Several studies have shown that

fluoxetine or carbamazepine exposures affect behaviour (reproductive, prey catching, prey avoidance, aggressive and exploratory behaviour, and locomotion), reproductive success (sexual development, egg release, hatching), embryonic development, serotonin and dopamine levels, oxidative stress, cytotoxicity, genotoxicity, immunotoxicity and many other physiological processes in various aquatic organisms like crustaceans, insects, molluscs, amphibians and fish (tables 1.9 and 1.10).

Table 1.9: Acute and chronic toxicity effects of carbamazepine on aquatic organisms

Class/group of organism	Species/common name of organism	End point/Responses	References
Alga	***Chlorella vulgaris***	48 hrs EC_{50} growth inhibition-37 mg L^{-1}	Jos et al., 2003
	Chlorella pyrenoidosa, Scenedesmus obliquus	144 hrs and 30 days EC50 Growth inhibition-33.11 and 7.00 mg L^{-1} (*C. pyrenoidosa)*and 54.60 and 0.88 mg L^{-1} (*S. obliquus)*, decreased chlorophyll content, inhibited chlorophyll *a* synthesis, increased catalase and superoxide dismutase activities	Zhang et al., 2012
	Pseudokirchneriella subcapitata/Selenastrum capricornutum	96 hrs growth inhibition NOEC and LOEC- >100 mg L^{-1}	Ferrari et al., 2003
	Desnodesmus subspicatus	3 days growth inhibition EC_{50}-74 mg L^{-1}	Cleuvers, 2003
Diatom	***Cyclotella meneghiniana***	4 days growth inhibition NOEC-10 µg L^{-1}	Ferrari et al., 2004
Duckweed	***Lemna minor***	7 days growth inhibition EC_{50}-25.5 mg L^{-1}	Cleuvers, 2003
Rotifer	***Branchionus calyciflorus***	48 hrs reproduction inhibition NOEC-377 µg L^{-1}, LOEC-754 µg L^{-1}	Ferrari et al., 2003
Cnidaria	***Hydra attenuata***	96 hrs LC_{50} and EC_{50}-29.4 and 15.52 mg L^{-1} respectively, reduced feeding, attachment to substrate and hydranth number	Quinn et al., 2008
Crustacea	***Thamnocephalus platyurus***	24 hrs immobilization- >100.0 mg L^{-1}	Kim et al., 2009
	Daphnia magna	48 hrs EC_{50}->13800 µg L^{-1}	Ferrari et al., 2003
	D. magna	48 hrs EC_{50}-98 mg L^{-1}	Jos et al., 2003
	D. magna	48hrs EC_{50}->100 mg L^{-1}	Cleuvers, 2003, Kim et al., 2007
	D. magna	Enhanced reproduction during 21-23 days, increased positive phototactic behaviour in adults and juveniles, responses were non-monotonic	Rivetti et al., 2016
	Cerodaphnia dubia	48 hrs EC_{50}-77.7 mg L^{-1}, 7 days reproduction inhibition NOEC-25 µg L^{-1} and LOEC-100 µg L^{-1}	Ferrari et al., 2003
	Gammarus pulex	Increased ventilation and reduced activity or locomotion at lower concentration (10 ng L^{-1}) while higher concentration resulted to decreased ventilation and increased activity or locomotion	De Lange et al., 2006, 2009
Insecta	***Chironomus riparius*** **(midge)**	28 days LC_{50}-0.16 to 0.21 mg Kg^{-1}, Suppressed emergence of pupa NOEC-0.033 to 0.14 mg Kg^{-1}, LOEC-14 to 0.234 mg Kg^{-1}	Oetken et al., 2005

Table 1.9: Acute and chronic toxicity effects of carbamazepine on aquatic organisms (continued)

Class/group of organism	Species/common name of organism	End point/Responses	References
Insecta	***C. riparius***	40 days LC_{50}-1.1 mg Kg^{-1}, suppressed emergence, reduced fertility by reduction in number of fertile egg clutches but not number of clutches produced	Heye et al., 2016
	***C. tepperi* (Embryo)**	7 days larval survival EC_{10}-4 µg L^{-1}	Kumar et al., 2016
Mollusca	***Corbicula fluminea* (Asian clam)**	Reduced filtering rate and induced oxidative stress in the gill and digestive gland (decreased superoxide dismutase and glutathione reductase activities and increased catalase activity, reduced Hsp22, Hsp40, Hsp70 mRNA levels in the gill, increased Hsp 60 and Hsp90 levels in the gill and Hsp60 mRNA levels in the digestive gland)	Chen et al 2014
	***Dreissena polymorpha* (zebra mussel)**	Increased tissue concentration with highest bio-concentration factor in tissue of snails exposed to lowest concentration after 7 days exposure; Increased mRNA level of hsp70 in the gill (up-regulated stress protein level in gill) after 1 day exposure but down-regulated mRNA levels of hsp70 superoxide dismutase in the gill and metallothionein in the digestive gland after longer exposure.	Contardo-Jara et al., 2011
	D. polymorpha	Induced cell cytotoxicity	Parolini et al., 2011
	***Elliptio complanata* (freshwater mussel, haemolymph)**	Increased phagocytic and intracellular esterase activities, inhibition of cell adherence, no effect on lipid peroxidation	Gagné et al., 2006
Pisces/Fish	***Danio rerio* (Zebrafish-adult and embryo)**	Decreased reproductive output by decreasing number of viable embryos, caused atretic oocytes and altered ovarian histology in females, altered kidney tubule morphology, decreased plasma II ketotestosterone concentration in males and females, increased embryo mortality but did not cause any developmental abnormality	Galus et al., 2013
	***D. rerio* (adult)**	Decreased embryo production in crosses with F1 male offspring of exposed parents, reduced courtship potential but increased sperm motility of F1 male offspring of exposed parents	Galus et al., 2014
	***D. rerio* (embryo and larvae)**	10 days mortality NOEC-25 mg L^{-1}, LOEC-50 mg L^{-1}	Ferrari et al., 2003
	***D. rerio* (embryo)**	72 hrs EC_{50}-86.5 mg L^{-1}, NOEC-30.6 mg L^{-1}, inhibited hatching, growth retardation, tail deformation, pericardial oedema and delayed heart beat (heart abnormality)	Van den Brandhof and Montforts, 2010

Table 1.9: Acute and chronic toxicity effects of carbamazepine on aquatic organisms (continued)

Class/group of organism	Species/common name of organism	End point/Responses	References
Pisces/Fish	***D. rerio* (brain)**	Inhibition of ATP (adenosine triphosphate) hydrolysis, inhibition of acetylcholinesterase activity at higher concentration	Siebel et al., 2010
	D. rerio	Increased DNA damage and formation of apoptotic cells leading to genotoxicity	Rocco et a., 2011
	***Lepomis gibbosus* (pumpkinseed sunfish)**	Increased glutathione reductase and glutathione S-transferase activities in the digestive tract/gland, increased time spent on locomotion with increasing conc and decreased time spent in the dark area with increasing conc [CBZ caused reduction in anxiety as fish preferred staying in the light area without seeking for refuge in the dark area]	Brandão et al., 2013
	***Oryzias latipes* (embryo late blastular stage)**	LC_{50}-13.1 ng L^{-1}, decreased survival of embryo, increased number of embryo with shrunk yolk-sac and haemorrhaged embryo, delayed embryonic development, reduced embryo hatching ability, increased hatching time and number of embryo that failed to swim upward and reduced larval upward swimming ability	Nassef et al., 2010a
	***O. latipes* (adult)**	Altered feeding behaviour by increasing time to eat midge larvae, reduced feeding rate and swimming speed	Nassef et al., 2010b
	***O. latipes*(larva)**	96 hrs LC_{50}-45.87 mg L^{-1}	Kim et al., 2009
	***Cyprinus carpio* (common carp)**	Decreased sperm motility and velocity, induced oxidative stress	Li et al., 2010
	***Macquaria ambigua* (golden perch)**	7 days, larval survival EC_{10}-1.1 µg L^{-1}	Kumar et al., 2016

Table 1.10: Acute and chronic effects of fluoxetine on aquatic organisms

Class/group of organism	Species/common name of organism	Responses	References
Crustacea	***Gammarus pulex***	Increased ventilation/reduced activity or locomotion at lower concentrations (10-100 ng L^{-1}) and reduced or increased activity at higher concentrations (10-100 µg L^{-1}) but no effects at 0.1, 1 and 1000 ng L^{-1}	De Lange et al., 2009
	G. pulex	Decreased activity or increased ventilation at lower concentrations (10 to100 ng L^{-1}) but no effect on activity at higher concentrations (1µg L^{-1}, 1 mg L^{-1})	De Lange et al., 2006
	Hyalella azteca	No effect on reproduction and number of newborn per female but reduced growth rate with LOEC and NOEC of 100 and 33 µg L-1 after 28 days exposure	Pery et al., 2008
	Daphnia magna	48 hrs EC_{50} immobility-6.4 mg L^{-1}	Christensen et al., 2007
	D. magna	48 hrs EC_{50} immobility-820 µg L^{-1}, reduced number of neonates with NOEC 56 µg L^{-1}	Brooks et al., 2003
	D. magna	Immobilization, reduction in reproduction but increased feeding rate after 21 days exposure with no enantiospecific differences	Stanley et al., 2007
	D. magna	Mortality, reduced reproduction and growth of 3rd brood newborn of parents and exposed offspring of exposed parents after 21 days exposure, NOEC and LOEC for reduction in growth were 8.9 and 31 µg L^{-1}	Pery et al., 2008
	D. magna	48 hrs immobilization test EC_{50}-7.6 mg L^{-1}, 21 days reproduction inhibition test EC_{50}-0.24 mg L^{-1}	Varano et al., 2017
	D. magna	6 days exposure to 36 µg L^{-1} had no effect on survival, reproduction morphology of adults and neonates, sex ratio but 30 days exposure caused increased number of neonates produced	Flaherty and Dodson, 2005
	D. magna	Increased positive phototactic response in adults exposed all through life, and adults and juveniles exposed for 48 hrs, behavioural response was non-monotonic	Rivetti et al., 2016
	Cerodaphnia dubia	48 hrs LC_{50}-510 µg L^{-1}, reduced number of brood per female, reduced number of neonate per female with NOEC-89µg L^{-1} and LOEC-447 µg L^{-1} during 7-8 days	Henry et al., 2004
	C. dubia	48 hrs LC_{50}-234 µg L^{-1}, reduced reproduction during 7 days exposure with NOEC-56 µg L^{-1}	Brooks et al., 2003

Table 1.10: Acute and chronic effects of fluoxetine on aquatic organisms (continued)

Class/group of organism	Species/common name of organism	Responses	References
Insecta	***Chironomus riparius*** **(Midge)**	7 days exposure caused larval mortality, reduction in growth and inhibition of emergence with LOEC-666 mg Kg^{-1} and NOEC-59.5 mg Kg^{-1}	Pery et al., 2008
	C. riparius	No effects on number of eggs per clutch, emergence and sex ratio after 28 days exposure to concentration ranges 0.15 to 5.86 mg Kg^{-1}	Nentwig, 2007
	C. riparius	Increased female/male sex ratio of F0 population and reduced adult emergence of F1 population	Sánchez-Argüello et al., 2009
	C. tepperi **(Embryo)**	7 days larval survival EC_{10}-59 µg L^{-1}	Kumar et al., 2016
Annelida	***Lumbriculus variegatus***	Increased reproduction at concentrations between 0.94 and 2.34 mg Kg^{-1} after 28 days exposure	Nentwig, 2007
Mollusca	***Physa acuta***	Mortality of 24 hrs old eggs and inhibition of hatching in 96 hrs old eggs at 0.5 mg L^{-1} while lower concentrations reduced hatching rate of exposed eggs, induced cytogenetic effects and altered embryonic development	Sánchez-Argüello et al., 2012
	P. acuta **(adult)**	Increased mortality of snails and reduced reproduction at higher concentrations but increased reproduction at lower concentration	Sánchez-Argüella et al., 2009
	Potamopyrgus antipodarum **(Mud snail)**	Reduced reproduction with EC_{10}-0.81 µg L^{-1} and NOEC-0.47 µg L^{-1} after 56 days exposure	Nentwing, 2007
	P. antipodarum	6 weeks exposure reduced reproduction with NOEC-13 µg L^{-1} and LOEC of 69 µg L^{-1}, increased energetic cost due to reproduction but no effect on growth	Pery et al., 2008
	P. antipodarum	Decreased embryo and egg production at higher concentrations while lower concentrations increased embryo production, increased growth but reduced reproduction in F1 than exposed parents	Gust et al., 2009
	Dreissena polymorpha **(zebra mussel)**	Low concentration 20 ng L^{-1} caused reduction in number of oocyte per follicle and spermatozoa per seminiferous tubule	Lazzara et al., 2012
	Elliptio complanata **(freshwater mussel)**	Induced release of non-viable glochidia larvae in females and spermatozeogmata in males, increased foot volume due increased uptake and storage of water	Bringolf et al., 2010
	Lampsilis fasciola, L. cardium **(freshwater mussel)**	Induced female sexual behaviour in form of mantle lure display behaviour and release of glochida larvae (viable), increased foot volume due to relaxation of foot muscle which aided uptake and storage of water	Bringolf et al., 2010

Table 1.10: Acute and chronic effects of fluoxetine on aquatic organisms (continued)

Class/group of organism	Species/common name of organism	Responses	References
Pisces/Fish	***Carassius auratus*** **(Goldfish, female)**	Increased serotonin and dopamine levels in the hypothalamus, increased norepinephrine levels in the telecephalon, reduced estrogen level in blood plasma, and expressions of estrogen receptors (ERα, ERß1) mRNA and isotocin mRNA levels in the brain	Mennigen et al., 2008
	Oryzias latipes **(Japanese medaka)**	Induced developmental abnormalities, increased level of circulating plasma estradiol in females	Foran et al., 2004
	O. latipes **(newly hatched)**	72 hrs LC_{50}-840 µg L^{-1}, reduced velocity and distance covered with NOEC-10 µg L^{-1} and LOEC-100 µg L^{-1}, altered locomotor behaviour as exposed larvae spent more time in the peripheral zone unlike the control	Chiffre et al., 2014
	O. latipes **(Larvae)**	96 hrs LC_{50}-5.5, 1.3 and 0.2 mg L^{-1} at pH 7, 8 and 9 respectively, accumulation of Fluoxetin in juvenile fish body and liver with bioaccumulation level increasing with increasing Fluoxetine concentration	Nakamura et al., 2008
	O. latipes	Induced developmental abnormalities, increased level of circulating plasma estradiol in females	Foran et al., 2004
	Pimephales promelas **(fathead minnow)**	48 hrs LC_{50}-705 µg L^{-1}	Brooks et al., 2003
	P. promelas **(juvenile)**	48 hrs exposure caused acute toxicity while 7 days exposure caused reduction in growth, survival and feeding rate, No enantiospecific effects during acute toxicity but enantiospecific effects occurred during survival, feeding rate and growth tests	Stanley et al., 2007
	Macquaria ambigua **(Golden perch/embryo)**	7 days exposure larval survival EC_{10}-260 µg L^{-1}	Kumar et al., 2016
	Carassius auratus **(Goldfish, female)**	Increased serotonin and dopamine levels in the hypothalamus, increased norepinephrine levels in the telecephalon, reduced estrogen level in blood plasma, and expressions of estrogen receptors (ERα, ERß1) mRNA and isotocin mRNA levels in the brain	Mennigen et al., 2008
Amphibia	***Rana pipiens*** **(larvae)**	Delayed development of larvae, reduced food intake leading to reduced weight gain	Foster et al., 2010
	Xenopus laevis **(African cawed frog/ embryo)**	Malformation during 4 days exposure with EC_{10}-3000 µg L^{-1}	Richards and Cole, 2006

Also, carbamazepine and fluoxetine have been shown to accumulate in the tissues of exposed organisms (Brooks et al., 2005, Ramirez et al., 2007, Bringolf et al., 2010, Schultz et al., 2010, Vernouillet et al., 2010). Carbamazepine was shown to bioaccumulate across trophic levels in a green alga *Pseudokirchneriella subcapitata*, a crustacean *Thamnocephalus platyrus* and a cnidarian *Hydra attenuate* exposed through food (Vernouillet et al., 2010). Almeida et al. (2014) also reported accumulation of carbamazepine in the tissues of bivalves *Venerupis decussate* and *V. Philippinarum* up to 0.011 µg g^{-1} and 0.01 µg g^{-1} respectively while Ramirez et al. (2007) reported up to 1.16 ng g^{-1} in muscle tissues of fish *Lepomis* species from a contaminated aquatic environment. Similarly, fluoxetine concentrations up to 0.11, 1.34 and 1.58 ng g^{-1} have been found in muscle, liver and brain tissues respectively of fish samples (*Lepomis macrochirus*-bluegill, *Ictalurus punctatus*-channel fish, *Pomoxis nigromaculatus*-black crappie) (Brooks et al., 2005), up to 0.6 ng g^{-1} in the brain of fish *Catostomus commersoni* (White sucker) (Schultz et al., 2010) and 79.1 ng g^{-1} in tissue of mussel *Elliptio complanata* from contaminated freshwater (Bringolf et al., 2010). Thus, there is potential for their bioaccumulation in exposed aquatic organisms.

These findings indicate the need to investigate the effects of these psychiatric pharmaceutical substances on more aquatic species at risk of exposure in their natural habitat for the development of more adequate water quality guidelines. Freshwater planarians as stated earlier might be ideal organisms to assess effects of these psychiatric pharmaceuticals. Importantly, some of these drugs targets such as serotonergic, dopaminergic, GABAergic, cholinergic, adrenergic, melatonergic, norepinephrinergic neurotransmission systems, voltage gated ion channels have been identified and quantified in freshwater planarians *Schmidtea mediteranea, Dugesia dorotocephala, D. gonocephala, D. tigrina, D japonica, Polycelis tenuis, Dendrocoelum lacteum* (Welsh and Moorhead, 1960, Morita and Best, 1966, 1993, Welsh and King, 1970, Welsh and Williams, 1970, Sarnat and Netsky, 1985, Eriksson and Panula, 1994, Reuters et al., 1995, 1996, Nogi and Levin, 2005, Umeda et al., 2005, Rawls et al., 2006, 2007, Cebria, 2008, Nishimura et al., 2007a, 2007b, 2008). Moreover, planarian nervous system has been shown to bear more resemblance to that of vertebrates than to invertebrates (Sarnat and Netsky, 1985, Buttarelli et al., 2002) and their neurotransmitters functionally and structurally is like those of humans (Buttarelli et al., 2008, Cebria, 2007, Nishimura et al., 2010).

Additionally, most studies on ecotoxicological effects of these psychiatric substances previously mentioned are based on single contaminant effects. Notably, in the natural aquatic

environment pharmaceuticals do not occur as single contaminant but with their metabolites, conjugates, other pharmaceuticals and chemical contaminants, and concomitantly with biotic and abiotic stressors, with which they may sometimes interact. Hence, it is possible that single exposure bioassays alone may not actually reveal the impact of these pharmaceuticals on aquatic organisms. Single exposure bioassays are helpful in revealing the specific effects of a given compound, but mixture exposures are relevant as they give additional information on the complexity of possible impacts of these contaminants on aquatic organisms in the natural environment.

1.5 Research objectives and thesis outline

The main objective of this work was to determine if freshwater planarians could be used as model species in the ecotoxicological assessment of psychiatric pharmaceutical substances in the freshwater environment. Initial ecotoxicological evaluation involved assessment of freshwater planarian responses to environmental relevant concentrations of the selected psychiatric substances. Exposure to concentrations usually detected in freshwater environment is necessary for better evaluation of responses of these organisms to effects of psychiatric substances in their natural habitat and determination of sensitive endpoints. In line with the thesis objectives, the freshwater planarian *Schmidtea mediterranea* responses were evaluated at the molecular, cellular, organismal and population levels of biological organization. This way *S. mediterranea*'s response to psychiatric substances exposures at different levels of biological organization may be correlated and sensitive endpoints determined.

In addition, assessment of the mediating effects of other stressors on drug toxicity necessary for better understanding of possible interactions between psychiatric substances and other environmental stressors and their consequences on aquatic organisms is vital. Consequently, this section of the study focused on the concomitant exposure of *S. mediterranea* to fluoxetine and carbamazepine (both of which are psychiatric pharmaceutical substances), fluoxetine and tributyltin (TBT) (a neurotoxic agent) or salinity as a model natural abiotic stressor.

The results are expected to contribute to ecotoxicological data necessary for the ecotoxicological risk assessment of these pharmaceutical substances in freshwaters.

Generally, this study addressed these questions:

a) Is *S. mediterranea* a suitable species to evaluate effects of low concentrations of environmental contaminants?

b) Are environmental concentrations of psychiatric pharmaceuticals toxic to *S. mediterranea*?

c) Are psychiatric drugs` effects on *S. mediterranea* altered by the presence of other psychiatric drugs or neurotoxic compounds?

d) Are psychiatric drugs` effects on *S. mediterranea* altered in the presence of natural stressors?

Initial tests addressed the sensitivity of *S. mediterranea* to low concentrations of a model environmental contaminant (tributyltin TBT). Mortality, behavioural, regeneration and DNA damage responses were evaluated to gauge their sensisitivity and reliability as ecotoxicological endpoints that can be used in routine bioassays (Chapter 2).

Responses of *S. mediterranea* to psychiatric pharmaceuticals fluoxetine and carbamezepine were addressed in Chapter 3. Results are discussed in terms of the toxicity of each of the compounds and the adequacy of freshwaters planarian responses for environmental risk assessment of these pharmaceuticals.

After single exposure assessment, the effects of fluoxetine under concomitant exposure to another psychiatric pharmaceutical (carbamazepine) or the neurotoxic contaminant TBT (Chapter 4) or a gradient of salinity (Chapter 5) were also assessed using genotoxicity, behaviour and reproduction as endpoints.

In the last chapter (Chapter 6), the major findings are discussed concerning the putative role of freshwater planarians as an ecotoxicological test organism as well as the ecological effects of psychiatric pharmaceutical in freshwaters.

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Chapter 2

Toxicity of tributyltin (TBT) to freshwater planarian *Schmidtea mediterranea*

Chapter 2.0

Toxicity of tributyltin (TBT) to freshwater planarian *Schmidtea mediterranea*

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Abstract

The freshwater planarian *Schmidtea mediterranea*, one of the best characterized animal models for regeneration research and developmental biology, is being recognised as a useful species for ecotoxicological studies. Sensitive endpoints related to planarians' behaviour and regeneration can be easily evaluated after exposure to environmental stressors. In this work the sensitivity of *S. mediterranea* to a gradient of environmentally relevant concentrations of TBT was studied using multiple endpoints like survival, locomotion, head regeneration and DNA damage. In addition, a feeding assay based on planarian's predatory behaviour was performed. Results indicated that TBT is toxic to planarians with an LC_{50} of 1.87 µg L^{-1} Sn and 1.31 µg L^{-1} Sn at 48 h and 96 h of exposure respectively. Sub-lethal exposures to TBT significantly reduced locomotion and feeding, delayed head regeneration and caused DNA damage in exposed planarians. The behavioural endpoints (feeding and locomotion) were the most sensitive parameters followed by head regeneration and DNA damage. Similar to other aquatic model organisms, *S. mediterranea* showed high sensitivity towards TBT exposure. Based on our results, and though further research is required concerning their sensitivity to other pollutants, the use of freshwater planarians as a model species in ecotoxicology is discussed.

Keywords: Tributyltin toxicity, Behavioural endpoints, Regeneration, Genotoxicity, *Schmidtea mediterranea*

2.1. Introduction

Tributyltin (TBT) is an organometallic compound, mainly used as biocide in antifouling paints applied to boat and ship hulls and submerged static structures to discourage attachment and growth of organisms (Turner, 2010). Also, TBT is used as molluscicide to control snail vectors of schistosomiasis, for wood preservation, slime control in paper mills and industrial disinfectant (Snoeij et al., 1987). Many countries have regulated the use of TBT (Abbott et al., 2000, IMO, 2001), but given its persistence in sediments (Dowson et al., 1996) and ability to diffuse into water column (Unger et al., 1988), sediments can act as long-term sources. Moreover, ineffectiveness of alternative products led to illegal use of TBT and is still permitted in International Maritime Organization (IMO) non-member countries (Barroso and Moreira, 2002, Okoro et al., 2011). Thus, environmental concentrations of TBT remain high enough to motivate concern.

The majority of studies on toxicity of TBT have focused on marine species and imposex in marine and freshwater molluscs leading to reproductive failure and population decline has been shown as one of the most deleterious effects of TBT exposure (Oehlmann et al., 1996, Barroso et al., 2000). However, TBT concentrations in freshwaters and freshwater sediments have been estimated to reach 7.1 µg l^{-1} and 3700 µg kg^{-1}, respectively (IPCS, 1990), indicating its potential impact on freshwater biota and the need for more ecotoxicological studies.

In non-target organisms, TBT toxicity has been studied using reproduction, mortality and growth as endpoints. Few reports deal with behavioural and regeneration endpoints. Behavioural responses are usually sensitive and suitable parameters to evaluate effects of low levels of contaminants (Pestana et al., 2007, Alonso and Camargo, 2011, Rodrigues et al., 2015). Similarly, regeneration occurs in many metazoans, and involves re-creation of lost parts (Sánchez-Alvarado, 2000). Since many contaminants are known to have cytotoxic and teratogenic effects (Mizuhashi et al., 2000, Hagger et al., 2002, Velma et al., 2009), they may alter the process of regeneration. Therefore, regeneration can also be useful in studying effects of toxicants on differentiation and growth (Weis and Weis, 1987).

Freshwater planarians are benthic invertebrates that occupy an important position in the food chain as abundant predators (Thorp and Covich, 2001). Planarians undergo blastemal regeneration, a process that could be used to understand regeneration in other studied model systems and metazoans, given the important position occupied by planarians in Metazoan evolution (Sánchez-Alvarado and Newmark, 1998).

Freshwater planarians have been suggested as useful indicators for water quality and pollution (Kent, 1974) and are sensitive to low concentrations of environmental toxins (Nano et al., 2002, Rodrigues et al., 2015). Although freshwater planarians are not model organisms in ecotoxicology, they have been used in various studies and simple protocols exist to measure locomotor behaviour and regeneration (Knakievicz, 2014). Additionally, feeding bioassays based on planarians' predatory behaviour may be useful in understanding the sub-lethal effects of contaminants (Rodrigues et al., 2015).

The aim of this study was to determine the effects of TBT on locomotion, regeneration and DNA (comet assay) using the freshwater planarian *S. mediterranea* as model organism. In addition, we tried to devise a convenient quantitative endpoint of planarian feeding bioassay that can be used to complement the ecotoxicological evaluation of a wide range of environmental stressors with this planarian species.

2.2. Materials and methods

Tributyltin (TBT)

Tributyltin chloride (TBTCl; 97%) from Fluka, Switzerland was used to prepare a stock solution of 0.1 M dissolved in absolute ethanol. Experimental treatments and dilutions from this stock solution were prepared using ASTM hardwater (ASTM, 2004) keeping concentration of ethanol below 0.01%. Experimental solutions and water samples were kept from light at 4 ºC to avoid degradation.

Organotin chemical analysis

The stock solution (100 µg L^{-1} TBTCl), and experimental treatments were analysed at the end of the exposure (after 48 h) to evaluate degradation of TBT. 10 mL of sample from each concentration was acidified with 5 mL of acetic acid (ultrapure grade) and then subjected to extraction using a microwave system (CEM Explorer, 3 min, 40 W) with tripropyltin as standard and a procedure blank. Extracts were kept at 4 ^{o}C before further analysis. Before derivatisation, extracts from each sample were pooled and appropriate volume of a standard solution containing TBT, DBT and MBT (1000 mg L^{-1} in methanol; LGC standards) added to help determine butyltin species. 5 mL of 1 M acetic acid/sodium acetate (ultrapure grade) was added to 1 mL of supernatant (extract + standard solution) and the pH was adjusted to 4.5 with ammonium

hydroxide (ultrapure grade). To this solution was added 1 mL of isooctane (ultrapure grade) and 1 mL of freshly prepared 1% aqueous sodium tetraethyl borate (99.8%). The mixture was shaken at 300 rpm for 20 min for phase separation, the organic layer was transferred into amber Gas Chromatography (GC) auto-sampler vials. These were stored at -20 ^{o}C until analysis with gas chromatograph-inductively coupled mass spectrometry (GC-ICPMS).

Estimation of TBT degradation efficiency

TBT degradation efficiency was obtained using the butyltin degradation index (BDI) according to Díez et al. (2002) and butyltin degradation index percentage (BT_{deg}) according to Díez and Bayona (2009) with slight modification. BDI is the ratio of TBT main degradation products [monobutyltin (MBT), dibutyltin (DBT)] and TBT

$$\mathrm{BDI} = \frac{\mathrm{MBT} + \mathrm{DBT}}{\mathrm{TBT}}$$

Similarly,

$$\mathrm{BT_{deg}} = \left(1 - \left[\frac{\mathrm{TBT}}{\mathrm{TBT} + \mathrm{DBT} + \mathrm{MBT}}\right]\right) \times 100$$

where MBT, DBT and TBT refer to the concentrations of the butyltins expressed as tin (Sn). BDI value less than 1 and BT_{deg} less than 50% signify poor or moderate TBT degradation while BDI greater than 1 and BT_{deg} higher than 50% imply efficient of high TBT degradation (Diez et al., 2002, Diez and Bayona, 2009).

Test organisms

S. mediterranea (asexual strain) cultures were maintained in plastic containers with ASTM hardwater (ASTM, 2004), under constant darkness and temperature of 20 ± 1 °C. Planarians were fed chicken liver once a week, with medium renewal after feeding and every 2 days.

Planarians used in experiments ranged from 0.5 cm to 1.0 cm in length. Before experiments, animals were starved for 1 week to prevent contamination due to food digestion (Wu and Persinger, 2011) and ensure uniformity in response to toxicant (Oviedo *et al*., 2008a). All exposures were performed in darkness at 20 ± 1 °C.

Acute toxicity of TBT

Based on range finding experiments, 7 TBT concentrations plus control (CTR) and solvent control treatments (SCTR, absolute ethanol) were chosen for the acute test. 10 replicates (1 planarian per 40 mL glass crystalizing dish) per concentration were used containing 20 mL of medium. Solutions were renewed after 48 h. Mortality was checked every 24 h up to 96 h and number of dead organisms recorded. Animals with degenerating body or without detectable movement under strong light were considered dead.

TBT effects on behaviour

Planarians were exposed to sub-lethal TBT nominal concentrations of 0.25, 1.0 and 4.0 µg L^{-1}, control and solvent control treatments for 48 h. Exposure was carried out in 40 mL glass vials with 20 mL of medium. 10 replicates (1 planarian per vial) per concentration were used. Solutions were not renewed during exposure. The same experimental procedure was applied to both behavioural parameters (locomotion and feeding).

Locomotion

After exposure, locomotor activity was measured according to Raffa et al. (2001) and Pagan et al. (2009) with slight modifications. Briefly, after exposure each planarian was transferred into a transparent dish of 21 cm by 17.5 cm dimension containing 50 mL of experimental medium, placed on a graph sheet with 0.5 cm grid spacing. Locomotor activity was calculated as number of grid lines crossed by each planarian over 3 mins observation in a well lit room between 11.30 am and 3.30 pm. Each planarian was used once and results were reported as the mean number of grid lines crossed in 3 min per concentration.

Feeding inhibition

After exposure, planarians were transferred to clean glass vials containing 20 mL of ASTM medium (ASTM, 2004) and 15 chironomid larvae. Since this is the first attempt to evaluate feeding inhibition in *S. mediterranea* using chironomid larvae, we carried out some trial tests to determine suitable larval stage/size. Based on our trials, six-day old (second instar) larvae were used. Number of larvae consumed per organism after 24 h was recorded.

Head regeneration

Planarians were decapitated above the pharynx. Each headless animal was immediately exposed to sub-lethal nominal concentrations of TBT (0.25, 1.0 and 4.0 µg L^{-1}), to the control and solvent control treatments. Exposure was carried out in 40 mL glass crystallizing dishes containing 20 mL of medium. Animals were exposed to TBT through the period of head regeneration (8 days) and medium was changed every 48 h. 10 replicates (1 planarian per vial) per concentration were used. Each replicate was examined daily under Zeiss stereo microscope (KL 300 LED) to follow the regeneration process. The time until formation of photoreceptors was recorded as well as abnormalities. Results were reported as mean time in days necessary for regeneration of photoreceptors.

Comet assay

Comet assay was performed to detect DNA strand breaks on planarian cells. After exposure (3 replicates of 5 planarians per concentration) to sub-lethal nominal concentrations of TBT (0.25, 1.0 and 4.0 µg L^{-1}), control and solvent control treatments in 100 mL glass crystalizing dishes with 50 mL of medium for 48 h, animals were used to evaluate DNA damage. Solutions were not renewed during exposure. The comet assay was performed with slight modifications as described by Nogueira et al. (2006) and Prá et al. (2005) under yellow light, to prevent UV-induced DNA damage. 5 planarians were pooled and disintegrated chemically and mechanically by puncturing with a pestle in 0.48% (w/v) of trypsin (Sigma). 10 µL of the cell suspension were mixed in 0.5% low melting point agarose at a ratio of 1:10 (v/v) were placed on 1% (w/v) normal melting point agarose pre-coated microscope slides. The embedded cells were immersed into a precooled lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM tris base and 1% triton X-100, 10% DMSO, pH 10) at 4 ºC for 120 min in the dark. The slides were then filled with freshly prepared cold alkaline buffer (0.3 M NaOH and 1 mM EDTA, pH 13). Electrophoresis was performed at 43 V, 300 mA for 10 min on alkaline buffer. Then, slides were neutralized with ice 0.4 M Tris-HCl (pH 7.5). Visual scoring of cellular DNA was based on the categorization into 5 classes, depending on DNA damage, of 100 cells/condition randomly selected using fluorescence microscope (Olympus BX41TF, China), as described by Garcia et al. (2004).

Statistical analysis

The effects of TBT exposure on *S. meditteranea* sub-lethal endpoints were assessed by one-way analysis of variance (ANOVA) followed by multiple comparison Dunnett's post hoc test. The latter test allowed to test for significant differences between TBT treatments and the solvent control treatment (SCTR). These calculations were performed with GraphPad Prism version 5.0 for Windows (GraphPad Software, La Jolla California USA) with results reported as mean ± S.D (standard deviation). The LC_{50}'s at 48 h and 96 h were obtained by probit analysis at 95% confidence interval (CI) using IBM SPSS statistics (version 21).

2.3. Results

Chemical analysis

The measured TBT concentration of the stock solution was lower than nominal concentrations. Consequently, TBT nominal concentrations used for the acute tests were adjusted using the measured concentration of TBT as tin (Sn) in the stock solution.

Similarly, results from the analysis of the experimental water after exposure showed poor degradation of TBT with the BDI values less than 1 and BT_{deg} values less than 50% for all samples as shown in table 2.1. TBT degradation among the experimental concentration increased with decreasing concentration since BDI and BT_{deg} values increased with decreasing TBT concentrations.

Table 2.1: Butyltin concentrations and degradation indexes for experimental water samples

Samples	**TBT nominal concentrations (ng L^{-1})**	**MBT (Sn ng L^{-1})**	**DBT (Sn ng L^{-1})**	**TBT (Sn ng L^{-1})**	**BDI**	**BT_{deg} (%)**
Stock solution	100000	72.5 ± 5	415 ± 20	24080 ± 470	0.0202	1.98
C1	250	3.9 ± 0.2	2.6 ± 0.3	8 ± 1	0.8125	44.83
C2	1000	5.3	2.8 ± 0.2	16 ± 1	0.5063	33.61
C3	4000	15.8 ± 0.2	11.9 ± 0.2	103 ± 15	0.2689	21.19

Concentration ± standard deviation of butyltin metabolites [monobutyltin (MBT), dibutyltin (DBT)] and TBT as Sn (ng L^{-1}) in the sub-lethal experimental water at the end of 48 hours exposure period and stock solution, and butyltin degradation index (BDI) and degradation percentage (BT_{deg}) for each sample.

Acute toxicity

The 48-h and 96-h LC_{50} (95% CI) were 1.87 Sn µg L^{-1} (1.36 – 2.58 Sn µg L^{-1}) and 1.31 Sn µg L^{-1} (0.95 – 1.8 Sn µg L^{-1}) respectively. In these acute experiments, planarians experienced head reduction and head loss followed by body degeneration before death.

Behavioural parameters

Exposure to TBT significantly reduced locomotor activity of planarians when compared with the solvent control treatment ($F_{(3, 10)} = 100.2$, $p < 0.05$, fig. 2.1). Reduction was dose dependent and the LOEC was 60.2 Sn ng L^{-1}. Locomotor activity reductions of 16.96, 25.61 and 85.81% were recorded at 8, 16 and 103 Sn ng L^{-1} respectively. Moreover, 10% of planarians exposed to 103 Sn ng L^{-1} were completely motionless while the rest showed no movement after crossing 2 to 7 grid lines.

Feeding was also significantly reduced in comparison with the solvent control treatment ($F_{(3, 10)} = 39.0$, $p < 0.05$, fig. 2.2) with a LOEC of 16 Sn ng L^{-1}. The reduction in feeding was dose-dependent with 33.33 and 84.33% reductions in feeding rates observed at 16 and 103 Sn ng L^{-1} respectively.

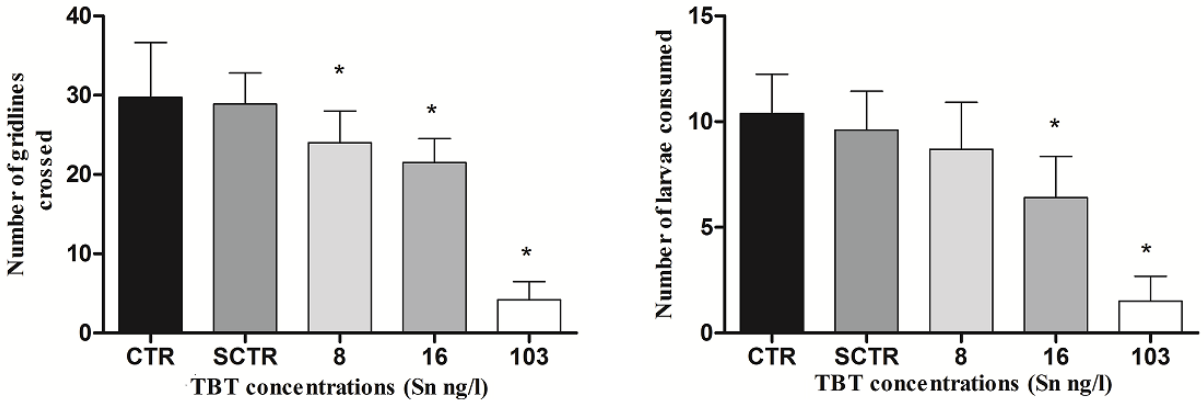

Fig. 2.1 and 2.2: Effects of TBT on planarian behaviour: 2.1- *S. meditteranea* locomotor activity, as number of gridlines crossed over 3 mins after short-term exposure (48 h) to sub-lethal TBT concentrations. 2.2- Feeding activity of *S. meditteranea*, as number of chironomid larvae ingested over 24 h, after short-term exposure (48 h) to sub-lethal TBT concentrations. Data presented as mean ± SD, n = 10. * Denotes a significant difference compared to the solvent control treatment, SCTR (Dunnett's test, $p < 0.05$).

Head regeneration

Head regeneration measured as days until photoreceptors formation was significantly delayed in planarians exposed to 8 and 16 Sn ng L^{-1} when compared to regeneration in the solvent control treatment ($F_{(3, 10)} = 944.4$, $p < 0.05$, fig. 2.3). However, while about 20% of planarians in the solvent control (SCTR) started forming photoreceptors by the fourth day, planarians exposed to 103 Sn ng l^{-1} remained acephalic throughout exposure. At the end of the exposure period planarians in the highest TBT treatment (103 Sn ng L^{-1}), failed to show the usual avoidance behavior to light stimulus, 30 % showed screw-like positions, 50% showed C-like position while 20 % of organisms were lying dorsally.

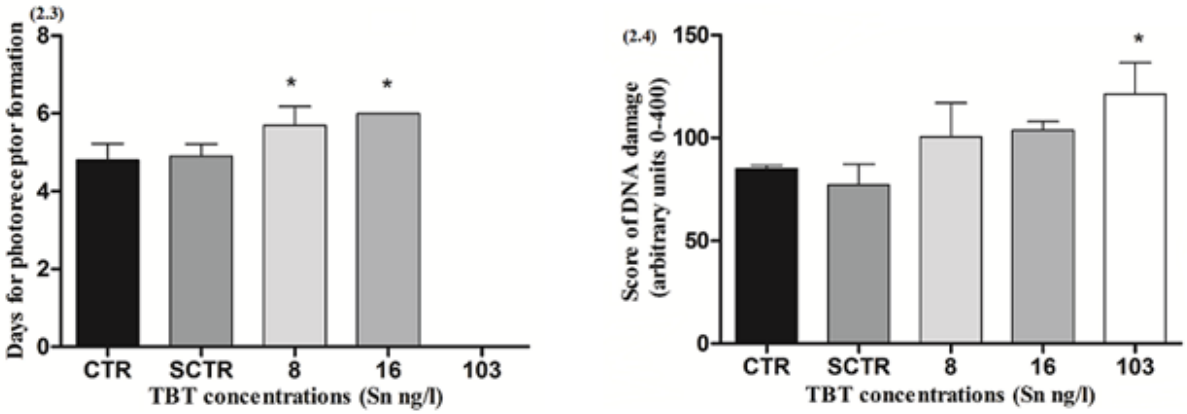

Fig. 2.3 and 2.4: Effects of TBT on planarian head regeneration and DNA well-being: 2.3- Effects of sub-lethal TBT concentrations on regeneration of *S. meditteranea*, measured as days until photoreceptor formation. Data presented as mean ± SD. n = 10. *Denotes a significant difference compared to the solvent control treatment, (SCTR) (Dunnett's test, $p < 0.05$). 2.4- *S. meditteranea* DNA damage, scored as arbitrary units ranging from 0-400, after short-term exposure (48 h) to sub-lethal TBT concentrations. Data presented as mean ± SD, n= 3. *Denotes significant differences compared to the solvent control treatment, SCTR (Dunnett's test, $p < 0.05$).

Comet assay

The results from the visual scoring used to evaluate DNA damage showed that TBT caused DNA damage. A significant DNA damage compared to solvent control was observed in planarians exposed to 103 Sn ng L^{-1} only ($F_{(3, 15)} = 6.28$, $p < 0.05$, fig. 2.4). More cells in the highest comet classes (classes 3 – 11% and 4 -13%) were seen in planarians exposed to 103 Sn

ng L^{-1} than in the solvent control treatment (classes 3 – 7% and 4 – 8%) indicating extensive DNA damage induced by TBT.

2.4. Discussion

S. mediterranea's potential as a test model for ecotoxicity testing, was under investigation in this study. Both lethal and sub-lethal effects of TBT (genotoxicity, behaviour and regeneration) were studied. TBT exposure resulted in a reduction of locomotion and feeding, a delay and/or suppression of regeneration and in an increase in DNA damage. Among the endpoints tested, behavioural responses showed to be highly sensitive.

Results from our study showed that TBT is not highly persistent in the water and TBT concentrations after exposure were much lower than nominal concentrations. Results showed significant degradation to its metabolites MBT and DBT as previously observed in other studies (Snoeji et al., 1987, Díez et al., 2002). Though not considered in this study, reductions in TBT concentrations could also be related to TBT accumulation by planarians, since it was shown that TBT accumulates in aquatic organisms (Ohji et al., 2002).

The 48 and 96 hours LC_{50}'s were lower than the reported concentration of 7.1 µg L^{-1} TBT in freshwaters (IPCS, 1990) indicating that environmentally relevant concentrations of TBT pose a threat to freshwater planarian populations. *S. meditteranea* showed to be as sensitive if not more when compared with other aquatic organisms. Previous studies by Thain (1983) reported 48 h LC_{50} of 1.6 and 2.3 µg L^{-1} TBTO (tributyltin oxide) for larvae of *Crassostrea gigas* and *Mytilus edulis* respectively and 96 h LC_{50} of 2.1 and 1.5 µg L^{-1} TBTO for larvae of *Solea solea* and *Crangon crangon* respectively. Toxicity to other aquatic invertebrates such as *Scrobicularia plana* with LC_{50} less than 1.3 µg L^{-1} Sn (Ruiz et al., 1994), *Daphnia magna* with 60% mortality at 2.5 µg L^{-1} after 21 d exposure (Oberdörster et al., 1998) and *Acetes intermedius* with LC_{50} of about 18.6 µg L^{-1} after 72 h exposure (Tang et al., 2009) have been reported.

TBT exposure caused a dose dependent reduction in planarian behavioural responses. Locomotor activity and feeding were significantly affected after 48 h. Similar behavioural effects, i.e., decreased swimming speed were reported for daphnids exposed to 7.1 µg L^{-1} of TBT for 21 d (Schmidt et al., 2006) or reduced tail movement or motionlessness for minnows larvae exposed to 4.45 and 9.96 µg L^{-1} TBT (Fent and Meier, 1992). In our study, reduction in locomotor activity was observed in response to lower TBT concentrations (8 ng L^{-1}). Previous studies showed that TBT affects behaviour of organisms by causing deterioration of body surface

epithelial, severe muscular alterations (Fent, 1992, Fent and Meier, 1992) and neurotoxicity (Yu et al., 2013). Similar effects are plausible in this study and reduced locomotor activity and motionlessness observed in planarians may be due to alteration to external body surfaces and neurotoxicity related to TBT exposure. This is because planarians move by gliding, which involves ventral ciliary activity, and crawling, which is purely muscular activity (Stringer, 1917, Talbot and Schötz, 2011). In fact, planarian locomotion has been used to test presence of neurotoxic compounds (Knakievicz and Ferreira, 2008), and studies with freshwater planarians have shown impairments in their gliding activity associated to damaged external body layers (epithelial and muscular) due to exposure to aluminium (Kovačević et al., 2009), and ammonium (Alonso and Camargo, 2011).

Reduction in planarian feeding activity due to TBT could be also associated to locomotor inhibition. Freshwater planarians are predators and according to Yu et al. (2013) predatory behaviour involves the nervous system and ability to sense, locate and capture prey. Planarians are known to be positively chemotaxis with well-developed sensory system which helps them detect presence of prey or food (Inoue et al., 2015). Failure to perceive, move, trap or capture prey will thus result in reduced predation and food intake.

These effects in planarian behaviour related to TBT exposure could have ecological implications. Reduction in locomotion could make them prone to attack by predators. Also, planarians with reduced locomotor ability could have difficulty feeding and moving away quickly from unfavourable conditions. Feeding inhibition on the other hand will affect planarian survival, growth and reproduction since freshwater planarians grow and reproduce when well fed and de-grow when starved (Oviedo et al. 2008b).

We also showed that head regeneration in *S. mediterranea* was delayed and in some cases suppressed by TBT exposure. These results were in accordance of previous studies where TBT exposure has been shown to cause retardation in limb regeneration of fiddler crabs (Weis et al., 1987) and inhibition of limb regeneration in *Ophioderma brevispina* (Walsh et al., 1986) and *Limulus polyphemus* (Itow et al., 1998). TBT has been reported to affect regeneration through neurotoxicity, effects on tissues at wound site (Walsh et al., 1986), or apoptosis of germ cells (Cheng et al., 2014). A recent study showed that TBTCl can inhibit cell proliferation (Fickova et al., 2015). Regeneration in planarians involves proliferation of the totipotent stem cells, neoblasts, to form new tissues at wound sites (blastema formation) and remodelling of old tissues to attain the relative size (Reddien and Sánchez-Alvarado, 2004, Reddien et al., 2005). Thus, it is

possible that TBT altered neoblast response to wounding signals in our test animals, causing apoptotic effects, or inhibition of neoblast proliferation. The implication to planarian populations could be the loss of ability to regenerate and even reproduce, in the case of asexual strains. Secondly, since regeneration is a process of cell renewal and growth, it is possible that TBT contamination can adversely alter these processes in aquatic organisms.

Finally, our study showed that TBT caused increased DNA damage in planarians. Increase in DNA damage due to TBT has been reported in *Caenorhabditis elegans* (Wang et al., 2012) and *M. edulis* (Hagger et al., 2005). Genotoxicity and mutagenicity of TBT have been reported in marine polychaete *Platynereis dumerilii* (Hagger et al., 2002) and fish *Hoplias malabaricus* (Ferraro et al., 2004). TBT genotoxicity has been associated to its ability to disrupt intracellular calcium homeostasis (Orrenius et al., 1992), resulting to a rapid increase in the level of intracellular calcium (Boelsterli, 2003). This can cause an activation of Ca^{2+} dependent degradative enzymes known to contribute to cell death (Orrenius et al., 1992) or cause damage to DNA by fragmentation (Mattioli et al., 2003). TBT genotoxicity may thus affect cell repair, growth and regeneration processes in planarians. DNA damage was however a less sensitive indicator of TBT toxicity when compared to regeneration and behavioural parameters.

Overall this study adds ecotoxicological data concerning effects of TBT to aquatic organisms. Moreover our results lend support to the use of freshwater planarians as promising model organisms, to study effects of environmental stressors using a range of organismal endpoints including regeneration. The fact that freshwater planarians can be easily maintained under laboratory conditions (Oviedo et al., 2008a) using similar artificial culturing media used to maintain daphnids and chironomids facilitates comparison with these models organisms and allows multispecies exposures. Future research should evaluate planarians' sensitivity to other chemical and natural stressors using a wider range of endpoints (biochemical biomarkers, reproduction, etc.), aiming to disseminate the use of planarians in ecotoxicological studies.

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Chapter 3.0

Effects of low concentrations of psychiatric pharmaceutical substances on the freshwater planarian, *Schmidtea mediterranea*

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Abstract

There is increasing knowledge about the presence of psychiatric pharmaceutical substances in the aquatic environment due to increasing number of ecotoxicological studies with sensitive species in addition to improved methods of analysis. Here, we assess the effects of two psychiatric substances carbamazepine and fluoxetine in the planarian *Schmidtea mediterranea* using endpoints such as survival, behaviour (feeding, locomotion), DNA damage and regeneration. Also, planarian asexual reproduction by fissioning as a sensitive endpoint was used to assess the reproductive effects of these compounds. Whereas for survival, no effect was observed for carbamazepine exposure, fluoxetine exposure was toxic to planarians with an LC_{50} of 357.93 and 160.01 µg L^{-1} at 48 and 96 hrs respectively. Time for head regeneration in decapitated planarians was not affected by either fluoxetine or carbamazepine exposures. Fluoxetine was more toxic than carbamazepine and caused an increase in locomotor activity and DNA damage (from 0.1 µg L^{-1}), and a decrease in feeding and fissioning which were concentration dependent. Despite some alteration on planarian locomotion observed in intermediate concentrations, no effects were observed in the other endpoints in response to carbamazepine exposure. The observations in the present study showed that freshwater planarians such as *Schmidtea mediterranea*, animal models in neuropharmacology are sensitive to low concentrations of psychiatric drugs, and that there is need to include non-model aquatic invertebrates and sensitive sub-lethal parameters in ecotoxicological assessment of psychiatric drugs. Future studies to determine effects of these psychiatric drugs on neurotransmitters and other biochemical biomarkers are required.

Keywords: Psychiatric pharmaceuticals toxicity, Behaviour, Regeneration, Reproduction, Genotoxicity, *Schmidtea mediterranea*

3.1. Introduction

The presence of human pharmaceuticals in surface waters and sediments has become an issue of concern with regards to their ecological effects. These pharmaceuticals products are released into the aquatic environment from sewage effluents entering the water bodies (Heberer, 2002a, Lindqvist et al., 2005, Roberts and Thomas, 2006). Several studies reviewed by Heberer (2002 b) and Fent et al., (2006) reported measurable concentrations of human pharmaceuticals in some sewage influent and effluents, and also in surface waters. Among these human pharmaceuticals the psychiatric drugs, carbamazepine and fluoxetine known to interact with the central nervous system are also detected.

Carbamazepine is a mood stabilizer used to treat epilepsy, neuropathic pain and maniac disorders (Ambrósio et al, 2002). Carbamazepine acts on the voltage-gated sodium ion channel, where it binds to the channel proteins, thus blocking the channel and reducing the frequency at which impulses are fired during epileptic crisis (Ambrósio et al, 2002). It can also interact with voltage-gated calcium and potassium ion channels, signaling pathways (serotonergic, dopaminergic, glutamergic) and receptors (Post et al., 1983, Ambrósio et al, 2002). Carbamazepine has been detected in many aquatic systems (Ternes, 1998, 2001, Heberer et al., 2002, Calisto et al., 2011, Sim et al., 2011, Fedorova et al., 2014) and due to its ubiquity it is considered as an indicator of man's/anthropogenic influence on aquatic ecosystems (Clara et al., 2004, Heberer et al., 2002). Environmental concentrations of carbamazepine of up to 21.6 µg L^{-1} in influent wastewaters (Sim et al. 2011), 21.0 µg L^{-1} in effluent wastewaters (Sim et al. 2011), or 12 µg L^{-1} in surface waters (Loos et al., 2009), 41.6 ng g^{-1} in water sediments (Thacker, 2005) and 1.1 µg L^{-1} in ground water (Ternes, 2001) have been reported. Carbamazepine is persistent in water and sediments (Oetken et al., 2005, Metcalfe, 2014) with a half-life of about 82 days (Sanderson et al., 2004). It has low removal rate by the sewage treatment plants (Heberer, 2002a, Heberer et al., 2002, Metcalfe, 2014) due to its inability to undergo biodegradation at lower concentrations and insignificant photodegradation (Zhang et al.,2008).

Fluoxetine is a selective serotonin re-uptake inhibitor used in the treatment of depression, anxiety, obsessive compulsive disorder, bulimia nervosa and obesity (Wong et al., 1995, David et al., 2009). It inhibits the re-uptake of serotonin to the pre-synapse resulting in increased levels of serotonin in the synaptic cleft (Wong et al., 1995, Fent et al., 2006). Also, fluoxetine can alter dopamine, melatonin and norepinephrine neurotransmission in the brain (Bymaster et al., 2002, Penttilä et al., 2004, Reierson et al., 2009, Kobayashi et al., 2012). Fluoxetine has been detected

in some aquatic environment (Heberer et al., 2002, Kolpin et al., 2002, Metcalf et al., 2003) with concentrations reaching 0.6 µg L^{-1} in influent wastewaters (Benotti and Brownawell, 2007), 0.59 µg L^{-1} in sewage effluents (Chen et al., 2006), 0.32 µg L^{-1} in surface waters (Weston et al., 2001), 19.37 ng g^{-1} in sediments (Schultz et al., 2010) and 0.056 µg L^{-1} in ground waters (Barnes et al., 2008). It is stable in aqueous solution, not readily degraded by hydrolysis, photolysis and biodegradation, being readily adsorbed and persistent in sediments (Kwon and Armbrust, 2006).

Previous studies have shown that both carbamazepine and fluoxetine cause mortality in non-target organisms at concentrations higher than those found in the environment (Brooks et al., 2003, Jos et al., 2003, Henry et al., 2004, Kim et al., 2007, Heye et al., 2016). Notwithstanding, their low environmentally relevant concentrations have been shown to alter reproduction, development, growth, behaviour and other physiological processes in aquatic organisms (Foran et al., 2004, Oetken et al., 2005, Galus et al., 2014, Rivetti et al., 2016). Carbamazepine exposure has been shown to inhibit pupal emergence in chironomids (Oetken et al., 2005), reduce breeding success in zebrafish (Galus et al., 2014), induce oxidative stress in mussels (Martin-Diaz et al., 2009) and teratogenic effects in zebrafish (Lee et al., 2013), and to alter reproduction and phototactic responses in daphnids (Rivetti et al., 2016). Similarly, fluoxetine exposure affected reproduction, feeding and predator avoidance behavior in fathead minnows (Weinberger and Klaper, 2014), caused disarranged movement and neurochemical alterations in zebrafish larvae (Airhart et al., 2004), aggression in toadfish (McDonald et al., 2011), increased fertility in *Daphnia magna* (Flaherty and Dodson, 2005) and altered embryonic development in *Physa acuta* (Sánchez-Argüello et al., 2012). These responses indicate the possible ecological consequences of these neuroactive drugs on aquatic organisms. Though effects of sub-lethal concentrations of fluoxetine and carbamazepine have been reported for a number of aquatic invertebrates, nothing is known of their effects on freshwater planarians.

Freshwater planarians are benthic organisms with wide distribution in many freshwater bodies, and with both prey and predatory roles. Planarians have been used as models in neuropharmacology and toxicology studies (Raffa et al., 2001, Raffa and Desai, 2005, Pagán et al., 2006, 2009), and have almost all neurotransmitters related to all neurotransmission systems of vertebrate especially mammals, including dopaminergic and serotonergic systems (Pagán et al., 2009, Ramakrishnan and DeSaer, 2011).

The present study aimed to evaluate the sensitivity of freshwater planarian *Schmidtea mediterranea* to low concentrations of each of the neuroactive drugs fluoxetine and

carbamazepine using survival, regeneration, behaviour, reproduction and also DNA damage as endpoints. The relevance of these parameters in ecotoxicology has been highlighted in previous study (Ofoegbu, et al., 2016). Moreover, the presence of serotonergic, melatonergic and dopaminergic systems and voltage-gated sodium ion channels in freshwater planarians are well documented (Morita and Best, 1993, Nishimura et al., 2007, Buttarelli et al., 2008, Oviedo et al., 2003). Serotonin and dopamine are involved in planarian neuromuscular activities and melatonin is known to be involved in planarian asexual reproduction by fissioning (Algeri et al., 1983, Morita and Best, 1984, 1993, Wu et al., 2015). Serotonin mediates in regeneration and other neuromuscular activities including that involving planarian pharynx while locomotion is regulated by dopamine (Algeri et al., 1983, Wu et al., 2015).

The endpoints namely locomotion and feeding were chosen for this evaluation since planarian behaviour is affected by drugs associated with monoaminergic systems of mammals (Carolei et al., 1975, Algeri et al., 1983). Also, the effects of fluoxetine and carbamazepine on fissioning were evaluated since the experimental animal is the asexual strain and reproduces only by transverse fission of the posterior end. Fissioning as a sensitive endpoint has been used to assess the effects of environmental stressors in planarians (Best et al., 1981a, b, Best and Morita, 1991, Levy and Miller, 1978, Rivera and Perich, 1994) and may also be regarded as a behavioural endpoint (Best and Morita, 1991). Head regeneration after decapitation as an endpoint was used to evaluate the effects of these pharmaceutical compounds on neoblast mitotic activities while the comet assay was used to assess the drugs' effects on planarians' DNA damage. Planarians' feeding, regeneration and DNA damage have been shown before to be affected by environmental contaminants (Best and Morita, 1982, 1991, Horvat et al., 2005, Ofoegbu et al., 2016, Rodrigues et al., 2016).

3.2. Materials and Methods

Chemicals

Carbamazepine (Sigma Aldrich) and fluoxetine (fluoxetine chloride, TCI, 98.0%) were used in the experiments. A stock solution of 5 g L^{-1} carbamazepine was dissolved in absolute ethanol and experimental concentrations were prepared ensuring that ethanol concentration in the highest concentration did not exceed 0.01 %. Fluoxetine was dissolved in miliQ water to obtain a stock solution of 10 mg L^{-1}. These stock solutions were covered from light and stored at 4ºC until use.

Experimental solutions were prepared by diluting stock solutions in ASTM hard water (ASTM, 2004).

Experimental animals

Schmidtea mediterranea (asexual strain) used in all experiments came from laboratory cultures established for more than two years. The animals were maintained in ASTM medium (ASTM, 2004) and fed raw bovine liver puree twice a week with water renewal immediately after and two days after feeding. Animals were maintained in the dark at a temperature of 20 ± 1.0 ºC in a temperature controlled room. All exposures were done in the dark and at the same temperature of the animals' culture room. Planarians used for tests showed no signs of wounds, and were starved for a week except those used for reproduction tests which were starved for 4 days before exposure. Also, the sizes of the animals used for reproduction tests ranged from 13.5 ± 1.5 mm and 5.0 ± 1.0 mm for the other endpoints.

Acute test

Planarians were exposed to carbamazepine nominal concentrations of 0.625, 1.25, 2.5, 5.0, 10.0 mg L^{-1} and to a solvent control (SCTR, 0.01 % absolute ethanol diluted in ASTM medium) and a control treatment (ASTM only) or fluoxetine nominal concentrations of 1.638, 4.096, 10.24, 25.6, 160.0, 400.0 and 1000.0 µg L^{-1} plus control treatment (ASTM only). A total of ten replicates per concentration each with 1 planarian were exposed in a 35 mL glass crystallizing dish containing 20 mL of experimental solution. The experiment lasted for 96 hours and experimental solutions were renewed every two days. The experiment was checked daily for mortality and number of dead organisms recorded. Animals with degenerating body or without detectable movement under strong light were considered dead.

Behavioural responses

Planarians were exposed to 0.01, 0.1, 1.0 and 10.0 µg L^{-1} nominal concentrations of carbamazepine and fluoxetine respectively, and a control treatment (CTR). Also, a solvent control (SCTR, 2 µl L^{-1} absolute ethanol diluted in ASTM) treatment in the case of carbamazepine exposures was used. In each concentration 15 organisms divided into 5 replicates were exposed in 150 mL glass crystallizing dish containing 50 mL of experimental solutions.

Exposure period was 9 days and experimental solutions were renewed every 3 days. The same exposures scheme was used to assess effects on locomotion and post-exposure feeding rate.

Locomotor activity: Locomotor activity in planarians was measured according to Raffa et al., (2001), Pagan et al., (2009) and Ofoegbu et al., (2016) with slight modifications. Concisely, each planarian was placed at the center of a clear acrylic container (21.2 cm x 18.5 cm x 1.9 cm dimensions) containing 50 mL of experimental solution and which was placed on top of a graph sheet with 0.5 cm gridlines spacing. The number of gridlines crossed and re-crossed during a period of 2 minutes after 30 seconds of acclimation period under an average of 718 lux of white light was recorded. Measurements were taken once for each planarian and results were expressed as mean number gridlines crossed or recrossed by planarians in each experimental concentration.

Post exposure feeding: After 9 days of exposure in either carbamazepine or fluoxetine, planarians from each replicate were transferred to a 35 mL glass crystallizing dish with 20 mL ASTM hard water and 60 3-days old *Chironomus riparius* larvae, and left for 24 hrs. Small crystallizing dish and 3-days chironomid larvae were used here because of the size the experimental animals (4-6 mm) and based on findings from preliminary tests. After 24 hrs the number of larvae consumed by planarians in each replicate was recorded. Results were expressed as mean number of larvae consumed per concentration after 24 hrs.

Head regeneration

The protocol for exposure was similar to that used for behavioural parameters. After 9 days exposure, each planarian was decapitated before the pharynx. 15 decapitated worms (3 worms in each replicate) were subsequently exposed for another 9 days in 150 mL crystallizing dish with 50 mL of the same experimental solutions. Solutions were also renewed every 3 days during this period. Planarians in each replicate were examined daily to follow process of head regeneration using Zeiss stereo microscope (KL 300 LED). The number of days for photoreceptor formation and any abnormality for each organism in each replicate were noted. The results are reported as mean number of days for photoreceptor formation in planarians per test concentration.

Reproduction

Fissioning in planarians following methods by Best et al. (1981a, b), Best and Morita (1991) and Sheiman et al. (2003) with slight modifications was used to determine the effects of carbamazepine and fluoxetine respectively on planarian asexual reproduction. 30 planarians with tails tapering to a point showing fissioning potential were divided into 5 replicates and exposed in 150 ml glass crystallizing dish containing 100 mL of experimental solutions. Experimental concentrations were the same as for behavioural tests. Also, animals were not fed during the test and test solutions were renewed every 3 days during the 9 days exposure period. Planarian pieces resulting from fissioning in each replicate were removed before experimental water renewal, and the number recorded. After the exposure period, the total number of planarian pieces from each replicate was counted and results were reported as mean number of planarian pieces per experimental concentration.

Comet assay

Comet assay was performed to detect DNA damage in planarians as described previously by Prá et al. (2005), Lourenço et al., (2011) and Ofoegbu et al. (2016) under yellow light. Freshwater planarians were exposed to sub-lethal nominal concentrations of carbamazepine or fluoxetine (0.01, 0.1, 1.0 and 10.0 µg L^{-1}), control and solvent control (for carbamazepine), in 150 mL glass crystallizing dish with 100 mL of experimental solution. Exposure period was 9 days and experimental solutions were renewed every 3 days. After exposure, animals in each replicate were pooled (3 replicates of 7 planarians per concentration) and subjected to chemical (using 0.48% trypsin) and mechanical disintegration, followed by centrifugation at 10,000 g for 2 mins at 4ºC. Afterwards, 10 µL of cell suspension from each replicate was mixed with 0.5% low melting point agarose and spread on microscope slides pre-coated with 1% normal melting point agarose. Then, slides were immersed in lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base and 1% Triton X-100, 10% DMSO, pH 10) at 4ºC for 120 mins, in the dark. After lysis, slides were subjected to denaturation in freshly prepared cold alkaline buffer (0.3 M NaOH and 1 mM EDTA, pH 13) for 15 min. Electrophoresis was performed in the same alkaline buffer at 0.7V/cm and 300 mA for 10 mins and then followed by neutralization of slides using Tris HCl (pH 7.5) solution. Slides were then stained using ethidium bromide and visualized with a fluorescence microscope (ZEISS Axio Scope A1). One hundred cells, per each slide condition,

were randomly selected and visually categorized and grouped into 5 classes (0 to 4) depending on DNA damage as described by Garcia et al. (2004).

Statistical analysis

The LC_{50} at 95% confidence interval (CI) of 48 and 96 hours for fluoxetine were calculated by probit analysis, and all data were checked for homogeneity of variances using Levene's test with IBM SPSS statistics (version 21). Also, all data were checked for normality with Kolmogorov-Smirnov normality tests. Effects of fluoxetine on behaviour and reproduction were analyzed with analysis of variance ANOVA followed by Dunnett's post hoc test for significant difference between exposed group and control group. Similarly, carbamazepine effects on locomotor activity data were analyzed with analysis of variance ANOVA followed by Dunnett's post hoc test for significant difference between exposed group and non-exposed group. Whereas there were no significant differences between the control and solvent control groups (student *t* test, $p > 0.05$), comparisons in the carbamazepine treatment were made with solvent control. The arbitrary units for comet assay were obtained from an Excel programmed template which automatically calculates them by multiplying the observed comet by comet classification and summing the values per replicate. Further, these comet assay data were analyzed with analysis of variance followed by Dunnett's post hoc. Significant level was set $p < 0.05$. Fluoxetine data on behaviour, asexual reproduction and DNA damage were also subjected to linear trend post test for significant linear trend in responses due to fluoxetine exposures. Calculations were made with GraphPad Prism version 5 for Windows and results were expressed as mean ± standard error of mean (SEM).

3.3. Results

Acute toxicity test

Carbamazepine exposure did not cause any mortality in planarians during 96 hrs, even at the highest concentration of 10 mg L^{-1}. However, fluoxetine exposure was lethal to planarians with 48 and 96 hrs $LC_{50's}$ of 357.93 µg L^{-1} (237.29 to 532.97 µg L^{-1}) and 160.01 µg L^{-1} (107.00 to 239.27 µg L^{-1}) respectively.

Behavioural effects

Carbamazepine exposure caused an increase in locomotor activity of the planarians compared to those in the solvent control (SCTR) and with significant difference at 0.1 and 1.0 µg L^{-1} ($F_{(4, 15)}$ = 6.63, p = 0.0014, R^2 = 0.57, fig. 3.1). Contrary to this, locomotor activity of planarians exposed to 10.0 µg L^{-1} was similar to the solvent control group revealing a non-monotonic response of planarians to carbamazepine exposure. Fluoxetine exposure resulted to an increase in locomotor activity in planarians in comparison to the control treatment with a LOEC of 1.0 µg L^{-1} ($F_{(4, 15)}$ = 7.15, p = 0.001, R^2 = 0.59, fig. 3.2). The increase in locomotor activity was dose dependent and followed a significant linear trend (slope = 2.03, r^2 = 0.55, p < 0.0001). Carbamazepine exposure caused 23.5 and 35.5% increases in locomotor activity of planarians at 0.1 and 1.0 µg L^{-1} respectively compared to unexposed organisms while fluoxetine exposure caused 29.19 and 47.57% at the two highest concentrations 1.0 and 10.0 µg L^{-1} respectively compared to control treatment.

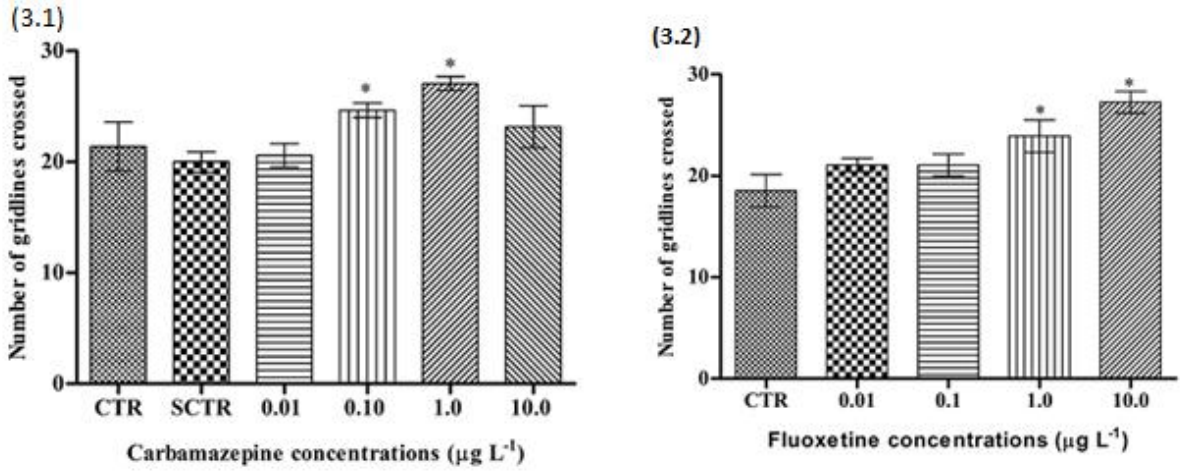

Fig. 3.1 and 3.2: Locomotor activity of *S. mediterranea* after 9 days exposure to carbamazepine (3.1) or fluoxetine (3.2). Locomotor activity measured as number of gridlines crossed and re-crossed in 2 mins after 30 secs acclimation. Values are expressed as mean ± standard error of mean (SEM), n = 15. Bars with * show significant difference from control (CTR) or solvent control (SCTR) (Dunnett's test $p < 0.05$).

Feeding rates of planarians exposed to carbamazepine did not vary significantly in comparison with solvent control treatment ($F_{(4, 15)}$ = 0.58, p = 0.682, R^2 = 0.10, fig. 3.3). Whereas, exposure to fluoxetine significantly reduced feeding activity in planarians with a LOEC of 1.0 µg L^{-1} ($F_{(4, 15)}$ = 4.36, p = 0.011, R^2 = 0.47, fig. 3.4). The reduction in the number

chironomid larvae consumed by planarians exposed to fluoxetine was concentration dependent and followed a significant linear trend (slope = -3.02, r^2 = 0.45, p = 0.0006). Fluoxetine caused 19.91 and 28.57% reduction in the number of larvae consumed by planarians exposed to 1.0 and 10.0 µg L^{-1} fluoxetine respectively compared to control treatment.

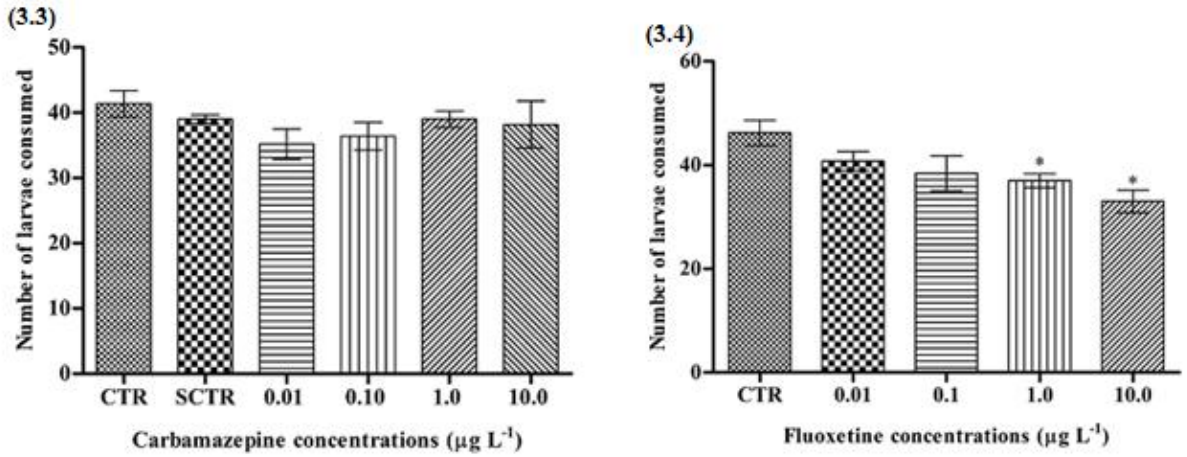

Fig. 3.3 and 3.4: Feeding activity in *S. mediterranea* expressed as number of chironomid larvae consumed during 24 hrs after 9 days exposure to carbamazepine (3.3) or fluoxetine (3.4). Values are mean ± standard error of mean (SEM), n = 15. Bars with * show significant difference from control (CTR) (Dunnett's $p < 0.05$).

Head regeneration

Planarians exposed to the different compounds formed photoreceptors before the 9th day and there were no effects of either carbamazepine ($F_{(4, 15)}$ = 0.73, p = 0.577, R^2 = 0.04, fig. 3.5) or fluoxetine ($F_{(4, 15)}$ = 1.43, p = 0.262, R^2 = 0.22, 0.05, fig. 3.6) in comparison with head regeneration observed for control treatments

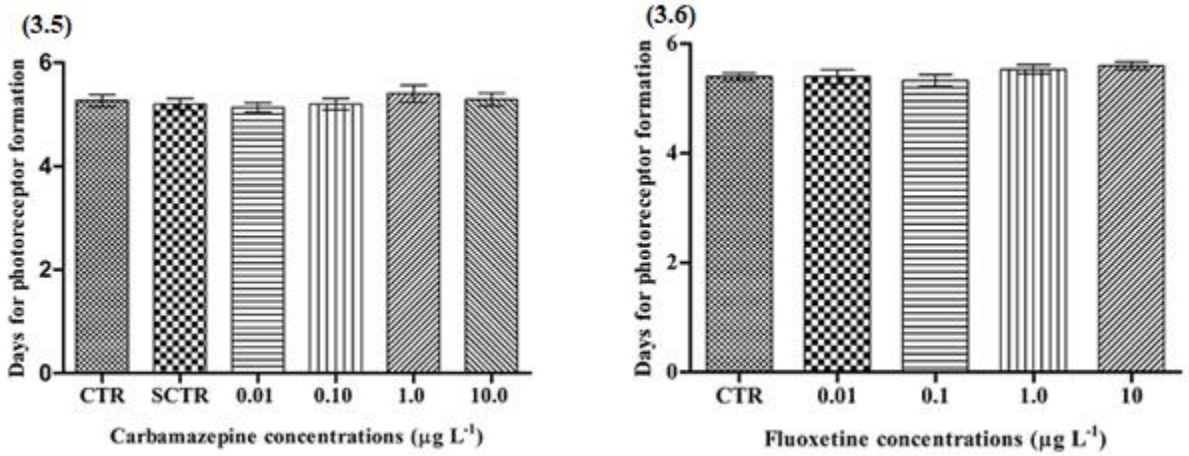

Fig. 3.5 and 3.6: Time for head regeneration expressed as days for photoreceptor formation in *S. mediterranea* exposed 9 days to carbamazepine (3.5) or fluoxetine (3.6) before and after decapitation. Values are mean ± standard error of mean (SEM), n = 15.

Asexual reproduction

Carbamazepine did not affect fissioning in *S. mediterranea* after 9 days of exposure ($F_{(4, 30)}$ = 1.07, p = 0.397, R^2 = 0.177, fig. 3.7). However, fluoxetine exposure significantly reduced fissioning in planarians exposed to 10.0 µg L^{-1} ($F_{(4, 30)}$ = 14.87, $p < 0.0001$, R^2 = 0.75, fig. 3.8)

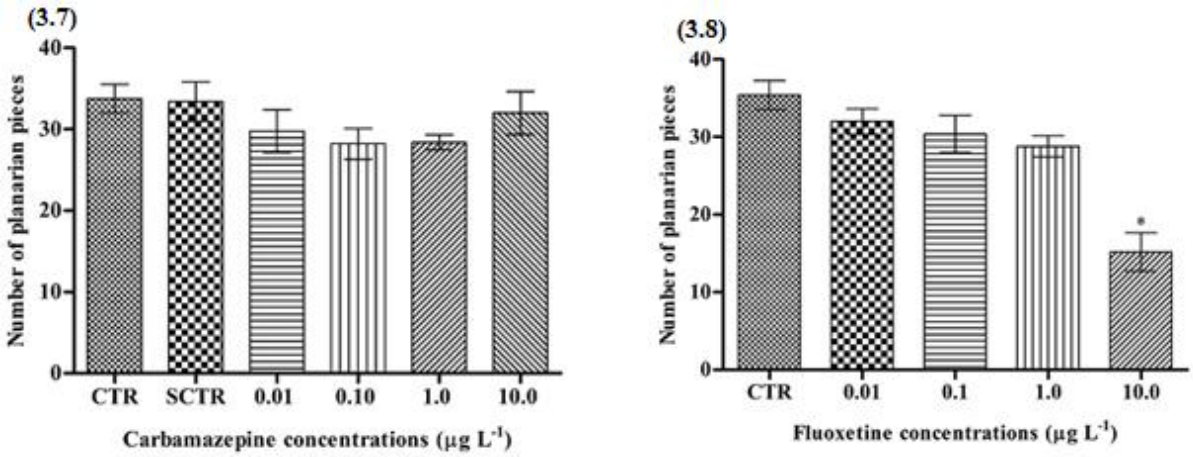

Fig. 3.7 and 3.8. Effects of carbamazepine (3.7) or fluoxetine (3.8) on fissioning in *S. mediterranea* measured as number of planarian pieces collected during 9 days exposure. Values are mean ± standard error of mean (SEM), n = 30. Bars with * show significant difference from control (CTR) (Dunnett's $p < 0.05$).

compared to those in the control group. The reduction in fissioning was concentration dependent with a significant linear trend (slope = -4.36, r^2 = 0.59, p < 0.0001). Planarian in each replicate of control treatment produced on average 5.9 smaller worms and only 2.5 worms per planarian in the 10.0 µg L^{-1} treatment resulting to a decrease of 57.63% from the control treatment.

DNA damage

Carbamazepine exposure did not increase DNA damage significantly in planarians ($F_{(4, 21)}$ = 1.53, p = 0.266, R^2 = 0.38, fig. 3.9) and the trend followed a non-monotonic pattern. Fluoxetine caused a significant increase in DNA damage in exposed planarians with a LOEC of 0.1 µg L^{-1} ($F_{(4, 21)}$ = 6.80, p = 0.007, R^2 = 0.73, fig. 3.10). The increase in DNA strand breaks due to exposure to fluoxetine was dose dependent and with a significant linear trend (slope = +19.57, r^2 = 0.71, p = 0.0004). Percentage of DNA damage due to fluoxetine at 0.1, 1.0 and 10.0 µg L^{-1} was 53.67, 59.07 and 70.34% respectively in comparison with 30.0% observed in control treatment.

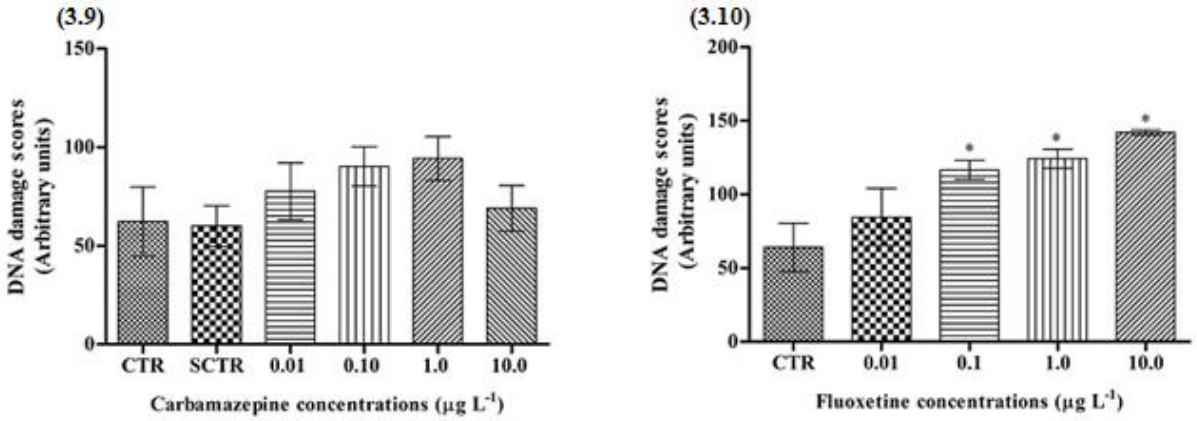

Fig. 3.9 and 3.10: Effects of carbamazepine (3.9) or fluoxetine (3.10) on DNA damage in *S. mediterranea* after 9 days exposure expressed as arbitrary units ranging from 0 to 400. Values are mean ± standard error of mean (SEM), n = 21. Bars with * show significant difference from control (CTR) (Dunnett's test p < 0.05).

3.4. Discussion

The present study examined the sensitivity of freshwater planarian, *S. mediterranea* to two psychiatric drugs, carbamazepine and fluoxetine using lethal and sub-lethal endpoints. Carbamazepine exposure caused an increase in locomotion at intermediate concentrations while

exposure to low fluoxetine concentrations caused mortality, increases in locomotor activity, reductions in feeding and fissioning, and an induction of DNA damage in *S. mediterranea*.

Carbamazepine exposure did not cause any lethal effects in *S. mediterranea* even at 10.0 mg L^{-1}. Previous studies with some aquatic organisms showed that carbamazepine is only lethal at very high concentrations which may not be found in aquatic environments. A 48 hrs LC_{50} of 77.7 mg L^{-1} for *Cerodaphnia dubia* (Ferrari et al. 2004), 96 hrs LC_{50} of 76.3 mg L^{-1} for *Daphnia magna* (Kim et al., 2007), 96 hrs LC_{50} of 19.9 mg L^{-1} for rainbow trout *Oncorhynchus mykiss* (Li et al., 2011), and 96 hrs LC_{50} of 45.87 mg L^{-1} for Japanese medaka *Oryzias latipes* (Kim et al., 2009) have been reported. Fluoxetine on the other hand, was toxic to planarians with a 48 and 96 hrs LC_{50s} of 357.93 and 160.01 µg L^{-1} respectively. Fluoxetine is known as the most acutely toxic human pharmaceutical (Fent et al., 2006) with a 48 hrs LC_{50} of 820.0 µg L^{-1} for *D. magna* and 234.0 µg L^{-1} for *C. dubia* (Brooks et al., 2003), 48 hrs EC_{50} of 27.0 µg L^{-1} for alga *Pseudokirchneriella subcapitata* (Christensen et al., 2007), 48 hrs LC_{50} of 705.0 µg L^{-1} for the fish *Pimephales promelas* and 15.2 mg kg^{-1} for *Chironomus tentans* (Brooks et al., 2003). Also, 7 days LC_{50} of 546 µg L^{-1} fluoxetine for mosquito fish *Gambusia affinis* (Henry and Black, 2008) and 500 µg L^{-1} of fluoxetine for 24 hrs old eggs of the pond snail *Physa acuta* (Sánchez-Argüello et al., 2012) have been reported. These findings of previous studies on the acute toxicity of these two drugs are in line with the results in this study reflecting the lesser toxicity of carbamazepine in comparison to fluoxetine. Also the results in this study suggest that *S. mediterranea* is one of the most sensitive invertebrates concerning fluoxetine acute exposures.

Fluoxetine and carbamazepine exposures affected the behaviour of planarians, especially their locomotor activity. Carbamazepine caused non-monotonic response in locomotion of exposed flatworms, with an increase in activity at 0.1 and 1.0 µg L^{-1} but these effects were no longer visible at the 10.0 µg L^{-1}. Non-monotonic behavioural responses by aquatic invertebrates to carbamazepine exposure have been observed in previous studies by De Lange et al. (2006) and Rivetti et al. (2016). De Lange and others reported a slight reduction in activity of crustacean *Gammarus pulex* exposed to 1 and 10 ng L^{-1} carbamazepine, while at higher carbamazepine concentrations activity was similar to that observed in control treatments. Similarly, in adult *D. magna* chronic exposures to carbamazepine decreased negative phototactic behaviour at concentrations 0.1 and 1.0 µg L^{-1} but behaviour at 10 µg L^{-1} was similar to non exposed animals (Rivetti et al., 2016). However, contradictory effects of carbamazepine on behaviour have also been reported for fish with exposures to carbamazepine causing a reduction in swimming speed

in Japanese medaka (Nassef et al., 2010) but an increase in swimming activity of *Lepomis gibbosus* (Brandão et al., 2013).

Fluoxetine however elicited a dose dependent increase in locomotor activity in exposed planarians from 1.0 µg L^{-1} and higher concentration. Alteration of behaviour due to fluoxetine exposure observed here is in line with effects of fluoxetine in other aquatic invertebrates. Exposure for 67 days to 22.3 µg L^{-1} fluoxetine increased behavioural activities (movement and burrowing) in fresh water mussel *Lampsilis fasciola* (Hazelton et al., 2014) while in *D. magna* exposure to concentrations of fluoxetine as low as 1.0 ng L^{-1} increased their positive phototactic behaviour, though response was non-monotonic (Rivetti et al., 2016). Also, exposure of *G. pulex* to fluoxetine resulted in a decrease in activity (locomotion and ventilation) at 10 to 100 ng L^{-1} while effects observed for higher concentrations (1 µg L^{-1} and 1 mg L^{-1}) are similar to non exposed animals (De Lange et al., 2006).

The results of locomotor activity of planarians exposed to carbamazepine can be related to time of action of the drugs and the ability of the animals to become non-responsive to the effects of the drugs at the highest concentration. Carbamazepine has been reported to act shortly after administration (Post, 1988, Ramakrishnan and DeSaer, 2011, Rivetti et al., 2016) with action being concentration dependent (Ramakrishnan and DeSaer, 2011) and time for maximum effect in mice was 1 hr after treatment (Bourgeois and Wad, 1988). Also, neurotransmitter receptors are known to be subject to becoming unresponsive or desensitized on long exposure to their neurotransmitters (Rivetti et al., 2016). It is possible that the highest concentration of carbamazepine tested here elicited effects in planarians shortly after exposure. Consequently, planarians may have recovered or become insensitive to the effects of the drug while those at lower concentrations were still responding to the effects of the drug at the time their locomotor activities were assessed. This non-monotonic carbamazepine effects on *S. mediterranea* locomotion agree with its effects on behavior of *Daphnia magna* (Rivetti et al., 2016) and *Gammarus pulex* (De Lange et al., 2006).

Also, the increase in locomotor activity may be a response of exposed planarians to escape from stress due to carbamazepine or fluoxetine exposure, or according to Rivetti et al. (2016) specific neurological response. Locomotion in planarians is controlled by dopamine (Wu et al., 2015) and increase in dopamine level in planarians has been associated to increased locomotor activity (Algeri et al., 1983). Also, both fluoxetine (Bymaster et al., 2002, Mennigen et al., 2008) and carbamazepine (Ambrósio et al., 2002, Post et al., 1983) have been shown to increase

dopamine level suggesting its involvement in this increased locomotor activity of exposed planarians observed in this study.

Carbamazepine exposure did not affect planarian feeding activity while fluoxetine exposures caused significant and dose dependent feeding inhibition. Although, reduction in feeding activities due to carbamazepine exposure have been reported for Japanese medaka *O. latipes* at 6.15 mg L^{-1} after 9 days exposure (Nassef et al., 2010) and *Hydra attenuata* at 50.0 mg L^{-1} for 96 hrs (Quinn et al., 2008). Fluoxetine exposure caused a decrease in feeding ability from 1.0 µg L^{-1} in exposed planarians. Fluoxetine exposure was reported to reduce feeding in fathead minnows (*Pimephales promelas*) (Weinberger and Klaper, 2014), Hybrid striped bass (Gaworecki and Klaine, 2008) and larvae of amphibian *Rana pipiens* (Foster et al., 2010). Also, feeding in polychaete *Hediste diversicolor* (harbor ragworm) was reduced with a LOEC of 10 µg L^{-1} fluoxetine (Hird et al., 2016).

Fluoxetine is known to affect food intake through its effects on serotonin levels (Wong et al., 1995, Halford et al., 2005). Earlier studies with rats showed that fluoxetine treatment resulting to increase in serotonin suppresses food consumption (Wong et al., 1995, Halford et al., 2005). Fluoxetine is assumed to be a satiation-inducing agent controlling appetite and satiation by reducing food intake without affecting frequency of feeding (Wong et al., 1995, Halford et al., 2005). In addition, Hird and others observed an increase in serotonin level with increasing concentration of fluoxetine, and a link between reduction in feeding rate with increasing serotonin level and fluoxetine concentration in the polychaete, habour ragworm (Hird et al., 2016). On the contrary, Gaworecki and Klaine, (2008) reported a reduction in ability to capture prey with decreasing serotonin concentration in hybrid striped bass fish. In planarians serotonin controls neuromuscular activities like excitatory effects on the pharynx and ciliated cells of ventral surface (Kabotyanski et al., 1991, Wu et al., 2015). Following these observations, potential alteration of serotonin levels in exposed planarians may have caused the reduction and monotonic effects in terms of feeding as observed for other invertebrates (Hird et al., 2016),. Carbamazepine exposure is also known to increase serotonergic activity (Ambrósio et al., 2002), but maybe the level of serotonin was not enough to produce significant effects on feeding in exposed planarians.

Nevertheless, the implications of these behavioural alterations in freshwater planarians are of ecological importance, since the concentrations are environmentally relevant. Increase in locomotor activity may be due to erratic movement which will make planarians vulnerable to

predation, increase their energetic costs and decrease fitness. In addition, the observed reductions in feeding rate might lead to impairments of growth and reproduction (Oviedo et al., 2008).

Regeneration in *S. mediterranea* was not affected by either carbamazepine or fluoxetine exposures at any of the concentrations tested. Fluoxetine treatment has been shown to reduce cell proliferation of cancer cells in mice and rats (in-vivo) (Kannen et al., 2011, 2012) and tumor cells in humans (in-vitro) (Krishnan et al., 2008). Lacaze et al. (2015) also reported that fluoxetine exposure induced cytotoxic effects on *Mytilus edulis* hemocytes (in-vitro) which they associated to an increase in reactive oxygen species and subsequent oxidative stress. Kannen et al. (2011) associated fluoxetine anti-proliferative potential to its influence on serotonergic activities while Lacaze et al. (2015) implicated oxidative stress due to increase in reactive oxygen species to cytotoxicity. However, lack of effect on regeneration in this study was not expected as regeneration in planarians has been shown to be controlled by serotonin and dopamine (Martelly et al, 1983, Wu et al., 2015). Serotonin and dopamine are known to control cell proliferation and differentiation in planarians (Martelly et al, 1983). Moreover, dopamine and serotonin levels have been shown to increase during regeneration in planarians, and treatment of sectioned planarians with dopamine or serotonin reduced time for regeneration (Martelly and Franquinet, 1984, Ribeiro et al., 2005). Planarian regeneration is associated to presence of totipotent stem cells neoblasts, and involves cell proliferation, migration and differentiation (Sánchez Alvarado, 2000). As such the concentrations of both compounds tested here might have been too low to elicit an effect. It would be interesting to address possible effects that higher concentrations or extended exposure periods might have on patterns of regeneration in planarians.

Asexual reproduction in *S. mediterranea* by fissioning was not affected by carbamazepine exposure, although a slight reduction in fissioning in planarians exposed to lower carbamazepine was observed. Carbamazepine have been reported to cause non-monotonic responses in *D. magna* reproduction with an increase at 1.0 and 10.0 µg L^{-1} but a decrease at 100.0 µg L^{-1} (Rivetti et al., 2016), decrease in emergence in *C. riparius*, no effects on reproduction in Oligochaete *Lumbriculus variegatus* and snail *Potamopyrgus antipodarum* (Oetken et al., 2005), and a reduction in reproduction in zebrafish (Galus et al., 2013).

The results of this study also showed a clear effect of fluoxetne in terms of planarian fissioning. Reductions in reproduction caused by chronic exposure to higher concentrations of fluoxetine have been reported before for freshwater molluscs *Potamopyrgus antipodarum* and

Valvata piscinalis (Netwing, 2007, Gust et al., 2009). However, reproduction increases caused by exposure to fluoxetine have also been reported for other freshwater invertebrates such as *Daphnia magna* (Flaherty and Dodson, 2005) and *Lumbriculus variegatus* (Netwing, 2007), while no effects of fluoxetine exposure was reported for *Chironomus riparius* (Netwing, 2007).

Planarian fissioning is controlled by longitudinal cords of the nervous system and brain (Best et al., 1969, 1975) and melatonin level (Morita and Best, 1984, 1993). Melatonin is a metabolite closely related to serotonin, and an increase in its level has been implicated in the suppression of fissioning in planarians (Morita and Best, 1984, 1993). Also, studies with rodents and humans showed an increase in melatonin level with fluoxetine treatment (Reierson et al., 2009, Kirecci et al., 2014). Fluoxetine exposure may have decreased fissioning in planarians through its influence on melatonin level. It is however clear that our results suggest an impairment of fissioning and thus of reproduction in *S. mediterranea* (asexual strain) exposed to fluoxetine and this suggests potential population level effects of chronic exposures to environmental relevant concentrations of fluoxetine.

Furthermore, the result showed that carbamazepine exposure did not induce significant DNA damage in planarians. Similar non significant increase in DNA damage by carbamazepine has been observed in other aquatic organisms *Ampelisca brevicornis*, *Mytilus galloprovincialis* and *M. edulis* (Martin-Diaz et al., 2009, Lacaze et al., 2015, Maranho et al., 2015), However, the possibility of carbamazepine to induce significant DNA damage in planarians cannot be ruled out. Exposure to fluoxetine on the other hand, resulted to an induction in DNA damage at concentrations as low as 0.1 µg L^{-1}. Similarly, fluoxetine exposure was shown to increase DNA damage in harbor ragworm *H. diverticolor* with LOEC 100 µg L^{-1} (Hird et al., 2016) and *M. edulis* (Lacaze et al., 2015) but a decrease in DNA damage in *A. brevicornis* after 10 days exposure (Maranho et al., 2015) and no effect on DNA in *Physa acuta* (Sánchez-Argüello et al., 2012). Findings from these previous studies and the present study showed freshwater planarian *S. mediterranea* to be one of the most sensitive aquatic organisms to genotoxicity due to fluoxetine. Genotoxicity due to fluoxetine exposure may be linked to oxidative stress (resulting from increased reactive oxygen species or increased oxidative metabolism of lipids) or immunotoxicity (Lacaze et al., 2015) and it has been shown that DNA damage may affect growth, development and reproduction in organisms (Lee et al., 2000). Moreover, similar to the result of the present study fluoxetine has been shown to be more genotoxic than carbamazepine (Lacaze et al., 2015).

Generally, the responses of *S. mediterranea* in this study support findings in other studies with sensitive aquatic organisms including daphnids, that carbamazepine is less toxic while fluoxetine is a very toxic human pharmaceutical in the aquatic environment (Fent et al., 2006, Lacaze et al., 2015).

In summary, our results support the use of freshwater planarians to evaluate effects caused by exposure to pharmaceutical substances in the aquatic environment. Low concentration of psychiatric pharmaceuticals and especially of fluoxetine induced genotoxicity, behavioural and reproductive detrimental effects in planarians showing the sensitivity of these organisms and of endpoints used. DNA damage was the most sensitive endpoint and its inclusion in standard ecotoxicological tests may prevent the under-estimation of the risks of the exposure to low concentrations of fluoxetine.'

Future studies evaluating the effects of psychiatric substances on biochemical biomarkers, and on neurotransmitter levels for a better understanding of mechanisms of action of these drugs on aquatic invertebrates are recommended. In addition, more studies on the effects of other pharmaceuticals and personal care products of ecotoxicological interest on freshwater planarians (sexual, asexual and parthenogenetic types) are needed to show their relevance as model organisms for ecotoxicity studies.

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Chapter 4

Influence of carbamazepine and tributyltin on the effects of fluoxetine in freshwater planarian, *Schmidtea mediterranea*

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Abstract

Neuroactive pharmaceuticals are common emerging contaminants within freshwaters, and like all pollutants may be present in complex mixtures including other neuroactive and neurotoxic compounds. In this investigation, the influence of another neuroactive pharmaceuticals (carbamazepine), or a neurotoxic compound (tributyltin chloride, TBT) on the effects of fluoxetine were evaluated using the freshwater planarian, *Schmidtea mediterranea* as a test organism. The effects of combined exposures to these compounds on planarians were assessed using endpoints such as planarian locomotor activity, asexual reproduction by fissioning and DNA damage using the comet assay. Results showed no interactions between both psychiatric drugs (carbamazepine and fluoxetine) on any of the tested parameters, re-enforcing at the same time the higher toxicity of fluoxetine. Concerning combined exposures of fluoxetine and TBT results revealed complex interactions on DNA damage and locomotion, suggesting that effects of fluoxetine can be mediated by the simultaneous exposure to TBT. Freshwater planarians are sensitive to pharmaceutical substances and to other contaminants in the freshwater environment and may be relevant in ecotoxicological risk assessment of chemical mixtures containing psychiatric pharmaceuticals, even lower concentrations.

Keywords: Combined exposure, Neuroactive pharmaceuticals, Neurotoxic compound, Behaviour, Reproduction, Genotoxicity, Freshwater planarian

4.1. Introduction

Different types of pharmaceutical substances have been detected in aquatic systems in many parts of the world. The presence of these chemical compounds in the aquatic environment has been associated to their wide usage in human and veterinary medicine, aquaculture and low removal rates by sewage treatment plants (Halling-Sørrenen et al., 1998). Measured environmental concentrations of these drugs in aquatic systems are usually in ng L^{-1} and µg L^{-1} ranges (Kolpin et al., 2002), but concentrations in mg L^{-1} in areas near hospitals and pharmaceutical production facilities have been reported (Kümmerer, 2001, Larsson et al., 2007, Philips et al., 2010). Though these environmental concentrations are lower than levels causing lethal effects, they may alter some life processes in aquatic organisms (Fent et al., 2006). Moreover, in natural ecosystem pharmaceuticals are usually present in complex mixtures with other pharmaceuticals, their metabolites and other chemical substances with which they can interact causing unexpected effects in non-target aquatic organisms (Wiegel et al., 2004, Fent et al., 2006). Thus, there is need to study effects of these complex mixtures in addition to studies addressing effects of single exposures. Several studies have shown that effects of mixtures of these pharmaceuticals on exposed organisms may be stronger than effects of individual drugs (Cleuvers, 2004, Flaherty and Dodson, 2005, Borgmann et al., 2007, Gust et al., 2013, Melvin et al., 2014), and that mixtures of these low environmental concentrations may be capable of causing deleterious effects (Flaherty and Dodson, 2005, Gust et al., 2013). Nevertheless, considering the variability in species responses to drugs and the fact that drugs have many mechanisms of action (Fent et al., 2006, Fong and Ford, 2014), studies on possible sub-lethal effects of these complex mixtures using non-target and non model aquatic species are essential for environmental risk assessment of pharmaceuticals. This is particularly important for psychiatric drugs because they are among the most prescribed and commonly used and detected pharmaceutical substances in the aquatic environment (Nentwig, 2008, Calisto and Esteves, 2009).

Fluoxetine is a neuroactive pharmaceutical and one of the widely used drugs (Nentwig, 2008). It is an antidepressant designed to inhibit the re-uptake of serotonin besides effects on uptake sites and receptors of other neurotransmitter systems such as dopaminergic, GABAergic, melatonergic, adrenergic etc systems (Wong et al., 1995, Bymaster et al., 2002, Reierson et al., 2009, Kirecci et al., 2014). Fluoxetine has been detected in water samples from some aquatic systems (Heberer et al., 2002, Kolpin et al., 2002), and of all human pharmaceuticals appears to

be the most toxic, causing acute toxicity at very low concentration (Fent et al., 2006). Fluoxetine concentrations may reach 590 ng L^{-1} in sewage effluents (Chen et al., 2006), 320 ng L^{-1} in surface waters (Weston et al., 2001) and 19.37 ng g^{-1} in sediments (Schultz et al., 2010). Acute and sub-lethal effects of Fluoxetine on different aquatic organisms have been shown (Brooks et al., 2003, Airhart et al., 2004, Henry et al., 2004, Flaherty and Dodson, 2005, Weinberger and Klaper, 2014, Rivetti et al., 2016)).

Carbamazepine is also a neuroactive drug used in the treatment of epilepsy and other psychiatric disorders, and acts on the voltage gated sodium ion channel as well as other voltage gated ion channels (calcium, potassium), neurotransmission systems (serotonergic, dopaminergic, glutamergic etc) and receptors etc (Post et al., 1983, Ambrósio et al., 2002). It is one of the most frequently detected pharmaceutical compounds in water samples from aquatic systems (Ternes, 1998, Heberer, 2002a, Clara et al., 2004, Yuan et al., 2013), being seen as a sign of human meddling in aquatic habitats (Clara et al., 2004) and of sewage contamination of surface waters (Heberer, 2002). Carbamazepine concentrations have been shown to reach 21000 ng L^{-1} in sewage effluents (Sim et al., 2011), 12000 ng L^{-1} in surface waters (Loos et al., 2009) and 41.6 ng g^{-1} in sediments (Thacker, 2005). Carbamazepine in the aquatic environment may be harmful to aquatic organisms (Fent et al., 2006) and its deleterious effects on various aquatic organisms have been reported (Oetken et al., 2005, Galus et al., 2014, Rivetti et al., 2016).

Tributyltin chloride (TBT), a model neurotoxic agent is an organo-metallic compound used mainly as biocide in antifoulant paint on boats, ship and dock, as preservative for paper, timber, wood, textile, leather and in wall paints (Fent and Müller, 1991, Fent, 1996). It is ubiquitous, widely found in many freshwaters, estuarine and coastal habitats (Fent, 1996, Sousa et al., 2013, Cruz et al., 2014) and persistent in sediments (Dowson et al., 1996, Fent, 1996) from where it can be released back into the water (Unger et al., 1988). TBT has been shown to inhibit voltage gated sodium ion-channel activities in guinea pig (Unno et al., 2002), decrease dopamine, serotonin and norepinephrine levels in the brain of rats (Elsabbagh et al., 2002), and induce state of depression in the central nerve activity of fish due to decrease in neurotransmitters levels and down-regulation of their receptors (Yu et al., 2013). TBT is very toxic to aquatic life, causing acute and chronic effects to some sensitive aquatic organisms at concentrations as low as 1 to 2 ng L^{-1} (Hoch, 2001). Though, TBT application on materials has been banned but its persistence nature in sediments, illegal use in some countries (Okoro et al, 2011) and toxicity at very low

concentrations makes it an environmental contaminant of relevance (Hoch, 2001, Aono and Takeuchi, 2008).

These 3 chemical substances are known to alter behaviour of exposed aquatic organisms in addition to other effects, and are commonly detected in aquatic environment. Moreover, they are known to interact with some neurotransmitters (dopamine, serotonin) and/or voltage gated ion channels (sodium ion channels) and produce effects in non target organisms.

Freshwater planarians have been recommended as models for neurotoxicology (Hagstrom et al., 2015) and neuropharmacology (Buttarelli et al., 2000, 2008). Their nervous system is more comparable to that of vertebrates than invertebrates and they possess almost all mammalian neurotransmitters (Buttarelli et al., 2008). They have been used to study behavioural alterations associated to various psychoactive drugs (Palladini et al., 1996, Raffa and Valdez, 2001, Raffa and Desai, 2005, Pagan et al., 2009, Ramakrishnan and DeSaer, 2011, Rawls et al., 2011). Also, planarians have been used to evaluate effects of some neurotoxic compounds present in freshwater environments such as cadmium (Wu et al., 2014, 2015, Plusquin et al., 2012), methyl mercury (Best and Morita, 1982, 1991), tetradecanoyl-phorbol-acetate (TPA) (Best and Morita, 1991), Chlorantraniliprole (Rodrigues et al., 2016) and tributyltin (TBT) (Ofoegbu et al., 2016). These studies suggest freshwater planarians as potential sensitive species for ecotoxicological assessment of neuroactive or neurotoxic substances in aquatic systems. However, investigations on their responses to mixtures of pharmaceuticals or mixtures of pharmaceuticals with other chemical contaminants present in the aquatic environment are lacking.

In the present study, the combined effects of psychiatric pharmaceuticals fluoxetine and carbamazepine on *S. mediterranea* locomotor activity and asexual reproduction, and combined effects of fluoxetine and TBT on *S. mediterranea* locomotor activity, asexual reproduction by fissioning and on DNA damage were evaluated. This study addresses possible interactions of carbamazepine or TBT with fluoxetine toxicity, and the relevance of the endpoints used as well as concentrations tested is based on previous work on single exposure of planarians to all compounds (Ofoegbu et al., 2016, 2018). Effects on locomotion have been observed in planarians under exposure to TBT, carbamazepine and fluoxetine while TBT and fluoxetine has been shown to also cause an induction of DNA damage, Moreover, exposures to fluoxetine have been shown to impair fissioning of planarians.

4.2. Materials and Methods

Chemicals

The chemicals used for this experiment were tributyltin chloride (TBT, 96%, Aldrich), carbamazepine (Sigma Aldrich) and fluoxetine (fluoxetine chloride, TCI, 98.0%). TBT stock solution of 0.1 M and carbamazepine stock solution of 5 g L^{-1} were prepared in absolute ethanol. Stock solution of fluoxetine 10 mg L^{-1} was prepared in miliQ water. All stock solutions were stored in the dark at 4ºC. Test solutions for all experiments were prepared by diluting the stock solutions in ASTM hard water (ASTM, 2004), ensuring that ethanol concentration in all treatments was kept below 0.01%.

Experimental animals

Experimental animals were taken from already established freshwater planarian cultures in the laboratory. Freshwater planarian, *Schmidtea mediterranae* (asexual strain) was used. Laboratory culture procedure with ASTM medium (ASTM, 2004) has been described elsewhere (Ofoegbu et al., 2018). Exposures were carried out at 20 ± 1.0 ºC and in the dark to avoid any photodegradation of the compounds. Animals used for the experiment were starved for 1 week before behavioural tests and comet assay, and 4 days before the reproduction test to ensure uniformity in metabolic status of exposed organisms (Oviedo et al., 2008) and prevent any interaction between food and test substances (Wu and Persinger, 2011). Planarians used for behavioural test and comet assay were 5.0 ± 1.0 mm while those used for the reproduction test were 13.5 ± 1.5 mm in length.

Fluoxetine and carbamazepine combined exposures

Locomotor activity

Locomotor activity was assessed to determine the effects of combined exposures of two psychiatric pharmaceuticals fluoxetine and carbamazepine on planarian behaviour. Planarians were exposed to nominal concentrations of fluoxetine (0, 0.1 and 10 µg L^{-1}) within two concentrations of carbamazepine (0 or 10 µg L^{-1}).

For each experimental condition 5 replicates with 3 planarians per replicate were used. The animals were exposed in 150 mL glass crystallizing dish with 50 mL of experimental solution for 9 days with experimental solutions renewal every 3 days. Locomotor activity in planarians after

exposure was measured as already described in previous work Ofoegbu et al., (2016) with slight modifications. Precisely, each planarian was transferred from the crystallizing dish to the center of a clear plastic container of 21.2 cm x 18.5 cm x 1.9 cm dimensions placed on a 0.5 cm-gridline graph sheet containing 50 mL of experimental solution. The animal was allowed 30 seconds acclimation period and after the number of gridlines crossed during a period of 2 minutes under an average of 718 lux of white light was noted. The number of gridlines crossed or re-crossed by worms was used to calculate mean number of gridlines crossed.

Reproduction

Reproduction test to determine the effects of fluoxetine and carbamazepine on planarian asexual reproduction was carried out following methods by Best et al. (1981a, b), Best and Morita (1991) and Sheiman et al. (2003) with slight modifications. Adult planarians (30 in number) with tails tapering to a point showing fissioning ability and divided into 5 replicates were exposed in 150 ml glass crystallizing dish containing 100 mL of experimental solutions. There were 5 replicates per concentration with each containing 6 planarians. Experimental solutions were similar to that used for locomotor activity and exposure lasted for 9 days. During exposure animals were not fed and experimental solutions were renewed every 3 days. Before experimental solution renewal, planarian pieces in each replicate were removed and counted. After 9 days, the total number of planarian pieces from each replicate was added and results were reported as mean number of planarian pieces per experimental concentration.

Fluoxetine and TBT combined exposures

Locomotor activity

The effects of TBT on fluoxetine toxicity to planarians were evaluated by monitoring planarians' behaviour (locomotor activity) after exposure to mixture of the two compounds. Planarians were exposed to nominal concentrations of fluoxetine (0, 0.1and 10.0 µg L^{-1}) within two concentrations of TBT (0 or 0.25 µg L^{-1}).

For each experimental condition 5 replicates with 3 planarians per replicate were used. The animals were exposed in 150 mL glass crystallizing dish with 50 mL of experimental solution for 9 days with experimental solutions renewed every 3 days. Locomotor activity in planarians after exposure was measured as already described in combined exposures to carbamazepine and

fluoxetine above. Precisely, each planarian was transferred from the crystallizing dish to the center of a clear plastic container of 21.2 cm x 18.5 cm x 1.9 cm dimensions placed on a 0.5 cm-gridline graph sheet containing 50 mL of experimental solution. The animal was allowed 30 seconds acclimation period and after the number of gridlines crossed during a period of 2 minutes under an average of 718 lux of white light was noted. The number of gridlines crossed or re-crossed by worms was used to evaluate locomotor activity.

Reproduction

Reproduction test to determine the effects of fluoxetine and TBT on planarian asexual reproduction was carried out following methods by Best et al. (1981a, b), Best and Morita (1991) and Sheiman et al. (2003) with slight modifications. Adult planarians (30 in number) with tails tapering to a point showing fissioning ability and divided into 5 replicates were exposed in 150 ml glass crystallizing dish containing 100 mL of experimental solutions. Experimental solutions were the same as that used for locomotor activity. All exposures were carried out in a 150 mL crystallizing dish with 100 mL of experimental solutions and exposure lasted for 9 days. 5 replicates per concentration with each containing 6 planarians were used. Animals were not fed during the exposure period and experimental solutions were renewed every 3 days. Before experimental solution renewal, planarian pieces in each replicate were removed and the number recorded. After 9 days, the total number of planarian pieces from each replicate was added and results were reported as mean number of planarian pieces per experimental concentration.

Comet assay

DNA damage was evaluated by the comet assay. Planarians were exposed to combined fluoxetine and TBT nominal concentrations as were used in locomotor activity and asexual reproduction tests. Animals (3 replicates of 7 planarians per concentration) were exposed in 150 ml glass crystallizing dish with 100 ml of experimental solution. Exposure period was 9 days and experimental solutions were renewed every 3 days. The comet assay was carried out with slight modifications of the protocol described in previous work (Ofoegbu et al., 2016) following the method by Lourenço et al. (2011) and Prá et al. (2005) under yellow light to prevent UV-induced DNA damage. After exposure, animals in each replicate were pooled and subjected to chemical (using 0.48% trypsin) and mechanical disintegration, followed by centrifugation at 10,000 g for 2

mins at 4ºC. Ten microliters of the cell suspension from each replicate was mixed with 0.5% low melting point agarose and spread on microscope slides pre-coated with 1% normal melting point agarose. Then, slides were immersed in lysing solution (2.5 M NaCl + 100 mM EDTA + 10 mM Tris–HCl + 1% DMSO + 10% TritonX-100) at 4ºC, for 120 mins, in the dark. After lysis the slides were subjected to denaturation in alkaline buffer (0.3M NaOH and 1mM EDTA, pH 13) for 15 minutes, followed by electrophoresis at 0.7V/cm and 300 mA for 10 min on the same buffer. After electrophoresis, slides were neutralized using an iced cold 0.4 M Tris HCl (pH 7.5) solution. Slides were then stained using ethidium bromide and visualized with a fluorescence microscope (ZEISS Axio Scope A1). One hundred cells, per each slide condition, were randomly selected and visually categorized and grouped into 5 classes (0 to 4) depending on DNA damage as described by Garcia et al. (2004).

Statistical analysis

Effects of the tested chemicals on locomotor activity, DNA damage and reproduction were analyzed with two-way analyses of variance (ANOVA) followed by a Dunnets' post-hoc for multiple comparisons whenever significant effects for any of the factors or their interaction were observed. These helped in testing altered effects of fluoxetine under the presence of either carbamazepine or TBT. The arbitrary units for comet assay used for two-way analyses were obtained from an Excel programmed template which automatically calculates them by multiplying the observed comet by comet classification and summing the values per replicate. As there was no significant difference between control and solvent control groups (student *t* test, $p > 0.05$), the solvent control groups were used for comparisons. All data were checked for homogeneity of variances using Levene's test with IBM SPSS Statistics (version 21) normality with Kolmogorov-Smirnov normality tests. Also, significant level was set at 95% confidence interval (95% CI or 0.05). Calculations were made with GraphPad Prism version 5 for Windows and results were expressed as mean ± standard error of mean (SEM).

4.3. Results

Effects of fluoxetine and carbamazepine on planarians

Locomotor activity

Fluoxetine exposure significantly increased locomotor activity. This effect was independent of carbamazepine exposure which also induced a non significant, albeit slighter increase on planarian locomotor activity (fig 4.1, table 4.1). Planarians exposed to 10 µg L^{-1} of fluoxetine and carbamazepine (combined) showed a 50% increase on the number of gridlines crossed during observation period in comparison with the control treatment. Moreover, planarians exposed to combined fluoxetine and carbamazepine showed some sign of hyperactivity/restlessness within an acclimation period of less than 30 secs compared to those in the single compound and control treatments.

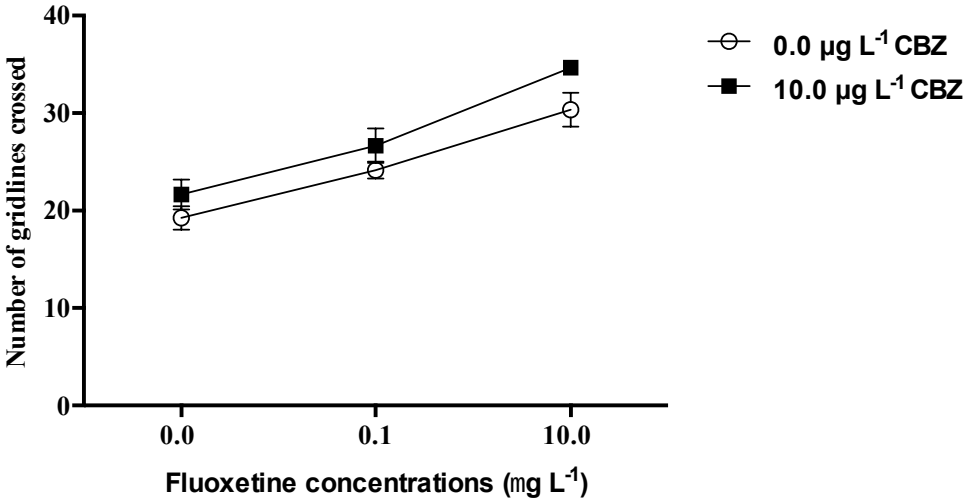

Fig. 4.1: Locomotor activity of *S. meditteranea* (mean ± standard error of mean SEM) under exposure to a gradient of fluoxetine concentrations and two levels of carbamazepine (CBZ).

Asexual reproduction

No significant interaction was observed between fluoxetine and carbamazepine exposures on planarians fissioning which was also not affected by exposure to carbamazepine alone (fig. 4.2, table 4.1). Planarians exposed to both concentrations of fluoxetine on the other hand showed a significant decrease on the number of fragments in comparison with the control treatments (fig. 4.2, table 4.1).

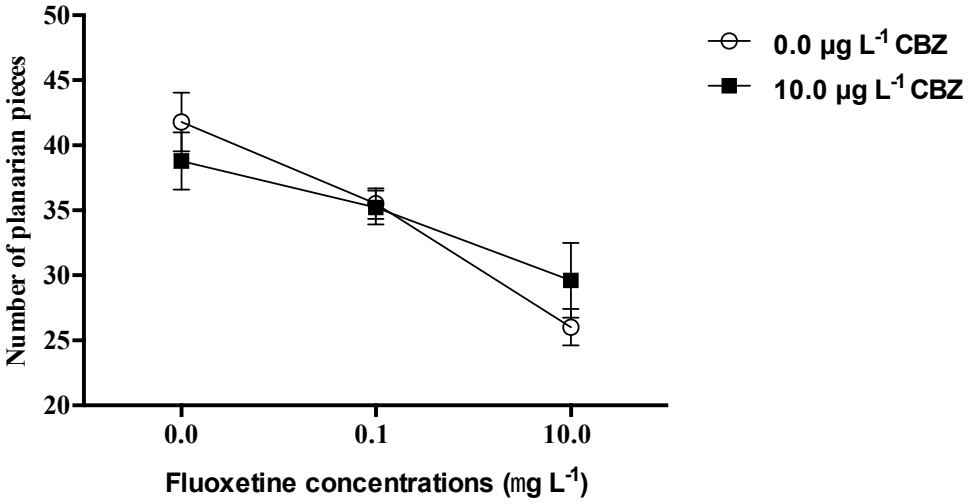

Fig. 4.2: Asexual reproduction of *S. meditteranea* (mean ± standard error of mean SEM) under exposure to a gradient of fluoxetine concentrations and two levels of carbamazepine (CBZ).

Table 4.1: Results of two-way ANOVAs testing for effects of fluoxetine (FLX), carbamazepine (CBZ) and of their interaction on *S. meditteranea* behavior and reproduction

	Degrees of Freedom	Sums of Squares	*F*	*p*-value	R^2
Locomotion					
FLX	2	732.00	39.79	<**0.001**	71.070
CBZ	1	71.46	7.77	**0.010**	6.937
FLX×CBZ	2	5.82	0.32	0.732	0.565
Fissioning					
FLX	2	791.70	19.77	<**0.001**	60.560
CBZ	1	0.07	0.00	0.953	0.005
FLX×CBZ	2	55.00	1.37	0.273	4.208

Effects of TBT and Fluoxetine on planarians

Locomotor activity

Fluoxetine and TBT had opposite effects on planarian locomotor activity. TBT exposure led to a significant decrease in the number of gridlines crossed by planarians within the observation period while significantly higher locomotor activity of *S. meditteranea* was observed under

exposure to 10 µg L^{-1} fluoxetine. However, these effects were not observed in treatments where organisms were simultaneously exposed to fluoxetine and TBT thus accounting for the significant interaction observed (fig 4.3, table 4.2).

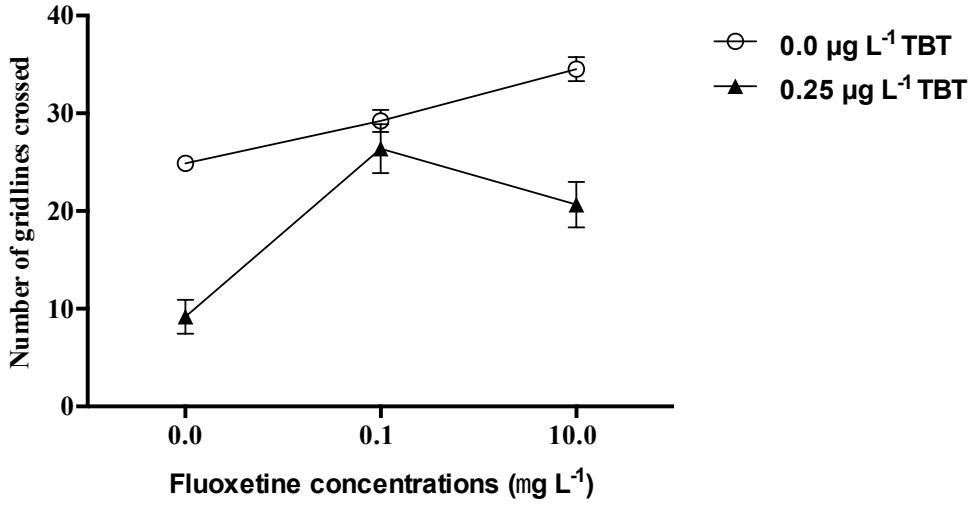

Fig. 4.3: Locomotor activity of *S. meditteranea* (mean ± standard error of mean SEM) under exposure to a gradient of fluoxetine concentrations and two levels of tributyltin (TBT).

Asexual reproduction

Despite the observed decrease in fissioning of planarians exposed to fluoxetine alone (fig 4) there was no significant effects of TBT and also no significant interaction between TBT and fluoxetine (table 4.2) meaning that significant reductions in fissioning caused by exposure to 10 µg L^{-1} of fluoxetine were independent of TBT.

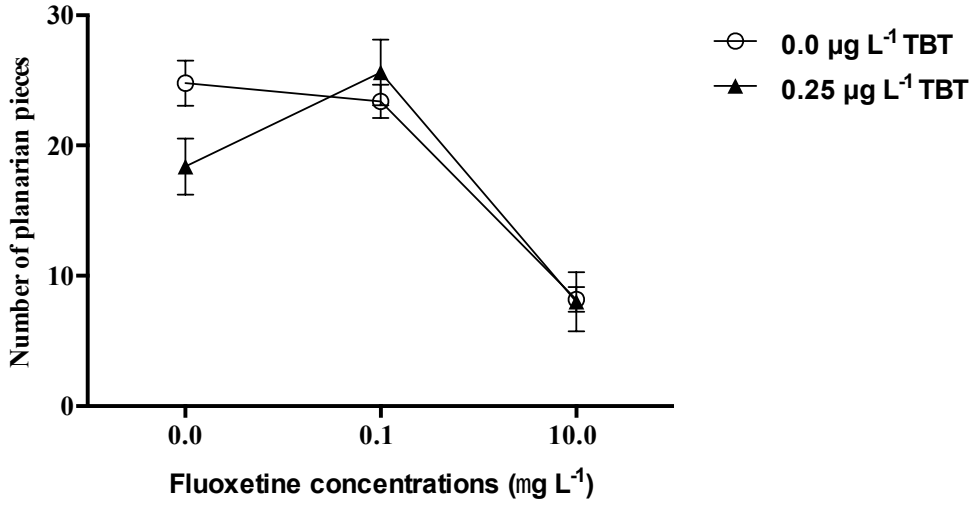

Fig. 4.4: Asexual reproduction of *S. meditteranea* (mean ± standard error of mean SEM) under exposure to a gradient of fluoxetine concentrations and two levels of tributyltin (TBT).

DNA damage

Exposure to fluoxetine led to a significant increase in DNA damage in planarians (fig 5, table II). However, there was a significant interaction between fluoxetine and TBT exposures as a decrease of DNA damage was observed in organisms exposed to both compounds simultaneously (fig 4.5, table 4.2). Despite no significant effects observed for TBT on main ANOVA the post-hoc test shows a significant increase in DNA damage caused by TBT (one-way ANOVA, $p < 0.05$) in treatments without fluoxetine

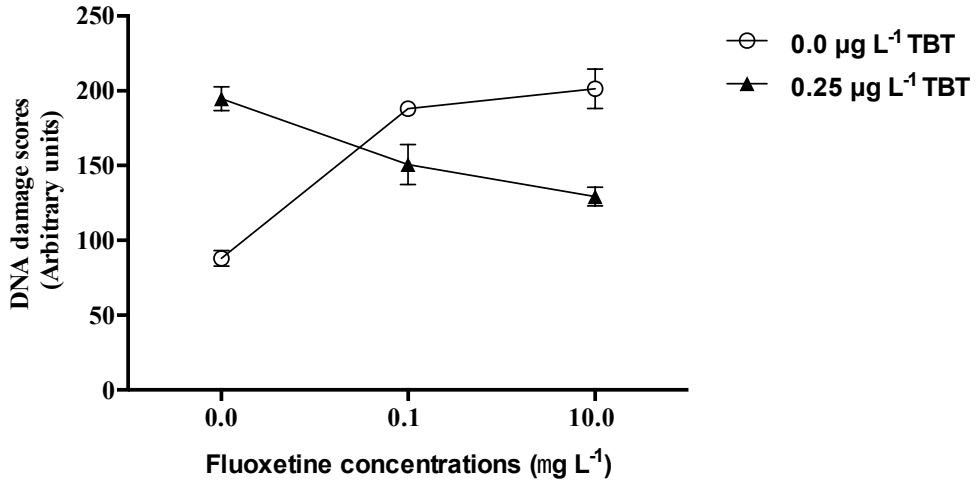

Fig. 4.5: DNA damage in S. *meditteranea* (mean ± standard error of mean SEM) exposed to a gradient of fluoxetine concentrations and two levels of tributyltin (TBT).

Table 4.2: Results of two-way ANOVAs testing for effects of fluoxetine (FLX), tributyltin (TBT) and of their interactions on *S. meditteranea* behavior, reproduction and DNA damage.

	Degrees of Freedom	Sums of Squares	*F*	*p*-value	R^2
Locomotion					
FLX	2	757.50	24.73	<**0.001**	33.860
TBT	1	869.50	56.78	<**0.001**	38.870
FLX×TBT	2	242.20	7.91	**0.002**	10.830
Fissioning					
FLX	2	1532.00	41.86	<**0.001**	73.450
TBT	1	16.13	0.88	0.357	0.774
FLX×TBT	2	98.47	2.69	0.088	4.721
DNA Damage					
FLX	2	2752.00	5.64	**0.019**	8.44
TBT	1	3.56	0.01	0.91	0.01
FLX×TBT	2	26930.00	55.21	<**0.001**	82.58

4.4. Discussion

Psychiatric drugs are nowadays commonly found in freshwaters and some such as fluoxetine can elicit adverse ecological effects. Research on different scenarios of exposure namely the concomitant presence of other compounds and how they mediate its toxicity, are needed. The results of this study showed that effects of low and environmentally relevant concentration of fluoxetine on a freshwater planarian species can be mediated by concomitant exposures to a neurotoxic compound such as tributyltin. These interactions showed a reduction of the effects induced by fluoxetine on planarians locomotor activity, and genotoxicity. At the same time no interactions were observed for exposures containing carbamazepine and fluoxetine.

Effects of each of the psychiatric drugs are consistent with research showing that neuroactive drugs can elicit increased locomotor activity in planarians (Ofoegbu et al., 2018). Fluoxetine and carbamazepine are known to increase dopamine and serotonin levels in organisms (Post et al., 1983, Wong et al., 1995, Ambrósio et al., 2002, Bymaster et al., 2002, Mennigen et al., 2008). Dopamine is involved in planarian locomotion (Wu et al., 2015) namely crawling (Nishimura et al., 2007), with an increase in level leading to increase in locomotor activity (Algeri et al., 1983). However, our results did not show significant interaction with respect to planarians' locomotor activity under combined carbamazepine and fluoxetine exposures. Possibly, both substances individually influenced planarian locomotor activity but under combined exposure the individual concentrations could not elicit prominent interactive effects on planarians. Another possibility may be associated to how each compound affects dopaminergic system. Fluoxetine is known to influence dopamine level through its interaction at the uptake sites and receptors (Wong et al., 1995) while carbamazepine influences dopamine level through activation of dopamine receptors (Post et al., 1983; Rivetti et al., 2016).

It is nonetheless important to note that we only scored locomotion by counting number of gridlines crossed by crawling planarians. Our observations however did detect some hyperactivity and restlessness in organisms exposed to combination of fluoxetine and carbamazepine after release in the measuring arena that obviously was not translated into numbers of gridlines crossed within the 2 mins observation period.

However, and despite the lack of research focused on the toxicity of mixtures of psychiatric pharmaceuticals towards invertebrates, studies have reported increases in risk of toxic effects of carbamazepine due to co-administration with fluoxetine in vertebrates (Levinson et al 1991, Pearson, 1990, Grimsley et al., 1991, Dursun et al., 1993) with hyper-activity and hyper-

reactivity among other effects due to co-treatment of carbamazepine with fluoxetine (Dursun et al., 1993) which authors associated to an increase in central serotonin and its relationship with various neurotransmitters. Additionally, overstimulation of dopaminergic neurotransmission in vertebrates, studies have shown may lead to hyperactivity (Staller and Faraone, 2007, Yu et al., 2013). This calls for additional behavioural endpoints to be pursued and also for the use of automated video tracking systems that are usually more convenient to evaluate alterations in behaviour such as locomotor velocity and activity in general (Rodrigues et al 2015).

However, a significant interaction on locomotor activity was observed in planarians exposed to a mixture of fluoxetine and TBT since the effects caused by fluoxetine (increased locomotor activity) were not observed when in the presence of TBT. This is probably because the opposite effects of both compounds in dopamine levels since TBT is known to interfere in dopamine and serotonin synthesis resulting to a decrease in their brain levels (Elsabbagh et al., 2002, Kim et al., 2002) and affect movement through its degenerative alteration of the surface epithelial layer and muscles (Fent and Meier, 1992).

Concerning asexual reproduction here measured by counting the number of planarian fragments resulting from fissioning in exposed organisms, there was no interactive effect of fluoxetine and carbamazepine or fluoxetine and TBT. Single exposures to carbamazepine did not affect fissioning of planarians while reduction in fission caused by TBT was observed despite lack of statistical significance. Asexual reproduction by fissioning in freshwater planarians is suppressed in the presence of increased melatonin level (Morita and Best, 1984, 1993) and controlled by longitudinal nerve cords and brain (Best et al., 1969, 1975). Additionally, DNA damage has been shown to also affect reproduction in organisms (Lee et al., 2000). Thus, reduction in fissioning due to exposure to fluoxetine might be associated to increased melatonin levels (Reierson et al., 2009, Kirecci et al., 2014) which have been implicated in suppression of fissioning in planarians (Morita and Best, 1984, 1993) or with DNA damage also induced by fluoxetine. TBT is also neurotoxic (Fent, 1996, Yu et al., 2013) and can affect planarian fissioning by its toxic effects on surface epithelial layer and muscles (Fent and Meier, 1992), and also possibly through DNA damage. In fact, preliminary experiments have shown that exposure to higher concentrations of TBT strongly reduced *S. mediterranea* fissioning (personal observation). The effects of combined exposure on fissioning were thus consistent with effects of exposure of planarians and other invertebrates to low levels of fluoxetine (Ofoegbu et al., 2018) given that carbamazepine or TBT have not been shown to influence melatonin levels.

The result showed an interactive effect of the TBT and fluoxetine on DNA damage in planarians under combined exposure conditions. Planarian exposures to single contaminant fluoxetine or TBT showed increased DNA damage but this effect was reduced in the combined exposure treatments. Induction of DNA damage caused by fluoxetine exposure has been associated to oxidative stress or immunotoxicity (Lacaze et al., 2015), while DNA damage due to TBT exposure has been linked to disruption of intracellular calcium homeostasis leading to increase in intracellular Ca^{2+} levels (Chow et al., 1992, Orrenius et al., 1992, Boelsterli, 2003), release of free radicals and disruption of mitochondrial activity (Gennarri et al., 2000, Mizuhashi et al., 2000, Wang et al., 2012). The interaction between fluoxetine and TBT might have led to decreased DNA damage through the interruption of the mechanisms of their individual genotoxicity and/or at the same time increased DNA synthesis. DNA synthesis in freshwater planarians has been shown to be triggered by increase in serotonin levels (Martelly et al., 1983, Martelly, 1984, Martelly and Franquinet, 1984). However, fluoxetine and TBT are known to have opposite effects on serotonin levels, whereas fluoxetine increases serotonin level to relieve depression (Wong et al., 1995), TBT decreases its level inducing a state of depression (Elsabbagh et al., 2002, Yu et al., 2013). Possibly, interaction between TBT and fluoxetine led to an increase in serotonin level with a resultant increase in DNA synthesis. This may have increased DNA repair rates, which might have contributed to the observed decrease in the amount of DNA strand breaks detected (Mazouzi et al. 2014).

In Summary, effects of exposure to mixtures of human pharmaceuticals or with other chemical contaminants on aquatic organisms may be very complex and not easily predictable. Thus, more ecotoxicological data is needed to unravel the unexpected but complex effects of mixtures of these environmental stressors. The freshwater planarian, *S. mediterranea* here displayed various responses to single and combined exposures depicting different levels of toxicity. The combined exposure to carbamazepine and fluoxetine is of ecological relevance since they are commonly co-occurring pharmaceuticals in the aquatic environments (Ternes, 1998, Heberer et al., 2002). However, these two psychiatric compounds are just a small portion of the large number of neuroactive pharmaceuticals and this alone calls for more research into their combined effects. Results also showed the possibility of neurotoxic compound like TBT to interfere with effects of neuroactive pharmaceuticals in situations of concomitant exposure in the wild, and the relevance of genotoxicity by comet assay and behavioural endpoint in the assessment of such complex interactions. This again calls for more research on mixture toxicity

assessments given the myriad of neuroactive pharmaceuticals and neurotoxic compounds found in the aquatic systems.

This research focused on wider ranges of concentrations of different compounds will benefit from the inclusion of planarian species such as *S. meditteranea* as test organisms for ecological risk assessment given their amenability to toxicity testing and ease of assessing genotoxic, behavioural and reproductive endpoints.

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Chapter 5

The influence of salinity on the effects of fluoxetine in freshwater planarian, *Schmidtea mediterranea*

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Abstract

Exposure to psychiatric pharmaceutical fluoxetine poses risk to freshwater planarian *Schmidtea mediterranea* behaviour and asexual reproduction. However, there is paucity of information on how these effects are influenced by natural stressors present in the freshwater habitats. Here, planarians which have been suggested as bio-indicator species in aquatic habitats were used to evaluate the influence of increasing salinity levels on effects of fluoxetine. To better understand the possible combined effects of these stressors on aquatic organisms, the effects of salinity, using sodium chloride (NaCl) as surrogate on *Schmidtea mediterranea* were first determined by evaluating survival, regeneration, behavioural and reproduction responses. Subsequently, the combined effects of NaCl and fluoxetine were assessed using behavioural and reproduction endpoints. Result showed that exposure to increased salinity is toxic to planarians with 48 and 96 hrs LC_{50s} of 9.15 and 7.55 g NaCl L^{-1} respectively. Also, sub-lethal levels of salinity reduced locomotor activity, feeding, reproduction, and delayed head regeneration. Moreover, some developmental malformations were observed in regenerating planarians exposed to salinity (1.5 and 3.0 g NaCl L^{-1}), as well as delayed wound healing after fissioning in adult worms and fissioned fragments which resulted into degeneration. Interaction between fluoxetine and salinity were only observed in *S. mediterranea* locomotor activity since fluoxetine exposures induced increased activity of planarians. Despite significant reduction caused by exposures to fluoxetine and salinity sub-lethal levels, no interactive effects were observed for asexual reproduction in the combined exposures. Additionally, unlike planarians exposed to fluoxetine alone, fissioned planarians and their pieces from combined exposure (fluoxetine and salinity 3.0 g NaCl L^{-1}) were unable to regenerate missing portions. Results show that *S. mediterranea* is sensitive to low levels of salinity, and that this stressor can alter the effects of fluoxetine and cause deleterious effects on regenerating organisms. The implication of these effects on the planarian population in the natural habitat is discussed as well as the need for more research on the effects of neuroactive pharmaceuticals under relevant exposure scenarios.

Keywords: Natural stressors, Fluoxetine, Behaviour, Asexual reproduction, Regeneration, Freshwater planarians

5.1 Introduction

Pharmaceuticals used in human medicine are usually excreted as parent compound or metabolite through urine and faeces into wastewater (Fent et al., 2006). The sewage treatment plants being inefficient to completely degrade or remove these substances results in their presence in effluents discharged in various freshwater bodies and subsequently in drinking water (Jones et al., 2001, Heberer, 2002a, Kolpin et al., 2002, Kümmerer, 2009, Huber et al., 2016). The concentrations of these chemical compounds have been reported to be in ng and µg L^{-1} (Heberer, 2002b, Kolpin et al., 2002) but may reach mg L^{-1} in places where there are pharmaceutical production facilities (Larsson et al., 2007, Philips et al., 2010) in some places. Nevertheless, these environmental concentrations have been shown to alter some physiological processes in aquatic organisms (Fent et al., 2006).

In addition to pollutants of pharmaceutical origin, the freshwater environment naturally is subjected to stress by environmental factors such as temperature, salinity, pH and ultra violet radiation, which are affected by climate change. Though, there is increased awareness on the impact of pharmaceuticals on aquatic organisms based on numerous studies with various species, little is known about the potential influence of environmental factors on the toxicity of pharmaceuticals. There are also evidences that environmental factors such as salinity, pH, temperature and UV can affect toxicity of pharmaceuticals (Nakamura et al., 2008, Donner et al., 2013, González-Ortegón et al., 2013). However, considering variability in responses of organisms to pharmaceutical substances, more studies involving mixtures of pharmaceuticals and these environmental factors on non target and non model aquatic organisms are needed for assessment of the impact of these compounds. Studies of this sort are important for fluoxetine known to be among the most commonly used pharmaceuticals, most commonly detected pharmaceutical substance in freshwater system and one of the most toxic to non target aquatic organisms (Heberer, 2002b, Kolpin et al., 2002, Fent et al., 2006, Nentwig, 2008)

Fluoxetine is an antidepressant which inhibits the re-uptake of serotonin from synaptic space to pre-synapse and affects uptake and receptors of other neurotransmitter systems such as dopaminergic, GABAergic, melatonergic, adrenergic systems etc (Wong et al., 1995, Bymaster et al., 2002, Fent et al., 2006, Reierson et al., 2009, Kirecci et al., 2014). Fluoxetine

concentrations may reach 590 ng L^{-1} in sewage effluents, 320 ng L^{-1} in surface waters (Weston et al., 2001) and 19.37 ng g^{-1} in sediments (Schultz et al., 2010). Studies with aquatic organisms from different phylogenies showed that environmentally relevant concentrations of fluoxetine altered their behaviour, reproduction, development and growth (Foran et al., 2004, Weinberger and Klaper, 2014, Rivetti et al., 2016).

Increasing salinity of freshwater systems is an ecological problem that has been neglected over the years. Salinity often times refers to sodium, calcium, magnesium, potassium, chloride, sulphate, carbonate and hydrogen carbonate ions, and their increases in freshwaters have been attributed to human activities and natural sources (Cañedo-Argüelles et al., 2013). Anthropogenic activities resulting to salinity increases include agricultural practices such as removal of vegetation/deforestation and irrigation, use of road salt as de-icer during winter and dust suppression during summer, mining (salt and coal), manufacturing activities and effluents (Hart et al., 1991, Kaushal et al., 2005, Sanzo and Hecnar, 2006, Cañedo-Argüelles et al., 2013). Natural sources of salinity increases include rise in sea level with increased coastal flooding and erosion, increased rate of evaporation from freshwater bodies, storm and tropical cyclones, weathering, dissolved salts in rainwater and sea sprays (Badjeck et al., 2010, Cañedo-Argüelles et al., 2013, Chand et al., 2015). Salinity level up to 300 g L^{-1} due to agricultural practices, 25% of sea water concentration due to use of salt as de-icer and in road construction, up to 40-fold increase due to mining and 13.64 to 17.08 g L^{-1} due to cyclone in freshwaters and more than 0.001 g L^{-1} in rainwater have been reported (Hart et al., 1991, Thunqvist, 2004, Kaushal et al., 2005, Mitra et al., 2011, Cañedo-Argüelles et al., 2013).

Sodium chloride, one of the most commonly used and detected salts, has been shown to be conserved and not subject to appreciable loss in aquatic system (Sanzo and Hecnar, 2006). When NaCl is released in water, it dissociates into its Na and chloride components. Na is highly soluble in water, adsorbs to soil particles/sediments and may be taken up in biological systems (Sanzo and Hecnar, 2006). Also, Na can alter freshwater ecosystem processes through cation competition by displacing Iron II (Fe^{2+}) and Ammonium (NH_4^+) ions resulting to their release from sediments into surface water (Cañedo-Argüelles et al., 2013). Chloride ion on the other hand, does not adsorb to soil particles/sediments but is known to accumulate in bottom sediments where it may take several years or centuries before it reaches steady state (Sanzo and Hecnar, 2006). Moreover, Cl^- in water sediment may form strong complexes with heavy metals like mercury and cadmium, and release them from sediment to surface water, or it may interact with

suspended particles and precipitate as solids in freshwaters systems (Sanzo and Hecnar, 2006, Cañedo-Argüelles et al., 2013). These heavy metal ions - Hg^{2+} or Cd^{2+} in media with non-stressful Cl^- or low salinity level may be less toxic to aquatic animals (Cañedo-Argüelles et al., 2013) but their effects at high salinity levels is uncertain. Environmental NaCl levels over 30 g NaCl L^{-1} (18 g Cl^- L^{-1}) due to de-icing, in road water run-off from snow-melt and 50 g NaCl L^{-1} (30 g Cl^- L^{-1}) due to mining activities in a freshwater system has been reported (Cañedo-Argüelles et al., 2013).

Effects of increasing salinity on exposed aquatic organisms include risk of high osmotic stress, which at very high salinity levels may lead to cellular damage or death (Cañedo-Argüelles et al., 2013, Chand et al., 2015). Increasing salinity can also cause formation of salt gradient in the aquatic system which interrupts oxygen transport to the benthos and a resultant reduction in oxygen concentration leading to anoxia and death of benthic organisms (Legovic et al., 1991, Cañedo-Argüelles et al., 2013). Furthermore, sedimentation of suspended particles on stream substrate due to increased salinity levels may reduce light filtration to water column with a resultant algal bloom (Cañedo-Argüelles et al., 2013) which may have negative consequences on aquatic animals. In addition, high NaCl levels can affect survival, behaviour, reproduction, development and growth in exposed freshwater organisms with a resultant alteration in species diversity (Sanzo and Hecnar, 2006, Ghazy et al., 2009, Cañedo-Argüelles et al., 2013, Chand et al., 2015, Venâncio, 2017).

Planarians are benthic organisms with a predator and prey role and have been recognized as bio-indicator species of freshwater environment (Knakievicz, 2014). They have been used in neuropharmacology studies in association to various psychoactive drugs (Palladini et al., 1996, Raffa and Valdez, 2001, Raffa and Desai, 2005, Pagan et al., 2009, Ramakrishnan and DeSaer, 2011, Rawls et al., 2011). Moreover, some freshwater planarians have been used in studies involving salinity effects on freshwater organisms (Webb et al., 1971, Legner et al., 1976, Rivera and Perich, 1994). Through these studies freshwater planarians appeared to be potential sensitive species for ecotoxicological assessment of pharmaceutical substances and salinity in aquatic systems.

Since fluoxetine or increasing salinity are known to cause deleterious effects on aquatic organisms, and may be common factors in some aquatic environments, the present study is aimed at evaluating the influence of increasing salinity on the effects of fluoxetine on freshwater planarian *S. mediterranea*, using locomotion and reproduction as endpoints.

For that, an initial evaluation of salinity toxicity to freshwater planarian *S. mediterranea* using mortality, behavioural (locomotor activity and feeding), regeneration and reproduction (asexual reproduction) endpoints was carried out. This initial test helped in establishing sensitivity of this planarian species to salinity gradients under laboratory conditions and in the selection of test concentrations for the combined exposure. The relevance of these endpoints as well as concentrations of fluoxetine tested is based on earlier work on single exposure of planarians to low concentrations of fluoxetine (Ofoegbu et al., 2018).

5.2 Materials and Methods

Chemicals

Fluoxetine (fluoxetine chloride, TCI, 98.0%) and salt, sodium chloride (NaCl, EMSURE®) were used in the experiment. A stock solution of salt 50 g NaCl L^{-1} was dissolved in milliQ water. Stock solution of fluoxetine was prepared by dissolving 10 mg L^{-1} in miliQ water. Stock solutions were protected from light and stored at 4ºC. Experimental solutions were diluted in ASTM hard water (ASTM, 2004).

Experimental animals

Freshwater planarians, *Schmidtea mediterranea* (asexual strain) used in this study were taken from planarian cultures in the laboratory. The protocol on how these animals were maintained in the laboratory with ASTM medium (ASTM, 2004) had been described in previous paper (Ofoegbu et al., 2016, 2018). Exposures were carried out in the dark to avoid any interference by changes in light and at 20 ± 1 ºC. Experimental animals were starved for 1 week before acute toxicity tests, behavioural and regeneration tests, and for 4 days before reproduction tests to ensure uniformity in metabolic status of animals and to avoid any interaction between food and the compounds. The size of animals used for reproduction (fissioning) tests were 13.5 ± 1.5 mm and 5.0 ± 1.0 mm for the other tests.

Salinity single exposures

Acute toxicity test

Planarians were exposed to salt nominal concentrations of 1.27, 1.79, 2.53, 3.57, 5.03, 7.09, 10.0 and 14.1 g NaCl L^{-1} and a control (ASTM medium only). A total of ten replicates per

concentration each with 1 planarian were exposed in a 35 ml glass crystallizing dish containing 20 ml of experimental solution. The experiment lasted for 96 hours and experimental water was renewed every 48 hrs. The experiment was checked daily for mortality and worms with degenerating body or no movement under strong light were considered dead.

Behavioural parameters

Planarians were exposed to nominal salt concentrations 0.375, 0.75, 1.5 and 3.0 g NaCl L^{-1} and a control (ASTM medium only). Five replicates per concentration with three planarians in each replicate were exposed in 150 ml glass crystallizing dish containing 50 ml of experimental solution. Exposure lasted 9 days with experimental solutions renewal every 3 days. Similar exposure procedure was applied to planarians used for locomotion and post exposure feeding tests.

Locomotion activity: Locomotor activity in planarians after exposure was measured as already described in earlier work Ofoegbu et al., (2016) with slight modifications. Precisely, each planarian was placed at the center of a clear plastic container of 21.2 cm x 18.5 cm x 1.9 cm dimensions on a 0.5 cm gridline graph sheet and containing 50 mL of experimental solution. The number of gridlines crossed during a period of 2 minutes after 30 seconds of acclimation under an average of 718 lux of white light was recorded for each individual animal and results are expressed as mean number of gridlines covered per experimental concentration.

Post exposure feeding: After exposure period of 9 days, planarians in each replicate were transferred to 35 mL glass crystallizing dish with 20 mL of ASTM hard water and 60 3-days old chironomid larvae. The age and size of the larvae were chosen based on results of range finding tests. The larvae and the planarians were left for 24 hrs and results are reported as mean number of larvae consumed per concentration after 24 hrs.

Head regeneration

Planarians similar in size to those used in behavioural endpoint were used. Experimental concentrations were similar to that used for behavioural test and each test concentration consisted of 5 replicates each with 3 planarians exposed in 50 mL of experimental solution for 9 days. After 9 days of exposure, each planarian was decapitated before the pharynx and exposed for another 9 days. The number of replicates per concentration, number of organisms and the

procedure for exposure were similar as before decapitation. Experimental solution was renewed every 3 days during this period (before and after decapitation). Process of head regeneration was followed daily by microscopic examination of decapitated worms with Zeiss stereo microscope (KL 300 LED). The number of days for photoreceptor formation and any abnormality for each organism in each replicate were noted. The result is reported as mean number of days for photoreceptor formation per experimental concentration.

Reproduction

Freshwater planarians used in this study are asexual (fissiparous) strains of *S. mediterranea*, as a result the effects of salinity on fissioning were investigated. Test concentrations used were similar to concentrations used in behavioural tests. Planarians asexual reproduction test following methods by Best et al. (1981a, b), Best and Morita (1991) and Sheiman et al. (2003) but modified slightly was used to determine the effects of salinity on fissioning. Planarians with pointed tail showing fissioning ability were exposed to nominal concentrations of NaCl and a control (ASTM only) for 9 days. Each concentration had 4 replicates, each with 6 planarians exposed in a 150 mL glass crystallizing dish containing 100 mL of experimental solution. Exposed animals were not fed during the test and test solutions were renewed every 3 days. Planarian pieces resulting from fissioning were removed and number noted before experimental water renewal. After the exposure period, the total number of planarian pieces from each replicate was added and results were reported as mean number of planarian pieces per experimental concentration. Any morphological changes in fissioning adults and fragments due to exposure were also noted.

Combined exposure to fluoxetine and salinity

The size of planarians used, the protocol for exposure and measurement of locomotor activity and asexual reproduction by fissioning were similar to the single salinity (NaCl) exposure. Planarians were exposed to three nominal concentrations of fluoxetine (0, 0.1 or 10 µg FLX L^{-1}) within three concentrations of salinity (0, 0.75 or 3.0 g NaCl L^{-1}) (table 5.1).

Table 5.1: Experimental concentrations for combined exposures of fluoxetine and salinity

	Experimental concentrations		
	0.0 g NaCl L^{-1}	0.75 g NaCl L^{-1}	3.0 g NaCl L^{-1}
0.0 µg FLX L^{-1}	0.0 µg L^{-1}	0.75 g NaCl L^{-1}	3.0 g NaCl L^{-1}
0.1 µg FLX L^{-1}	0.1 µg FLX L^{-1}	0.1 µg FLX L^{-1} + 0.75 g NaCl L^{-1}	0.1 µg FLX L^{-1} + 3.0 g NaCl L^{-1}
10.0 µg FLX L^{-1}	10.0 µg FLX L^{-1}	10.0 µg FLX L^{-1} + 0.75 g NaCl L^{-1}	10.0 µg FLX L^{-1} + 3.0 g NaCl L^{-1}

Statistical analysis

The 48 and 96 hours 50% lethal concentration, LC_{50} at 95% confidence interval (CI) for salinity were calculated by Probit analysis with IBM SPSS Statistics (version 21). Effects of salinity (single exposure) on locomotion, feeding, regeneration and reproduction were analyzed with one way analysis of variance (ANOVA) followed by Dunnett's post hoc for significant difference between exposed and non exposed control or linear trend post hoc. All data were checked for normality with Kolmogorov-Smirnov normality tests and homogeneity of variances using Levene's test. Effects of combined exposures (fluoxetine and salinity) on locomotor activity and fissioning were analyzed with two way ANOVA followed by Dunnett's post hoc tests for multiple comparisons whenever significant effects for any of the factors or their interaction were observed. These helped in testing altered effects of fluoxetine under the influence of NaCl. Significant level was set at 95% confidence interval (95% CI or 0.05). All calculations were made with GraphPad Prism version 5 for Windows and results were expressed as mean ± standard error of mean (SEM).

5.3 Results

Salinity single exposure

Acute toxicity

Increased salinity in culture medium was toxic to exposed planarians. The estimated 48 and 96 hrs LC_{50s} (95% confidence interval [CI]) were 9.15g NaCl L^{-1} (7.73 - 10.85 g NaCl L^{-1}) and 7.55 g NaCl L^{-1} (6.55 - 8.82 g NaCl L^{-1}) respectively. In addition, planarians showed signs of acute toxicity such as increased secretion of mucous which made experimental solutions to

appear cloudy, loss of head, degeneration of pharyngeal part and lesions/ulcerative wounds on the body before death.

Behaviour

Locomotor activity

Although the analysis of variance did not detect any significant differences in locomotor activity caused by exposure to NaCl ($F_{(4, 15)} = 1.97$, $p > 0.05$, fig. 5.1a), a post hoc linear trend analysis revealed a significant monotonic and linear decrease in locomotor activity with increasing concentrations of NaCl (slope = - 1.33, $p = 0.017$, $r^2 = 0.24$).

Feeding

Feeding activity in planarians was significantly reduced in planarians after 9 days exposure to NaCl ($F_{(4, 15)} = 5.07$, $p < 0.05$, fig. 5.1b). Exposed planarians fed less compared to the control treatment with a LOEC (lowest observed effect concentration) of 0.75 g NaCl L^{-1} salinity. There was a significant linear reduction in feeding (slope = -3.182, $p = 0.0004$, $r^2 = 0.458$) and feeding rates were reduced by 24.32, 34.27 and 32.43% in planarians exposed to 0.75, 1.5 and 3.0 g NaCl L^{-1} respectively in comparison with the control treatment.

Head regeneration

Head regeneration measured as days for photoreceptor formation in planarians exposed to salinity (NaCl) before and after decapitation was delayed ($F_{(4, 15)} = 6.63$, $p < 0.05$, fig. 5.1c). Number of days for photoreceptor formation in exposed decapitated worms was significantly increased with a LOEC of 1.5 g NaCl L^{-1}. Days for head regeneration of planarians exposed salinity also followed a significant linear increase with increasing concentration (slope = +0.34, $p < 0.0001$, $r^2 = 0.548$). The wound at decapitation site of 4 planarians exposed to 3.0 g NaCl L^{-1} failed to heal. Consequently, the site where the new blastema was to form degenerated by the third day (plate 5.1a) compared to non exposed planarians (control) which formed blastema by 3rd day (plate 5.1b). Also, an additional 4 planarians that managed to form blastema had it degenerating by 5th day. By the end of 9 days post head decapitation exposure period, all 4 planarians with delayed decapitation-wound healing formed photoreceptors but on malformed/underdeveloped blastema (plate 5.1d) and 1 of them had just one photoreceptor (plate

5.1e) compared to the control (plate 5.1c). On the other hand, all 4 planarians with degenerating blastema, degenerated completely by the end of day 9 after decapitation but only 1 managed to form photoreceptor while 3 degenerated without forming any photoreceptor.

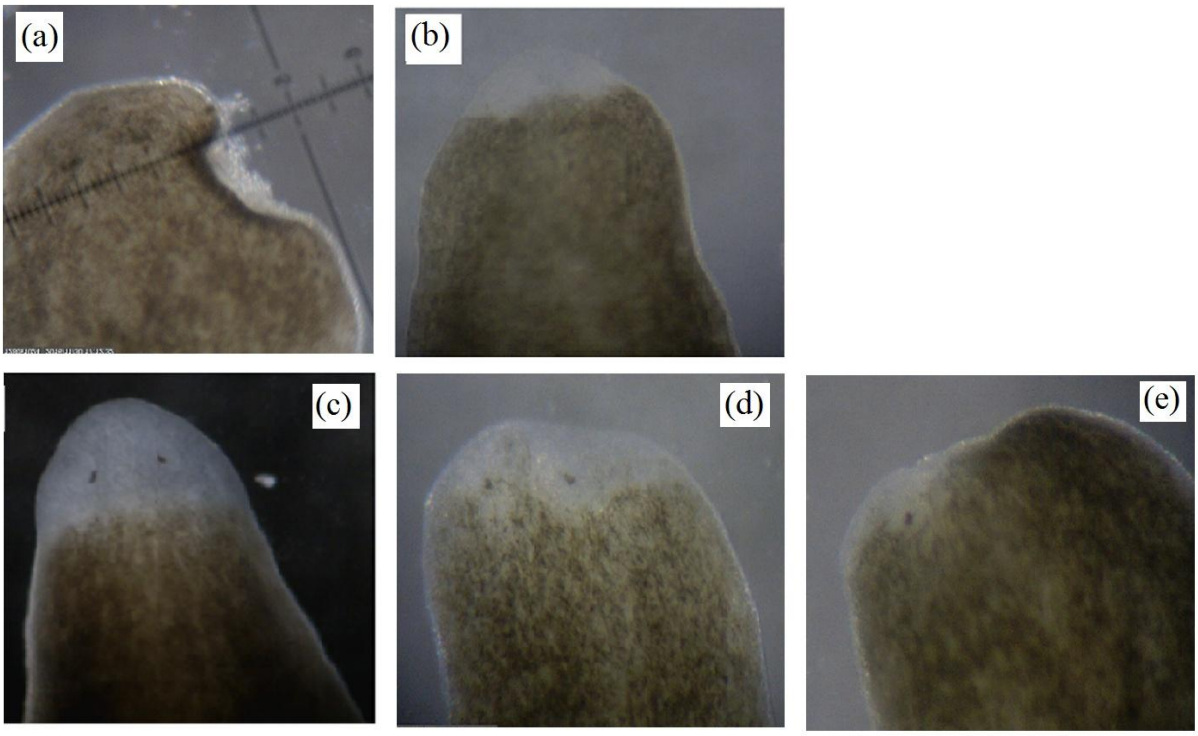

Plate 5.1: Pictures showing planarian *S. mediterranea* after decapitation under control and salinity (NaCl) exposures: **(a)** Decapitated *S. mediterranea* exposed to 3.0 g NaCl L^{-1} showing degenerating anterior end right after decapitation (day 3); **(b)** Decapitated *S. mediterranea* from control conditions with newly formed blastema (day 3); **(c)** *S. mediterranea* from control conditions with newly formed photoreceptor on normally shaped blastema; **(d)** *S. mediterranea* exposed to 3.0 g NaCl L^{-1} with newly formed photoreceptors on underdeveloped/abnormally shaped blastema; **(e)** *S. mediterranea* exposed to 3.0 g NaCl L^{-1} with underdeveloped/abnormally shaped blastema and only 1 photoreceptor on the body.

Asexual reproduction by fissioning

The effects of exposure to NaCl on fissioning followed a non-monotonic trend, with a slight increase at lower concentration (0.75 to 1.5 g NaCl L^{-1}) while at 3.0 g NaCl L^{-1} there was a significant decrease of 28.19% of number of planarian pieces produced in comparison with the control treatment ($F_{(4, 24)} = 8.46$, $p < 0.05$, fig. 5.1d). Generally, *S. mediterranea* pieces and the adults exposed to salinity 1.5 g NaCl L^{-1} (plate 5.2e, f) and 3.0 g NaCl L^{-1} (plate 5.2h, i) had their site of disintegration degenerating compared to non exposed organisms (plate 5.2a, b, c). At the

end of 9 days and as a result of the unhealed wounds observed in the fissioning adults, complete degeneration (death) was recorded for 1 planarian exposed to 1.5 g NaCl L^{-1}, and 3 in 3.0 g NaCl L^{-1}, while 1 in 1.5 g NaCl L^{-1} (plate 5.2d) and 2 in 3.0 g NaCl L^{-1} (plate 5.2g) showed tears on their bodies. During the 9 days exposure period, mortalities of 4.17% and 12.5% were observed for the two highest salinity concentrations respectively, but no mortality was observed among non exposed planarians.

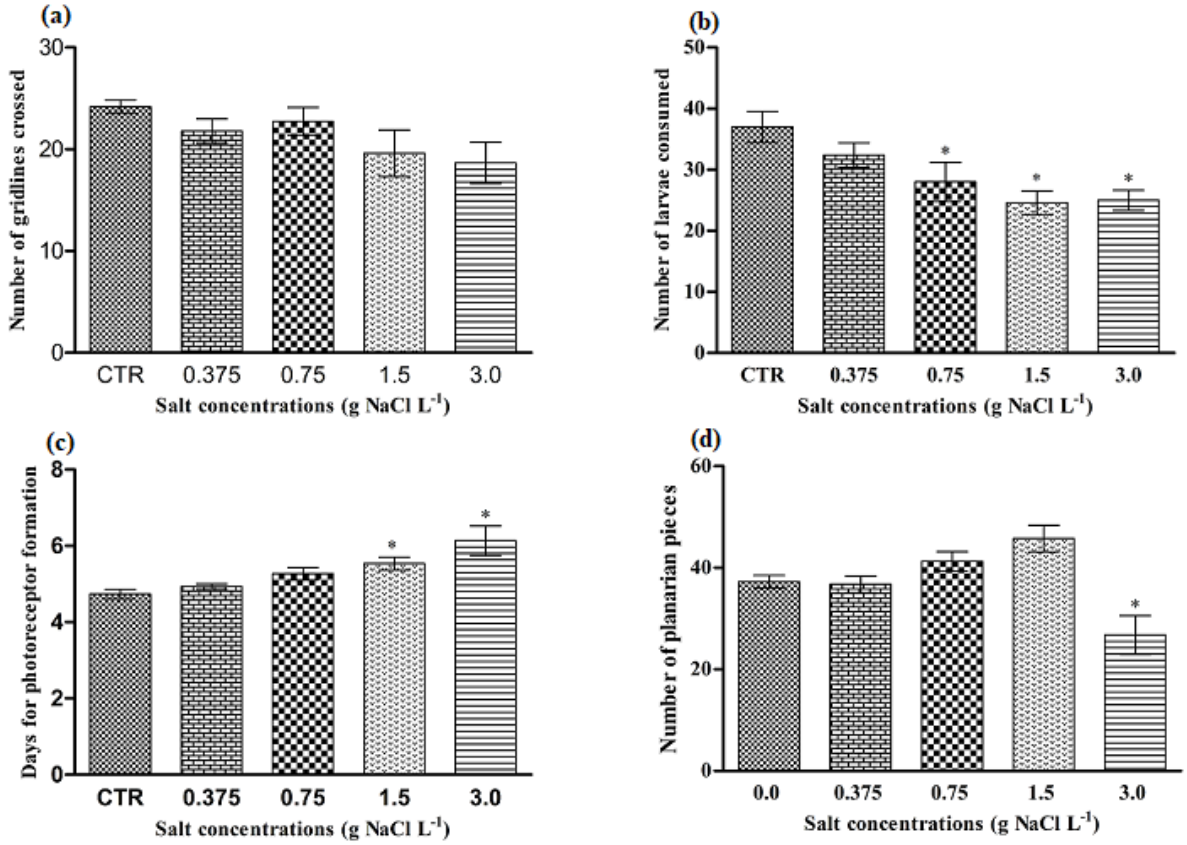

Fig. 5.1: Effects of salinity (NaCl) on *S. mediterranea* responses (mean ± standard error of mean SEM): **(a)** Locomotor activity; **(b)** Feeding rate; **(c)** Head regeneration; **(d)** Fissioning. * denotes significant differences compared to the control (CTR) treatment (Dunnett's test $p < 0.05$).

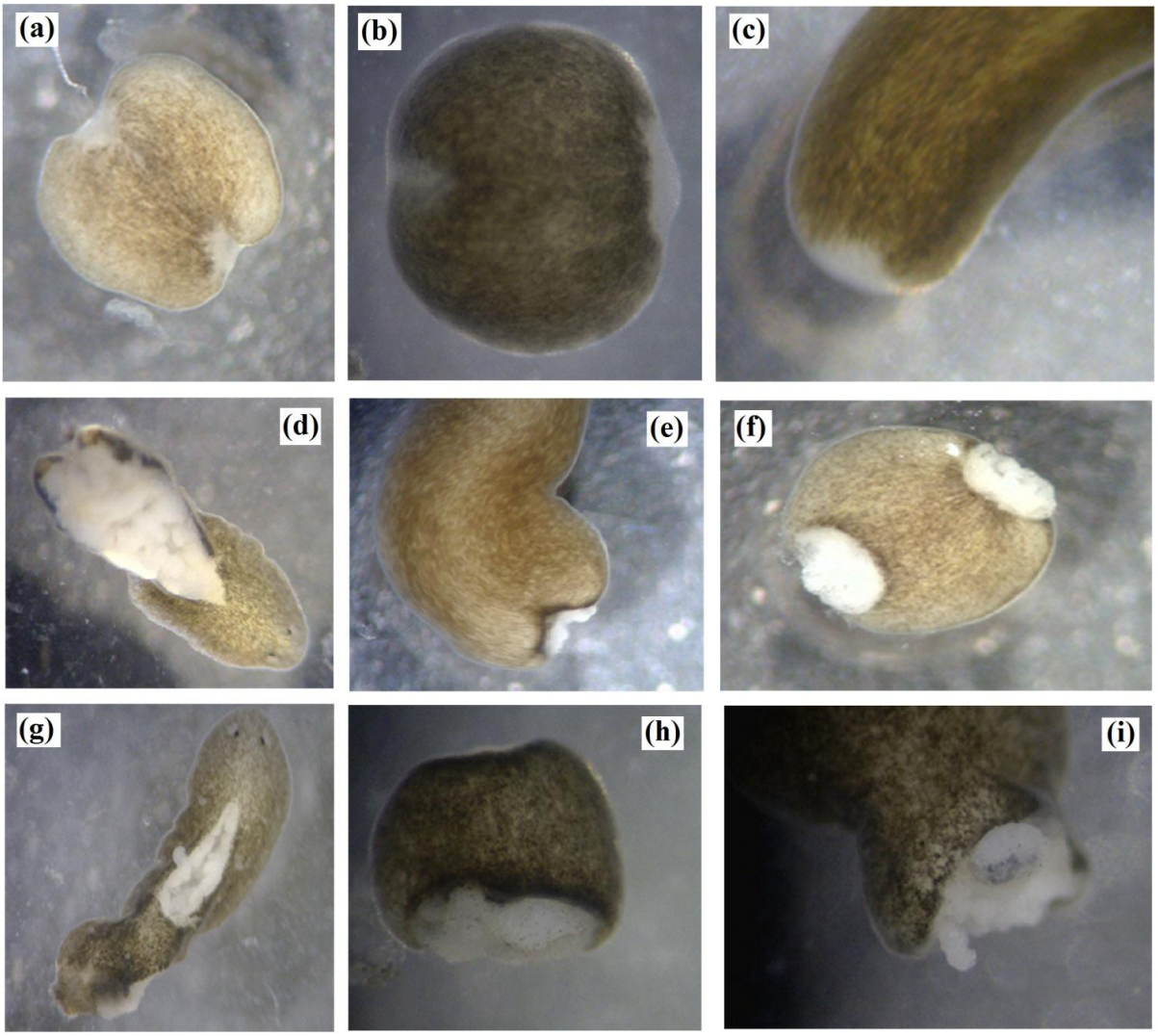

Plate 5.2: Pictures showing *S. mediterranea* during fissioning under control and salinity (NaCl) exposures: **(a, b)** *S. mediterranea* fissioned pieces regenerating missing parts under control conditions; **(c)** Regenerating posterior end of fissioned *S. mediterranea* under control conditions; **(d)** *S. mediterranea* exposed to 1.5 g NaCl L^{-1} with tear on the body; **(e)** *S. mediterranea* fissioned adult exposed to 1.5 g NaCl L^{-1} showing degenerating posterior end; **(f)** *S. mediterranea* fissioned piece under exposure to 1.5 g NaCl L^{-1} showing degenerating ends; **(g)** *S. mediterranea* adult exposed to 3.0 g NaCl L^{-1} with tear on the body; **(h)** *S. mediterranea* fissioned piece exposed to 3.0 g NaCl L^{-1} with degenerating ends; **(i)** *S. mediterranea* fissioned adult exposed to 3.0 g NaCl L^{-1} showing degenerating posterior end.

Combined exposure to fluoxetine and salinity

Locomotor activity

Fluoxetine and NaCl had opposite effects on planarian locomotor activity. NaCl exposure led to a significant decrease in the number of gridlines crossed by planarians within the observation

period while higher locomotor activity of *S. mediterranea* was observed under exposure to 10 µg L^{-1} fluoxetine (fig 5.2, table 5.2). However, effects of salinity were more pronounced under concomitant exposures to fluoxetine thus accounting for the significant interaction observed (fig 5.2, table 5.2).

Asexual reproduction

Both salinity and fluoxetine exposures significantly reduced reproduction of *S. mediterranea* and no significant interaction was observed between the two stressors (fig 5.3, table 5.2). Fissioning in planarians exposed to 0.75 g NaCl L^{-1} and 10.0 µg L^{-1} fluoxetine, and 3.0 g NaCl L^{-1} and the two concentrations of fluoxetine were significantly different from those under control conditions.

Generally, the pieces and the adults exposed to 3.0 g NaCl L^{-1} in mixtures with fluoxetine (0.1 and 10.0 µg L^{-1}) had their site of fissioning degenerating similar to planarians exposed to 3.0 g NaCl L^{-1} only (plate 5.2h, i). Although these planarians under combined exposure to fluoxetine and NaCl were in very bad condition, no mortality or tear on their bodies was observed.

Table 5.2: Results of two-way ANOVAs testing for effects of fluoxetine (FLX), Salinity (NaCl) and of their interaction on *S. mediterranea* behaviour and asexual reproduction

	Degrees of Freedom	Sums of Squares	*F*	*p*-value	R^2
Locomotion					
FLX	2	48.39	1.87	0.169	3.81
NaCl	2	607.6	23.50	< **0.001**	47.87
FLX× NaCl	4	147.8	2.86	**0.0373**	11.65
Fissioning					
FLX	2	2659	49.21	< **0.0001**	51.96
NaCl	2	1502	27.81	< **0.0001**	29.36
FLX× NaCl	4	227.0	2.101	0.108	4.44

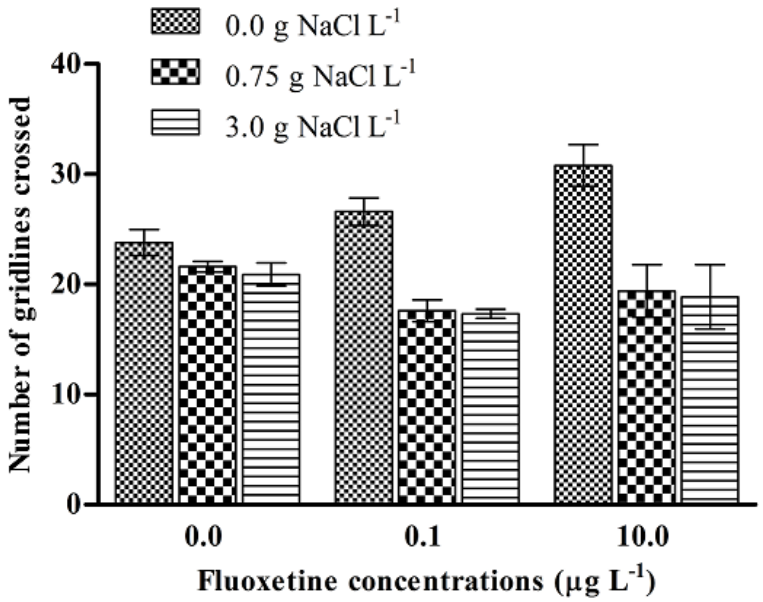

Fig. 5.2: Locomotor activity of *S. meditteranea* (mean ± standard error of mean [SEM]) under exposure to a gradient of fluoxetine and salinity (NaCl) concentrations.

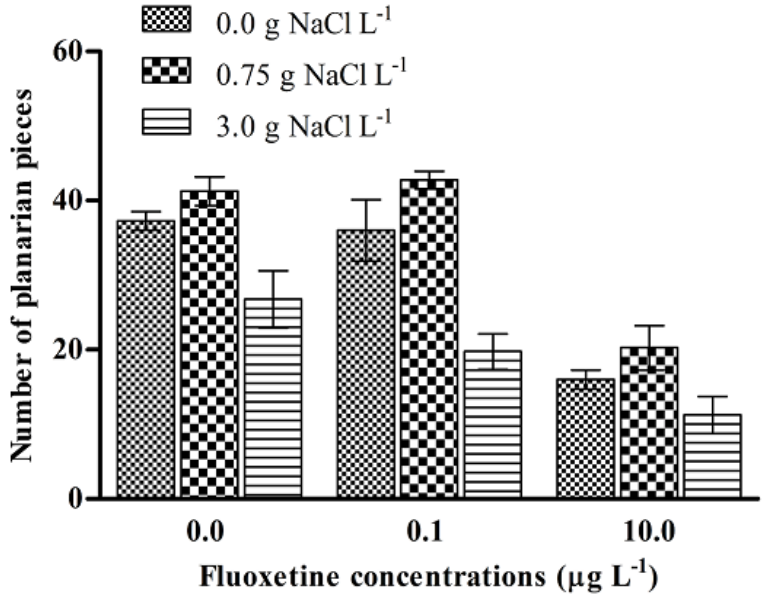

Fig. 5.3: Asexual reproduction of *S. meditteranea* (mean ± standard error of mean [SEM]) under exposure to a gradient of fluoxetine and salinity (NaCl) concentrations.

5.4 Discussion

The results from this study show that exposure to increased salinity (NaCl) is toxic to freshwater planarians and effects include reduced behaviour (locomotor activity and feeding),

increased time for head regeneration and also a decrease in reproduction. Also an interactive effect of salinity on fluoxetine effects was observed in relation to *S. mediterranea* behaviour.

S. mediterranea showed to be highly sensitive to increasing NaCl concentrations levels and these results are in accordance with the general sensitivity of freshwater planarians to salinity. Previous study by Rivera and Perich (1994) reported salinity (NaCl) toxicity to 4 planarian species such as *Cura foremanii* (100% mortality at 3 NaCl g L^{-1}), *Dendrocelopsis vaginatus* (100% mortality at 4 g NaCl L^{-1}), *Dugesia dorotocephala* and *D. tigrina* (100% mortality at 5 g NaCl L^{-1}) after 14 days exposure. Likewise, salinity toxicity in other freshwater organisms has been reported. Salinity 48 hrs LC_{50}s for *D. magna* were 2.99 g NaCl L^{-1} (NaCl) (Ghazy et al., 2009) and 5.9 g NaCl L^{-1} (Gonçalves et al., 2007), 2.7 g NaCl L^{-1} for *D. longispina* (Gonçalves et al., 2007), 96 hrs LC_{50} for embryo and tadpole of *Pelophylax perezi* were 3.79 and 7.4 g NaCl L^{-1} respectively (Santos et al., 2013) and 6.78 (10.6 mS cm^{-1}) and 6.5 (13 mS cm-1) g NaCl L^{-1} respectively (Venãncio, 2017), 24.6 g NaCl L^{-1} for freshwater prawn *M. rosenbergii* (Chand et al., 2015), and 10.65 (21.3 mS cm^{-1}) g NaCl L^{-1} for juveniles of fish *Lepomis gibbosus* (Venãncio, 2017). Also, 4.5 g NaCl L^{-1} salinity caused 100 % mortality in the rotifer *Brachionus patulus* after 2 days of exposure and to *Cerodaphnia dubia* after 2 weeks of exposure (Sarma et al., 2006). Moreover, effects on surface epithelium and increased secretion of mucous observed in exposed planarians have been reported in gills of tadpoles of amphibians (Bernabo et al., 2013, Venãncio, 2017) while effects on the head may be an indication of neurotoxicity which have to verified in future studies. These data suggest that acute effects due to increased salinization of freshwaters are plausible with obvious consequences for biodiversity. Also freshwater planarians are one of the most sensitive invertebrates in relation to acute exposures.

S. mediterranea behaviour was affected by increasing salinity levels. Reduced locomotion and feeding were observed in response to NaCl exposure which is in agreement with effects of salinity towards other freshwater species. Impairment of feeding due to exposure to salinity has also been reported in planarian *D. dorotocephala* (Legner et al., 1976). Legner and others observed that salinity below 0.25 g L^{-1} and above 1.0 g L^{-1} impaired feeding in these planarians. Similarly, study with cladoceran *Daphnia dentifera* showed that feeding rate was reduced with increasing salinity (Searle et al., 2016). Studies with tadpoles of frogs *Litoria ewingii* and *Rana sylvatica*, and other amphibians reported reduction in behaviour (locomotion and feeding rate) due to salinity (NaCl) exposures (Sanzo and Hecnar, 2006, Squires et al., 2008, Collins and Russel, 2009).

The reduction in behaviour (locomotor activity and feeding) in exposed planarians could be linked to salinity effects on planarians muscle activity, epithelial membrane, nerves or sensory ability or osmoregulatory imbalance as have been reported for other organisms (Sanzo and Hecnar, 2006, Squires et al., 2008, Bernabo et al., 2013, Venãncio, 2017). Exposure of planarians to NaCl may have interfered with muscle activity, surface membrane, nerves or sensory ability resulting to their inability to move or sense, search and capture prey. Also, exposure to NaCl may have led to increased costs of osmoregulation which is energetically demanding such that energy for locomotion and feeding are diverted to osmoregulation (Squires et al., 2008). This may have a negative impact on planarian asexual reproduction and population increase as there is a positive relationship between metabolic status and fissioning in asexual planarians. Fissiparous planarians that are well fed grow and fission more compared to starved ones (Oviedo et al., 2008). Besides effects in feeding, the reduction observed for locomotion might also have ecological consequences such as a diminished avoidance response to predators or other unfavourable conditions.

Salinity exposure also delayed head regeneration or time for photoreceptor formation in *S. mediterranea* exposed to sub-lethal salinity levels. Also associated to salinity toxicity in regenerating planarians, a failure of wound healing at decapitation site and blastema formation, degeneration of blastema, development of abnormally shaped blastema and abnormal photoreceptor regeneration were observed. Alteration in time for regeneration of missing parts due to increased salinity exposure has been reported previously for the polychaete *Diopatra neapolitana* (Freitas et al., 2015) and crab *Uca pugnax* (Shock et al., 2009). Although, these studies were on marine organisms their findings agree with ours that changes in salinity levels (NaCl) can affect regeneration processes in aquatic organisms. Regeneration in planarians after injury starts by rapid closing of wound which involves contraction of smooth muscle cells and spreading of epidermal cells, and initiation of differentiation within the regeneration blastema that also involves direct contact between the epithelial cells and parenchyma (Schὺrmann and Peter, 1998). Any interference or interruption in these processes could result in delay or inhibition of wound healing which may affect proper regeneration (Schürmann and Peter, 1998). Toxic effects on surface epithelial membrane and muscles associated to exposure to NaCl have been reported in tadpoles of amphibians (Sanzo and Hecnar, 2006, Squires et al., 2008, Bernabo et al., 2013, Venãncio, 2017). Another chloride salt, magnesium chloride was also shown to affect head regeneration in planarians by impairing muscle contraction thereby obstructing

wound healing and sealing-up of wound at the decapitation area (Schùrmann and Peter, 1998). Possibly, a similar mechanism may be responsible for the failure of wound healing and subsequent failure of blastema formation and degeneration of cut site in some decapitated planarians exposed to the highest NaCl concentration tested here. Similarly, effects of NaCl on surface epithelial membrane of blastema may account for their degeneration while toxic effects on cells within the regeneration blastema may have also contributed to the observed delay in photoreceptor formation. Moreover developmental malformations and delayed or inhibition of metamorphosis due to exposure NaCl in amphibians have been reported (Sanzo and Hecnar, 2006, Squires et al., 2008, Collins and Russel, 2009). Such toxic effects on regenerates and malformations may threaten normal development and activity in freshwater planarians, and adversely affect their population dynamics in their natural freshwater habitats.

Asexual reproduction here measured by looking at the number of fragments resulting from fissioning was also impaired in planarians exposed to increased salt concentrations. Similarly, salinity was shown to reduce fissioning in freshwater planarians *D. tigrina*, *D. dorotocephala*, *C. foremanii* and *D. vaginatus,* and reductions in the sexual reproduction in parthenogenetic planarian *D. schubarti* were also observed (Rivera and Perich, 1994, Knakievicz et al., 2006). Studies with other aquatic organisms like cladocerans showed that exposure to increased NaCl reduced number of progeny per female in *D. magna* (Ghazy et al., 2009), population density of *D. dentifera* (Searle et al., 2016), fecundity in *D. magna* and *D. longispina* (Gonçalves et al., 2007), Also, exposure to increased NaCl reduced population growth in rotifers *Anuraeopsis fissa*, *Brachionus calyciflorus,* and *B. Havanaensis,* decreased population density of cladocerans and reproduction in freshwater crustacean *Branchipus schaefferi* (Sarma et al., 2005, 2006).

The effects of NaCl exposure on *S. mediterranea* fissioning may be associated to salinity toxic effects on surface membrane, nervous system and muscles, since NaCl exposure have been shown to affect these in larval forms of amphibians (Sanzo and Hecnar, 2006, Squires et al., 2008, Bernabo et al., 2013, Venãncio, 2017). Asexual reproduction by fissioning in freshwater planarians is controlled by longitudinal nerve cords and brain, and involves neuromuscular activities among others (Best et al., 1969, 1975, Morita and Best, 1984). During fissioning planarian's posterior portion adheres firmly to a substrate while the anterior portion pulls away and the constricted area stretches becoming thinner until it ruptures (Morita and Best, 1984). The effects of salinity on the surface epithelium, nerves and muscles may have made it difficult for the planarians to attach firmly to substrate (surface of containing vessel), stretch and disintegrate.

The effects may have been felt more in the highest concentration of 3.0 g NaCl L^{-1}. However, slight increases in fissioning of planarians exposed to 0.75 and 1.5 g NaCl L^{-1} may be a response to toxicity as this type of response have been reported in fissioning of asexual *D. dorotocephala* exposed to other contaminants (Best et al., 1981a). It may be that these lower salinity levels presented an increased osmotic pressure on exposed planarians which resulted to increased fissioning as they tried to cope with or regulate it while at 3.0 g NaCl L^{-1} because of collapse of osmoregulatory mechanism and subsequent cellular damage (Cañedo-Argüelles et al., 2013), fissioning rate reduced dramatically.

Moreover salinity also caused degeneration in planarians after fissioning which can be related with effects observed for regeneration. Additionally, tears/wound on the body of some adult planarians exposed to higher concentrations of NaCl may hasten their degeneration. Similar effects on planarian body were reported by Harrath et al. (2004) in field populations of sexual *S. mediterranea* in a freshwater habitat with salinity level of 2.9 g L^{-1}. The resultant effect of tears on planarian body, inability to regenerate lost parts after fission and degeneration of the fragments and fissioning adults will obviously lead to strong negative effects in terms of survival and reproduction of planarians.

Concerning the effects of the combined exposure, the results showed significant interactive effects of fluoxetine and salinity on planarian locomotor activity. Individual exposures to fluoxetine led to an increase in locomotor activity which may be related to fluoxetine effects on dopamine levels. Increase in dopamine level in organisms has been implicated in fluoxetine exposures (Bymaster et al., 2002, Mennigen et al., 2008) and increase in locomotor activity in planarians (Algeri et al., 1983). Salinity on the other hand induced a weaker but opposite response and a decrease in locomotor activity which may be linked to its effects on muscles, surface epithelium, nerves or increased osmotic demand and its resultant high energetic cost (Sanzo and Hecnar, 2006, Squires et al., 2008, Bernabo et al., 2013, Venãncio, 2017). However, the outcome of the combined exposures depicts effects of salinity on fluoxetine resulting to locomotor activities lower than that of planarians in fluoxetine single exposures. Studies have shown that sodium (Na) is required for neural uptake, that fluoxetine binding on serotonin uptake site is sodium ion (Na^{+}) dependent and that fluoxetine binding to serotonin uptake site was highest in the presence of NaCl than chloride salts of other metals like potassium, rubidium, lithium and cesium (Wong et al., 1995). However, the effects of this association between Na+ and fluoxetine on dopamine level which may influence locomotor activity in planarians under

combined exposure are not known. Possibly, the combination of fluoxetine and salinity exposed planarians to high toxicity stress. Studies have shown that planarians display different behaviuoral responses with respect to toxicity (Grebe and Schaeffer, 1991, Rawls et al., 2011, Wu et al., 2014). Planarians under toxicity stress may become hyperactive, reduce locomotion, withdraw, become motionless etc. According to Grebe and Schaeffer (1991) classification of planarians responses to toxicity, hyperactivity or increased locomotor activity is a sign of altered behaviour (locomotion) while reduced/laboured movement is a sign of morbidity or increased toxicity. This shows that increased salinity concentrations have the potential of increasing toxicity due to fluoxetine on planarians' behaviour in the wild.

However there was no interactive effect of fluoxetine and salinity exposure on planarian asexual reproduction by fissioning. Fluoxetine and salinity induced strong reductions in fissioning. Planarian asexual reproduction by fissioning is under neuromuscular control and suppressed by increased melatonin level (Best et al., 1969, 1975, Morita and Best, 1993). Fluoxetine has been shown to increase melatonin level (Reierson et al., 2009, Kirecci et al., 2014) while NaCl have been shown to affect muscular and nervous activities, and surface membranes of exposed organisms (Sanzo and Hecnar, 2006, Squires et al., 2008, Bernabo et al., 2013, Venãncio, 2017).

The results from the mixtures of salinity and fluoxetine on planarian locomotor activity and fissioning corroborated with views from other studies (Nakamura et al., 2008, Donner et al., 2013, González-Ortegón et al., 2013), that environmental factors may influence the effects of pharmaceuticals on aquatic organisms. Here the results show that, in general, planarians exposed to fluoxetine a neuroactive pharmaceutical, and salinity (NaCl) showed an increased toxic response in terms of locomotion and possibly on reproduction.

Overall, the results showed that the freshwater planarian *S. mediterranea* showed sensitivity to increasing salt concentrations and that the effects of exposures to fluoxetine may be affected by salinity resulting to increased stress. *S. mediterranea* behaviour, asexual reproduction by fissioning and regeneration are sensitive endpoints that can be used in ecotoxicological assessment of chemical compounds and of natural stressors and they are rapid and sensitive parameters to use for mixture toxicity assessments.

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Chapter 6

General conclusions

Chapter 6

General conclusions

Various human activities have resulted in the release of different chemical substances to the freshwater environment, leading to alteration of water quality and freshwater biodiversity due to pollution (Dudgeon et al., 2006, Strayer and Dudgeon, 2010). Human pharmaceutical substances including psychiatric drugs are among these chemical compounds released and now commonly detected in the aquatic ecosystems (Nentwig, 2008, Calisto and Esteves, 2009). In addition to chemical pollution, freshwater environments are also under effects of different natural stressors associated to global environmental changes (Vaughn, 2010). Aquatic organisms are as a result subjected to stress due to chemical and natural stressors. Moreover, since pharmaceutical compounds are bioactive and released on a daily basis into the environment (Fent et al., 2006), there is need for their environmental risk assessment under relevant exposure scenarios including concomitant presence of other stressors.

Generally, pharmaceutical substances are different from pesticides or other toxic chemical formulations. This is because for many pharmaceutical compounds their mode of action are not well known, and sometimes not only one specific mode of action but many side effects too (Fent et al., 2006). Pharmaceuticals are also made to produce beneficial effects at low concentrations but become toxic at higher concentrations, thus high concentrations used for short-term acute toxicity and chronic tests may not actually reveal the impacts of low and environmentally relevant concentrations of these pharmaceuticals on aquatic organisms.

Traditionally, environmental risk assessment and ecotoxicological studies have been based on the use of established protocols, few established ecotoxicological model organisms and endpoints such as mortality (Fent et al., 2006). These have hampered effective toxicity testing of pharmaceutical substances in aquatic organisms. Nevertheless, it has been suggested that pharmaceutical ecotoxicity assessments should be designed with a focus on specific targets of the pharmaceutical compound in different test organisms, and on the basis of similar modes of action (Fent et al., 2006).

Consequently, the main objective of this study was to investigate the feasibility of using the freshwater planarian *Schmidtea mediterranea,* a non-model species, for the ecotoxicological assessment of psychiatric pharmaceuticals in the aquatic environment. To carry out this investigation, several endpoints of interest for ecotoxicological assessment were first tested using

tributyltin (TBT) as a model contaminant. *S. mediterranea* was then subjected to single contaminant exposures of environmentally relevant concentrations of two psychiatric compounds fluoxetine and carbamazepine. *S. mediterranea* was also subjected to combined exposure of both psychiatric drugs, and also fluoxetine and TBT (used a model co-occuring neurotoxic contaminat). In addition, and due to the emergent ecological issues associated with salinization of freshwaters, *S. mediterranea* was also subjected to combined exposures of fluoxetine under different salinity levels.

The research developed in the present work and the obtained results are summarized in the following highlights:

***S. mediterranea* is sensitive to chemical and natural stressors and a suitable model species for ecological risk assessment in freshwater environment**

Risk assessment of freshwater environmental contaminants using freshwater invertebrates have always involved established model organisms such as *Daphnia magna*. However, the results from this study reveal that freshwater planarians like *S. mediterranea,* non-model invertebrates are sensitive and good model organisms for ecotoxicity assessment of environmental contaminants. In this study, 48 hrs LC_{50}s of 1.87 µg TBT L^{-1}, 9.18 g NaCl L^{-1} and 357.93 µg fluoxetine L^{-1} were obtained for *S. mediterranea*, against 2.5 µg TBT L^{-1} (LC_{60} after 21 days exposure), 5.9 g NaCl L^{-1} and 820 µg fluoxetine L^{-1} for *D. magna* (Oberdörster et al., 1998, Brooks et al., 2003, Gonçalves et al., 2007). These results showed *S. mediterranea* to be more/or as sensitive to the neuroactive pharmaceutical fluoxetine and TBT as *D. magna,* and also that *S. mediterranea* is among the most sensitive freshwater invertebrates concerning salinity.

The results of the different tests performed using *S. meditteranea* clearly showed that a wide range of responses can be used as sensitive and reliable ecotoxicity endpoints to assess effects of different stressors on various levels of biological organization. Freshwater planarian behaviour (locomotor activity and the feeding assay here developed), regeneration and asexual reproduction by fissioning are simple, cheap and easy to measure parameters that can be used in addition to survival endpoint for ecotoxicity studies. These responses are also easily adjusted to other freshwater planarian species (see Annex1).

In addition, evaluation of freshwater planarian DNA damage by comet assay may be used to evaluate genotoxic effects of environmental contaminants at sublethal levels. Moreover, other

studies such as effects on biochemical responses or bioaccumulation studies using planarians can be a fruitful area of research within toxicity assessment of environmental contaminants.

Psychiatric pharmaceuticals can pose ecological risk for the freshwater environment

This study contributes to psychiatric pharmaceuticals toxicity data on non-model freshwater organisms. The results of Chapter 3 showed that *S. mediterranea* is sensitive to environmental concentrations of psychiatric pharmaceuticals and that behaviour, asexual reproduction and genotoxicity are sensitive endpoints that can be used to assess their effects. Also, the result showed that fluoxetine is more toxic than carbamazepine which is in line with otrher studies performed with other freshwater invertebrates including *D. magna* (Fent et al., 2006, Lacaze et al., 2015). Exposure to low and environmentally relevant concentrations of fluoxetine induced genotoxic, behavioural and reproductive effects in *S. medittreanea* supporting the general knowledge that freshwater planarians are good model organisms for neurotoxicology and neuropharmacology (Buttarelli et al., 2008, Pagan et al., 2009, Rawls et al., 2011, Hagstrom et al., 2015), and providing insight on the possible mode of action and toxic effects of low environmental concentrations of neuroactive drugs on non-target aquatic organisms (Fent et al., 2006).

Fluoxetine effects in organisms may be influenced by other environmental stressors

There have been speculations of possible mediation of other environmental stressors including pharmaceuticals on the effects of pharmaceuticals and the dangers these may pose to freshwater organisms in the wild. However, most studies considering effects of mixtures cointaining pharmaceuticals have often used very high concentrations and focused on acute lethal effects that may not be environmentally relevant (Cleuvers, 2004, Henry and Black, 2007, Melvin et al., 2014, Varano et al., 2017). The results from Chapters 4 and 5 showed that the effects of low concentrations of fluoxetine may be altered in the presence of other chemical contaminants and natural stressor (salinity - NaCl), as shown by various responses displayed by exposed planarians. The complexity of the responses displayed by planarians and the sensitivity of the sublethal endpoints used, further stresses the need to test effects of mixtures containing low environmentally relevant concentrations of these stressors. It is clear that these mixture toxicity assessments need to be performed using a wider range of concentrations and including other

exposure scenarios (presence of other chemicals/natural stressors). It is however also clear that using planarians and responses such as behaviour, regeneration and asexual reproduction (fissioning) can be instrumental to gather relevant toxicity data for these complex mixtures containing pharmaceutical compounds.

Behavioural parameters proved to be early-warning indicators

Behavioural alterations in exposed organisms have been shown to occur early and at lower concentrations than mortality, and thus are suitable sensitive endpoints to assess toxic effects of contaminants at environmentally relevant concentrations (Pestana et al., 2007, Alonso and Camargo, 2011, Rodrigues et al., 2015). Moreover, planarian locomotor activity has been used in toxicology to evaluate toxic effects of psychiatric drugs (Thumé and Frizzo, 2017). The results from the single and combined exposures to psychiatric drugs and other stressors presented in chapters 3, 4 and 5 showed alterations of *S. mediterranea* locomotor activity even at very low environmental concentrations which is in line with results of other studies in which behavioural alterations are in fact early warning indicators of stress (Pestana et al., 2007, Alonso and Camargo, 2011, Rodrigues et al., 2015). Also, the fact that planarians can exhibit a wide range of behavioural responses to stressors calls for further research on planarian behaviour for better understanding of effects of these drugs on planarians and other organisms in the wild.

In conclusion, this work focused on the need to involve more sensitive aquatic invertebrate species in the risk assessment of psychiatric pharmaceuticals and other stressors of freshwater environment. The freshwater planarian *S. mediterranea,* a non-model species hereby displays important sensitive responses supporting the benefits of involving wide range of organisms from different phyla in toxicity testing. This may assist in alleviating some of the challenges hampering effective ecological assessment of pharmaceuticals in the freshwater environment.

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Annex 1

Ecotoxicity assays using freshwater planarians

Annex 1

Ecotoxicity Assays using Freshwater Planarians

Abstract

Freshwater planarians are free living flatworms known for their regenerative ability. Being easily cultured under laboratory conditions, they are recognized test model organisms in regeneration, developmental biology and neuro-pharmacological research. Also, they have been recently employed in toxicity testing where they displayed an array of sensitive and reliable responses to environmental stressors. Here we outline simple and easy to-follow protocols to evaluate effects of environmental contaminants and other stressors on survival, behavior (feeding and locomotor activity) and regeneration of freshwater planarians. These endpoints are comparable with responses of well-established ecotoxicological model species.

Keywords: Freshwater planarians; Lethal and sub lethal endpoints; Mortality; Behavior; Regeneration

Introduction

Environmental toxicants present in the freshwater ecosystem do not occur isolated but rather simultaneously with other stressors with which it may interact additively, antagonistically or synergistically (Altenburger et al., 2015, Holmstrup et al., 2010). Thus, chemical analyses alone cannot predict the ecological effects of different levels of these chemical substances. In addition, evaluation of harmful effects of pollutants on the environment requires the use of sensitive species and endpoints (Alonso and Camargo, 2011). The responses of these sensitive species at different levels of biological organization can be helpful to understand the mechanisms of action of pollutants and the effects of natural biotic and abiotic factors and how they can mediate toxicity (Holmstrup et al., 2010).

Moreover, bioassays with sensitive species ensure a correct assessment and monitoring of the ecological effects of different stressors on populations, communities and on ecosystem functioning (Brodin et al., 2014, Boxall et al., 2002.).

A number of animals such as annelids, nematodes, crustaceans, mollusks, insects and some vertebrates like amphibians and fish have been used as model species in bio-monitoring and

laboratory ecotoxicity studies. However, some of these model species are limited in their responses to some environmental contaminants (Best and Morita, 1991), research with some organisms may be disrupted due to shortage of experimental animals and studies with vertebrate species are restricted to developmental stages due to ethical concerns. Additionally, responses of different organisms and at different life stages to certain environmental stressors may vary (Gray, 1989, Best and Morita, 1991). Moreover, extrapolating results of one group of organisms to that of another from a different phylum/group may sometimes not be appropriate. Consequently, involving sensitive species from wide range of phylogenies in ecotoxicity bioassays has been deemed essential (Gray, 1989).

Freshwater planarians are free living flatworm triclads with wide distribution in many ecosystems in different parts of the world. Recent advances in molecular biology and morphological studies have been used to place them into 3 families – Planariidae, Dendocoelidae and Dugesiidae (Riutort et al., 2012). A lot is known about the morphology, biochemistry and physiology of freshwater planarians and information on their ecology and distribution in natural and artificial freshwater bodies abound. Freshwater planarians are commonly found in springs, lakes, rivers, ponds and streams, they are epibentic predators and scavengers eating small invertebrates such as crustaceans and insect larvae. They trap prey with mucous and eat them using a retractable pharynx which can extend out of the ventral mouth opening. Secreted enzymes immediately start to digest the prey tissues and digestion is completed intracellularly after the food has been sucked through the pharynx. Planarians exhibit unique developmental plasticity being able to grow or shrink based on food availability, and can survive without food for long periods (Saló et al., 2009).

Freshwater planarians are known to involve diverse reproduction forms such as sexual, asexual, physiological and parhenogenic forms. Sexual planarians are hermaphrodites, exhibiting cross and internal fertilization, though reproduction is seasonal. Physiological types are those which may reproduce sexually or asexually depending on season. Asexual reproduction occurs by transverse fission of the tail posterior end, producing true clones. When fission occurs in planarians, they divide themselves into two creating a head and a tail piece, each subsequently regenerating all missing body structures. Regeneration of missing parts after fission, is mediated by the presence of adult stem cells known as neoblasts, conferring planarians an astonishing regenerative capacity and morphological plasticity (Sánchez-Alvarado 2006, Newmark and Sánchez-Alvarado, 2001), including the rapid and complete head and cerebral ganglia

regeneration upon decapitation (Cebrià, 2007, Cebrià et al., 2002, Gentile et al., 2011). This reproductive strategy allows researchers to use clonal populations of both intact and regenerating planarians in parallel experiments to investigate developmental toxicity of different substances which due to the structurally and molecularly similarity between the planarian and mammalian brain, is relevant to vertebrate species (Hagstrom et al., 2015). It is also advantageous for producing large sets of genetically identical organisms making planarians excellent animal models for developmental and regeneration biology and stem cell research (Hagstrom et al., 2015, Newmark and Alvarado, 2002).

Planarians can detect environmental stimuli very efficiently using photoreceptors and chemoreceptors located at the head region (Davidson et al., 2011, Newmark and Alvarado, 2002, Oviedo et al., 2008b). Freshwater planarians are mobile, using cilia on their ventral surface to glide while crawling is aided by muscular contraction. They avoid sunlight (negative phototaxis) and are commonly found under rocks and leaves. Since specific and sensitive behavioural endpoints such as motility changes, seizure like movements or hyperkinesia are easily quantifiable in planarians, they are also considered good animal models to test the effects and mechanisms of psychoactive agents and drugs (Raffa et al., 2013, Pagán et al., 2013)

Freshwater planarians can be easily maintained under laboratory conditions and have been suggested as useful indicators of water quality (Kent, 1974, New, 1995), considering the array of their potential responses to pollutants (Best and Morita, 1991, Schaeffer, 1993, Plusquin et al., 2012, Ofoegbu et al., 2016, Rodrigues et al., 2016)

Here, some simple and inexpensive protocols for culturing freshwater planarians under laboratory conditions and assessing acute and chronic effects of contaminants using freshwater planarians are highlighted encompassing sensitive endpoints such as survival, behaviour and head regeneration. The following protocols are based on standard tests and standard protocols and on previous work with planarians so as to allow more comparable results with ecotoxicity model species such as *Daphnia* sp and *Chironomus* sp. Examples are given for the use of *Schmidtea mediterranea or Dugesia tigrina* (fig. 1) but similar protocols can be used with other freshwater planarians.

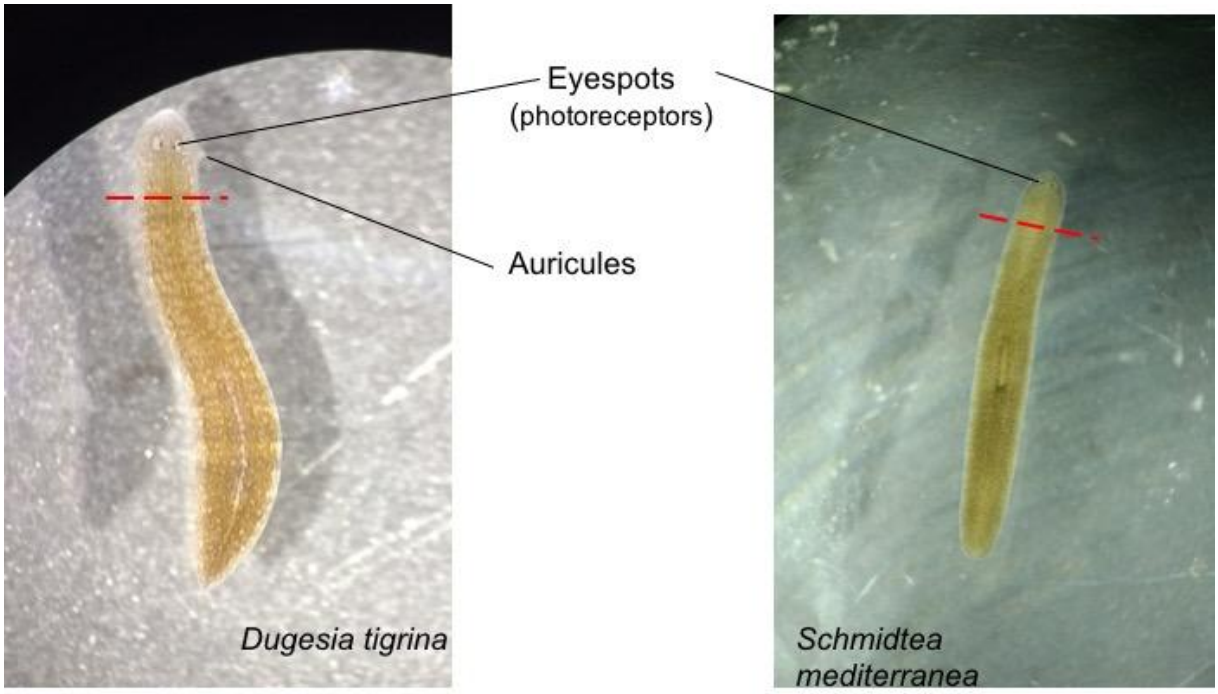

Fig. 1: Pictures showing intact planarians with dotted red line depicting area for decapitation

Materials

Milli-Q water or distilled water

ASTM hardwater (ASTM, 2004)

Containers for maintaining planarians

Bovine liver (used as food for planarians)

Manual/electric food grinder

Pasture/disposable pipettes

Graph sheets with 1.0 and 5.0 mm (0.1 and 0.5 cm) line spacing

50 -500-ml crystallizing dishes (glass or other inert material)

Petri dishes

Soft paint brush

Ice block/cold plate

Micro pipettes and tips

Volumetric flasks/conical flasks/measuring cylinders/beakers

Timer

Scalpel or razor blade

Dissecting microscope

pH / oxygen / conductivity meters

Environmentally controlled room or environmental chamber

Methods

Laboratory culture of planarians

1- A permanent culture ensures a sufficient number of organisms in similar physiological conditions and adapted to laboratory conditions. Freshwater planarians (*Schmidtea mediterranea*, *Dugesia tigrina*, etc) can be maintained in the laboratory under constant temperature 20-22ºC, with ASTM artificial hard water (see note 1). Worms should be kept in plastic containers in a density below 200 organisms/L of medium and kept in the dark (see note 2).

2- No artificial aeration is necessary.

3- Bovine liver is used as food for planarians (see note 3). Feeding is done once or twice per week by placing several pieces of previously ground liver in the containers.

4- After feeding (1-3 hours under light conditions), remove any food that has not been eaten and gently transfer planarians to clean medium in clean containers (see note 4).

5- Medium renewal is done twice a week as soon as feeding period ends

6- Separate containers should be used to keep planarians of similar sizes and isolate planarians to be used in tests. Planarians to be used in ecotoxicity assays should have similar sizes (body length), no visible wounds and no signs of de-pigmentation. Test organisms selected should be ones crawling or gliding normally in culture medium. Recently fissioned planarians should not be used.

7- Measure planarians body length by transferring them one at a time into a Petri dish or transparent container placed over a graph sheet with 1.0 mm or 0.1 cm line spacing, allow planarians to stretch before measurement. Planarians total body length can also be measured under a stereo dissecting microscope

8- Always starve planarians used for ecotoxicity tests for at least 4-7 days before the beginning of tests to prevent food interaction with contaminants and for uniformity in metabolic status of test organisms (Wu and Persinger, 2011, Oviedo et al., 2008a).

9- If sexually reproducing planarians are cultured, eggs and recently ecloded worms can be transferred periodically to new containers.

Acute survival test

To assess acute, lethal effects of contaminants**,** planarians are exposed to a range of concentrations of the test substance for up to 96 hours in static or semi static exposures. Percent Survival is recorded at 24 hours, 48 or 96 hours and compared with values in control treatments in order to estimate the median lethal concentration (LC_{50})

1. Experimental solutions are usually prepared by dilution of a stock solution. Stock solutions should preferably be prepared by dissolving the test substance in distilled water or Milli-Q water or in the artificial medium to be used. For acute tests, at least seven concentrations of the test substance (plus control treatments) should be chosen ranging from concentrations eliciting no observable effects to concentrations causing 100% mortality (see note 5)

2. At least 4 replicates containing 5 organisms each should be used in each test concentration and in control treatments. Freshwater planarians used for acute toxicity tests usually are juveniles with size ranging between 4.0 mm to 6.0. Other sizes can also be used but size rage should be kept to a minimum (± 1 or 1.5 mm)

3. Test vessels are filled with appropriate volumes of experimental solutions ensuring a minimum volume of 10 ml / planarian.

4. Gently transfer planarians to test vials using a plastic pipette or a soft brush. Care should be taken to ensure that planarians do not crawl out of vials immediately after transfer

5. Tests are conducted under the same conditions as in cultures (20 ± 1º C) and under darkness. No food or aeration is provided during exposure.

6. The test can be static with no medium renewal or semi static where partial renewal of medium can be performed periodically whenever tests substances aren't stable under experimental conditions

7. Planarians mortality should be checked every 12 or 24 hours. In addition to mortality, any abnormal behavior or appearance such as degeneration of body parts or immobilization under strong light or gentle mechanical stimulation should also be reported (see note 6).

8. For the acute lethal assay to be valid no more than 10% of the control organisms should be dead or show signs of de-pigmentation, wounds or immobilization.

9. Percent mortality is plotted against test concentrations and statistical methods (probit analysis or dose response curves) are used to estimate the LC_{50} with 95% confidence limits.

Planarian locomotor activity assay

Behavior alterations have been suggested as sensitive measures of sub-lethal effect of different contaminants showing higher or similar sensitivity as other classic endpoints such as, growth or reproduction (Alonso and Camargo, 2011; Hellou, 2011). Behavioral patterns under exposure to environmental stress are a result of a series of biochemical and physiological processes which in turn can be linked to alterations at upper levels of biological organization (Alonso and Camargo, 2011, Pestana et al., 2009). To assess effect of contaminants on locomotor activity, planarians are exposed to a range of concentrations of the test substance for 4-8 days in static or semi static exposures. Planarian locomotion patterns after or under exposure to the test substance are visually recorded in short intervals and compared with values in control treatments

1. For sub-lethal tests, at least five concentration of the test substance (plus control treatments) should be chosen ensuring that low or no mortality occurs due to exposure
2. At least 3 replicates with 5 planarians should be used in each test concentration and in control treatments (See note 8). Freshwater planarians used for sub-lethal toxicity tests should not evidence any signs of stress such as depigmentation areas or wounds and should be gliding or crawling normally in cultures. Size range of organisms can be similar to those used on acute tests or bigger always ensuring that size range is kept to a minimum (± 1 or 1.5 mm)
3. Test vessels are filled with appropriate volumes of experimental solutions ensuring a maximum volume of 20 ml per planarian.
4. Gently transfer planarians to test vials using a plastic pipette or a soft brush. Care should be taken to ensure that planarians do not crawl out of vials immediately after transfer.
5. Exposure is conducted for 4-8 days under the same conditions as in cultures (20 ± 1º C) and under darkness. No food is added during exposure.
6. Solutions should not be aerated during exposure.
7. The test can be static with no medium renewal or semi static where partial renewal of medium can be performed periodically whenever tests substances are not stable under experimental conditions
8. At the end of the exposure remove planarians with brush or disposable pipette one at a time from each replicate and immediately place them in the center of the measuring arena (dimension 20 x 20 x 2 cm) with a fine layer of the respective exposure solution. This transparent

measuring arena is placed on top of a graph sheet with 0.5 cm line spacing (fig 2) and should be illuminated evenly with no shadow areas (see note 7).

9. Allow the animal to acclimate for at least 5 secs and count the number of lines crossed or re-crossed by each planarian for 2-5 min (see note 8). Measure locomotor activity of each organism only once to avoid subjecting it to additional stress.

10. Report results of planarian locomotor velocity (*p*LMV) as numbers of lines crossed/ min) or as cumulative crosses per concentration. Results are plotted against test concentrations and dose-response curves can be used to estimate EC_{50} i.e. the concentration of the tested substance giving half-maximal response with 95% confidence limits (see note 9).

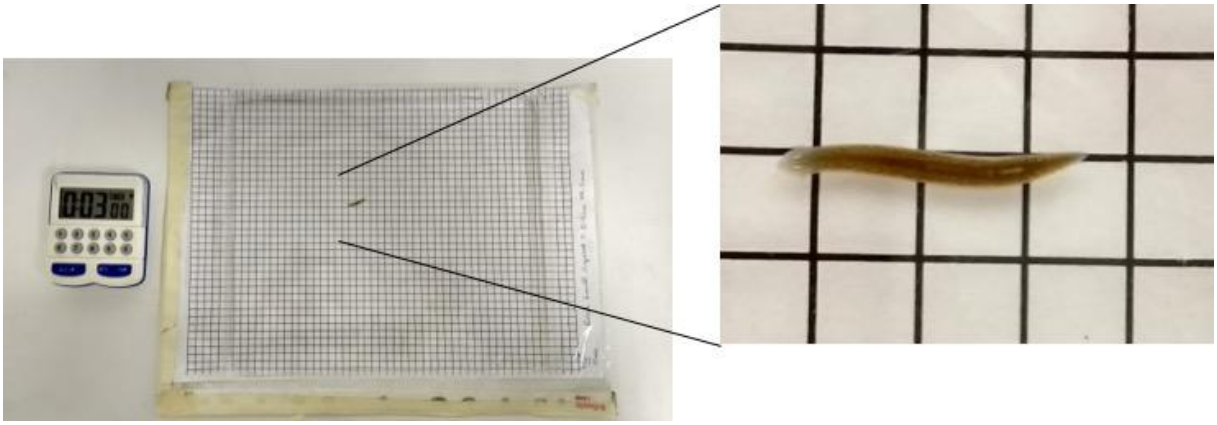

Fig. 2: Picture showing set-up for visual scoring of planarian locomotor activity.

Post-exposure feeding assay

Alterations in feeding behavior constitute a general stress response to different contaminants, and are used as relevant, sensitive and robust ecotoxicological endpoints to complement to traditional parameters such as growth and reproduction in different invertebrate species (Maltby et al., 2002, Pestana et al., 2007, Campos et al., 2016). Feeding rates after exposure to a gradient of concentrations of the test substance are compared to feeding rate in control conditions. Although planarians are given beef liver in cultures, they actively seek and prey on live invertebrates such as crustaceans and insect larvae. Given that many aquatic insect larvae such as chironomids are also easily cultured in the laboratory, they are an excellent option to use as food for feeding trials with planarians.

1. After exposure (follow steps 1- 7 described for locomotor activity assay above) transfer planarians of each replicate to a crystallizing dish with clean medium (50-100 mL). Experimental designs may vary but at least 5 replicates with 5 planarians should be used in each concentration of the test substance plus control treatments for the assessment of effects on feeding rate. Label the crystallizing dishes so as to maintain labeling used during exposure period

2. Randomly assign similar size live mosquito larvae into each of these crystallizing dishes with clean medium. Make sure to have 15-30 live mosquito larvae per planarian (see note 10).

3. Planarians are left to feed on mosquito larvae for 24 hours. No aeration is provided. Crystallizing dishes can be covered with lids, aluminum foil or left in a dark room.

4. After the feeding period count the mosquito larvae remaining and express results as number of larvae consumed by planarians per concentration. Results are plotted against test concentrations and dose-response curves can be used to estimate EC_{50} (see note 9).

Planarian head regeneration assay

Since many planarian species reproduce by binary fission, followed by a rapid process of regeneration of missing body structures any developmental effects caused by exposure to contaminants can be investigated by inducing would healing, regeneration and neurodevelopment through decapitation (Van Huizen et al., 2017). Moreover, we can compare effects of contaminant exposure in genetically identical adult and developing (regenerating) animals, excluding the variability of genetic factors (Hagstrom et al., 2015, Kalafatić et al., 2004). Times for complete head regeneration under exposure to a gradient of concentrations of the test substance are compared to control conditions (see note 11).

1. To decapitate planarians, transfer the animal to a glass slide or Petri dish with thin film of experimental medium that is sitting on ice and wait until the worm stops moving.

2. Under a dissecting microscope and as soon as the organism begins to stretch out use a sharp scalpel blade to make a clean single cut below the auricles for *Dugesia* species or above the pharynx for *Schmidtea mediterranea* (fig. 1, see note 12). Keep the posterior piece for tests while heads from control group can be returned to culture boxes.

3. Within 1 hr post-decapitation, planarians (posterior end) are gently transferred with a soft brush to 6-well plates, 1 worm per well, with 5 ml of the respective concentration of test substance (and dilution water) added to each well (see note 13).

4. At least 10 decapitated planarians should be used per concentration and control treatments

5. With the exception of observation periods, planarians are left to regenerate under darkness and at constant temperature (20 ± 1º C) for up to 15 days. No aeration or food is provided during regeneration period. Multi-well plates can be covered with lids, aluminium foil or left in a dark room.

6. Experimental solutions can be partially renewed in each well using micropipettes

7. Monitor regeneration daily under a dissecting microscope and visually inspecting and scoring the appearance of photoreceptors (*Schmidtea mediterranea*) or photoreceptor and auricles regeneration (eg *Dugesia tigrina*) (fig 3, See note 13). Number of days/hours for photoreceptor or auricles formations are plotted against test concentrations (see note 9).

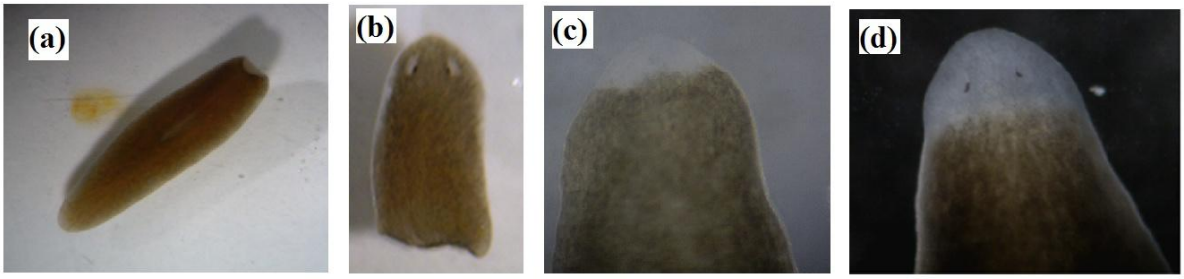

Fig. 3: Regeneration in planarian **(***S. mediterranea***)**: (a) newly decapitated worm (Day 0), (b) head piece from decapitated worm, (c) Newly formed blastema on prior decapitated worm (Day 3), and (d) Newly formed head with photoreceptor (Day 7)

Notes

1. ASTM artificial hard water is suitable for culturing *S. mediterranea* and *Dugesia* species (brown and black *Dugesia*). Using standard artificial medium for culturing and testing allows for comparison with results obtained with other established model species in ecotoxicity assays. To prepare ASTM hard water, add 200 ml of each of the following solutions to 19.2 L of Milli-Q water: 24.57 g L^{-1} $MgSO_4.7H_2O$; 19.20 g L^{-1} $NaHCO_3$; 0.8 g L^{-1} KCl and 2.4 g L^{-1} $CaSO_4.2H_2O$. Correct the pH to 7.6 ± 0.3. Other guidelines and artificial mediums for maintaining planarian cultures in the laboratory are also available (Oviedo et al 2008a).

2. Planarians show photophobic response to light and are most active in the dark. Planarian cultures are thus better maintained in the dark except for feeding periods. Planarians can be kept

in containers shed from light with aluminum foil in the absence of dark room or opaque containers. Cover the container partially or make holes on the container cover to allow oxygenation exchange with medium.

3. The bovine liver can be pureed manually with hand grinder/hand-cranked food mill by first removing the fat, veins and connective tissues after washing or with an electric blender. If the grinder is used, there will no need to sieve and centrifuge liver puree as any remaining vein, connective tissue is separated from the puree as you grind. Liver aliquots can be stored at -20ºC. Planarians can also be fed with chicken liver, mosquito larvae, egg yolks, etc.

4. Feeding can be performed twice a week if necessary with water renewal immediately after feeding (to prevent bacteria from fouling the water). Containers should not be cleaned with soap, detergents, disinfectants etc., as they are toxic to planarians. Planarians are gently transferred to clean containers with plastic Pasteur pipettes or with the help of a soft brush

5. If solvents are used to dissolve the test substance a control series containing the solubilizing agent (solvent control) at the level used in treatments must be run in addition to the treatment series and the negative control (dilution water control). To determine the concentrations to use in the definitive acute test range-finding tests may be conducted. Concentrations should be arranged in a geometric series.

6. Death is easily scored in planarians since the worms disintegrate after dying. However other morbidity signs such as head disintegration or lesions on the body might also be used as acute lethal endpoints

7. Any transparent container with a flat base and smooth surface can be used to measure planarian locomotion. Ensure that the volume of experimental solution used is only enough for the planarians to crawl and glide. The volume used is adjusted to the size of measuring arena and of test organisms. Measuring arena should be evenly illuminated so that photophobic behavior of planarians does not affect locomotion patterns

8. Planarians when released from the pipette/brush sometimes may curl or land dorsally, acclimation period gives them time to uncurl or turn. The time for acclimation and measuring locomotor activity may vary but it is important not to have very long time intervals between the first group and last group measured. Also time and readings can be stopped whenever planarians stop moving or reach edges of measuring arena.

9. Where the standard methods of calculating the EC_{50} are not applicable to the data obtained, other statistical methods can be used to report the effects of the tested concentrations on the sub-lethal endpoint (e.g. Testing for significant trends, Analysis of variance, etc.)

10. The size of the chironomid larvae used depends on the size of the planarians used in the assay and the feeding period chosen. Ensure that all are not totally consumed in control treatments within the feeding period chosen as the food availability can affect feeding rates. Other food items such as daphnids or frozen insect larvae can also be used.

11. Effects on planarians regeneration can also be evaluated by exposing of intact planarians to the test substance followed by decapitation and regeneration in clean medium (post-exposure regeneration), or alternatively with exposure of intact planarians to a gradient of concentrations of a test substance followed by regeneration in those same experimental solutions.

12. Reduction in activity of planarians is achieved by cooling them. Given that planarian decapitation occurs under the dissecting microscope it is advisable to keep the light at a distance so as to maintain the animal as immobile as possible

13. It is necessary that decapitated worms are transferred to experimental solution soon after cutting because regeneration starts less than an hour after amputation. Regeneration after decapitation starts with wound closure by muscular contractions of the body wall within the first 10 min. The epithelium then heals over the wound, forming a blastema (fig 3). The blastema is an accumulation of undifferentiated stem cells, the neoblasts that will differentiate into the missing parts including eyespots and cerebral ganglia (fig. 3). Blastema formation and size can also be used as an endpoint for these regeneration studies but photography and image analysis of all test organisms is time consuming.

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