Universidade de Aveiro Departamento de Biologia
2018

Diana Marina Gomes de Campos

Avaliação dos efeitos ecológicos de filtros ultravioleta em ecossistemas lóticos portugueses

Assessment of the ecological effects of UV-filters in Portuguese freshwaters

Universidade de Aveiro Departamento de Biologia
2018

Diana Marina Gomes de Campos

Avaliação dos efeitos ecológicos de filtros ultravioleta em ecosistemas lóticos portugueses

Assessment of the ecological effects of UV-filters in Portuguese freshwaters

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

Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio através da atribuição de uma bolsa de doutoramento atribuída a Diana Marina Gomes de Campos (SFRH/BD/87370/2012)

É preciso perder
Para depois se ganhar
E mesmo sem ver
Acreditar!

Ângelo Firmino (Boss AC)

o júri

presidente

Doutor João Manuel da Costa e Araújo Pereira Coutinho
professor Catedrático da Universidade de Aveiro

Doutor Rui Godinho Lobo Girão Ribeiro
professor associado com agregação da Universidade de Coimbra

Doutora Susana Patrícia Mendes Loureiro
professora auxiliar com agregação da Universidade de Aveiro

Doutor João Luís Teixeira Pestana
Investigador auxiliar da Universidade de Aveiro

Doutora Sara Calçada Novais
Investigadora em pós-doutoramento do Instituto Politécnico de Leiria

Doutora Paula Inês Borralho Domingues
Investigadora em pós-doutoramento da Universidade de Aveiro

agradecimentos

Queria começar por agradecer ao meu orientador, Doutor João Pestana por tudo o que me ensinou, por todo o apoio dado, pela enorme ajuda prestada ao longo destes 4 anos e pela paciência que mostrou. Ao Prof. Amadeu pela oportunidade de trabalhar neste grupo.

Um agradecimento muito grande a todas as pessoas que me ajudaram e que de certa forma contribuíram para este trabalho, Andreia, Carla Q., Carlos S., Diogo, Fátima, Gravato, Hugo M., Janeco, Luísa, Masha, Patrícia, Rita R. Rita S., Rui M. e Sónia.

Não poderia também deixar de agradecer ao melhor técnico de sempre. Obrigado Abel pela tua paciência e disponibilidade para ajudar sempre em tudo, mereces um bilião de bolos de chocolate.

Aos meus amigos do Alboi e arredores, em especial às mais pequeninas. Obrigado à Sara e ao Diogo pelos momentos de lazer e pela paciência. E um Obrigado muito especial à minha Rita, foste tão importante nesta caminhada, tu sabes o quanto te estou grata.

Á minha família, vocês são os melhores. Aos meus pais obrigado por lutarem todos os dias sempre a pensar em tornar a minha vida melhor. Não sou pessoa fácil de demonstrar afeto mas sei bem o valor incalculável que vocês têm. Obrigada Filipa por seres minha irmã. Adoro-vos a todos.

E por fim… Obrigado Tiago por tudo, por me fazeres feliz, por estares lá sempre, por não me teres deixado fracassar e sobretudo por me mostrares que existe muito mais para além disto e que há todo um mundo novo à espreita. Because you are my best friend, the one that I love until the end.

palavras-chave

Macroinvertebrados aquáticos, produtos de higiene pessoal, multigerações, misturas, mesocosmos, diferentes níveis de organização biológica.

resumo

Os Filtros ultravioleta (filtros UV) são compostos químicos usados em vários produtos de higiene pessoal e materiais (plásticos, tintas, etc.) de modo a proteger contra danos causados pela radiação ultravioleta. O uso destes compostos tem vindo a aumentar nos últimos anos sendo já considerados contaminantes emergentes dos ecossistemas aquáticos. De facto, estes compostos têm vindo a ser detetados em lagos e rios, mas até à data pouca informação existe sobre os seus efeitos nos ecossistemas aquáticos, sendo que os estudos existentes se centram em ensaios laboratoriais e num reduzido número de espécies. Contudo, devido às suas propriedades físico-químicas é esperado que os filtros UV acumulem nos sedimentos e nos organismos e coocorram com outros contaminantes. No entanto, embora o modo de ação dos filtros UV não seja totalmente conhecido, estudos prévios têm mostrado disrupção endócrina causada pela exposição a estes contaminantes em organismos aquáticos. Posto isto, uma avaliação dos efeitos ecotoxicológicos dos filtros UV é urgente para uma correta avaliação do risco ambiental. Assim, os estudos ecotoxicológicos devem-se focar em organismos bentónicos considerando parâmetros e cenários de exposição relevantes, o que nos permitirá determinar potenciais efeitos a longo prazo assim como efeitos ao nível das comunidades dos ecossistemas. Este trabalho propôs avaliar e perceber quais os efeitos dos filtros UV em organismos e comunidades de água doce usando uma abordagem baseada em diferentes níveis de organização biológica e tendo como intuito gerar dados ecotoxicológicos robustos e mais abrangentes. Para tal, foram estabelecidos vários objetivos específicos: 1) avaliar os efeitos de diferentes filtros UV (Benzofenona-3 (BP3); 3-(4-methylbenzylidene)camphor (4-MBC) e Octocrileno (OC)) usando duas espécies de invertebrados aquáticos detritívoros, o díptero *Chironomus riparius* (uma espécie modelo amplamente utilizada em ensaios ecotoxicológicos) e o tricóptero *Sericostoma vittatum* (uma espécie endémica da Península Ibérica), ambos com grande importância nos ecossistemas lóticos portugueses; 2) avaliar os efeitos de misturas de diferentes filtros UV, e de filtros UV juntamente com um repelente de insetos (N,N-diethyl-3-methylbenzamide - DEET) em parâmetros relacionados com a reprodução de *C. riparius*; 3) avaliar os efeitos multigeracionais decorrentes de exposição a BP3 em *C. riparius;* 4) usando sistemas de rios artificiais avaliar os efeitos do 4-MBC na estrutura e funcionamento dos ecossistemas.

resumo (cont.)

Os resultados obtidos mostraram que, de facto, os filtros UV selecionados causaram efeitos tóxicos em ambas as espécies usadas a concentrações ambientalmente relevantes, observando-se reduções ao nível do crescimento e das taxas alimentares assim como atrasos no desenvolvimento. Ao nível subcelular *C. riparius* e *S. vittatum* mostraram diferentes padrões de resposta à exposição aos filtros UV e apesar de se observarem custos metabólicos relacionados com os processos de destoxificação, não se observaram efeitos em termos de stress oxidativo nem neurotoxicidade. Adicionalmente, os resultados obtidos na exposição às misturas entre químicos revelaram interações sinergísticas para alguns parâmetros reprodutivos de *C. riparius* para as ambas as misturas testadas (BP3 – 4-MBC; BP3 - DEET). Estes resultados sugerem que exposições individuais aos filtros UV podem subestimar a toxicidade destes contaminantes em condições naturais. Nos ensaios multigeracionais observou-se também uma forte redução na fertilidade de *C. riparius* expostos a BP3. Observou-se ainda que a segunda geração foi mais afetada que a geração parental mesmo se mantida em condições controlo, mas cujos pais tinham sido previamente expostos a BP3. Todos estes resultados indicam que a BP3 provoca efeitos latentes na geração parental que são posteriormente observados nos descendentes, apontando assim para efeitos epigenéticos/transgeracionais. Por último, nos ensaios dos mesocosmos, não foram observados efeitos do 4-MBC na estrutura das comunidades de macroinvertebrados nem na degradação da matéria orgânica, em contraste com o forte efeito observado na produção primária. Estes resultados, embora não concordando com os resultados obtidos em ensaios laboratoriais sugerem potenciais efeitos indiretos da presença dos filtros UV nos sedimentos. Em conclusão, os resultados obtidos nesta tese indicam que os filtros UV apresentam risco para os organismos bentónicos e reforçam a necessidade de utilizar abordagens integradas com maior relevância ecológica para uma melhor avaliação do risco ambiental destes contaminantes emergentes.

keywords

Aquatic macroinvertebrates;Personal care products; multigenerations; mixtures; mesocosms; different levels of biological organization

abstract

Organic UV-filters are chemicals present in several personal care products and on other materials (plastics, paints, etc) to protect against ultraviolet radiation. The use of these compounds has been increasing throughout the years and, consequently, they are now considered global emergent contaminants of the aquatic environment. In fact, UV-filters have been frequently found in river and lake waters but to date, only scarce information exists about their effects and it is mostly based in acute or chronic toxicity data for a limited number of species. Due to their physico-chemical properties, UV-filters accumulate in biota and sediments and are expected to co-occur together with other persistent contaminants. Moreover, and despite uncertainties about their specific mode of action, research has shown endocrine disruption caused by exposure to organic UV-filters in several species. Investigation on the ecological effects of organic UV-filters is urgent for a correct environmental risk assessment. For that, studies should be focused on ecotoxicological data from benthic organisms considering relevant endpoints and exposure scenarios including binary mixtures. This will allow to address potential long-term as well as community and ecosystem level effects of organic UV-filters. Thus, the purpose of this thesis was to evaluate the effects of UV-filters using an integrated approach combining sub-organismal, organismal, population and community level responses, aiming to produce comprehensive and sound ecotoxicological data for freshwaters. With that purpose four specific objectives were proposed: i) assess the effects of selected UV-filters (Benzophenone-3 (BP3); 3-(4-methylbenzylidene)camphor (4-MBC) and Octocrylene (OC)) on two aquatic species, the dipteran *Chironomus riparius* (a model organism widely used in ecotoxicological assays) and the trichopteran *Sericostoma vittatum* (an endemic species of Iberian peninsula), both aquatic detritivores with an important role in the streams and rivers; ii) assess the effects of binary mixtures of different UV-filters and UV-filters combined with an insect repellent, DEET (N,N-diethyl-3-methylbenzamide) in *C. riparius* reproductive traits ; iii) assess the long-term effects of BP3 exposure over two consecutive *C. riparius* generations and iv) assess the effects of 4-MBC in the structure of macroinvertebrates community and functioning of ecosystem using a mesocosms approach. Obtained results showed that selected UV-filters indeed caused deleterious effects on both insect species at environmental relevant concentrations with reductions in the growth, feeding and development rates.

abstract (cont.)

At the sub-organismal level, *C. riparius* and *S. vittatum* showed different patterns of response to UV-filters exposure and, despite evidences of metabolic costs related with detoxification, no evidences of oxidative stress or neurotoxicity were found. Additionally, results obtained in mixture exposures showed synergistic interactions for some *C. riparius* reproduction related traits for binary mixture of BP3 and 4-MBC as well as for mixture containing BP3 and DEET. These suggest that individual chemical testing can underestimate toxicity of organic UV-filters under natural conditions. Concerning *C. riparius* long-term multigenerational exposure to BP3, our results showed that *C. riparius* fertility is strongly reduced by BP3 exposure being the filial (F1) generation more sensitive than the parental (P) generation. Moreover, the F1 generation exposed under control conditions but whose parents were exposed to BP3 showed to be affected. All these results indicate carry-over effects, pointing out to possible epigenetic/transgenerational effects. Finally, community ecotoxicological experiments using artificial streams (mesocosms) showed no effects of 4-MBC on the structure of macroinvertebrate community nor on leaf litter decomposition. However, primary production was strongly reduced due to 4-MBC exposure. These results, although not in agreement with the previous laboratory assays performed with detritivore species, suggest potential bottom up indirect effects caused by the presence of organic UV-filters in sediments. In conclusion, the results obtained in this work suggest that UV-filters present risk to freshwater benthic invertebrate communities and reinforce the need of using complex and higher tier ecotoxicity studies to a better environmental risk assessment of these emergent contaminants.

Table of Contents

Chapter 1: 15

General Introduction 17

1.1 Personal Care Products: Organic Ultraviolet filters and their presence in aquatic ecosystems 17

1.1.1 Organic Ultraviolet-filters 17

1.1.2 Fate and behaviour of organic UV-filters in aquatic ecosystems 19

1.2 Organic Ultraviolet filters in freshwaters: the need for tiered stepwise ecotoxicological approach 21

1.2.1 Addressing effects of organic UV-filters towards benthic invertebrates 21

1.2.2 Infra-organismal effects of organic UV-filters 23

1.2.3 Effects of mixtures containing organic UV-filters 25

1.2.4 Relevance of long-term, multigenerational exposures to organic UV-filters 26

1.2.5 High-tier ecotoxicity testing to address Community and ecosystem level effects of organic UV- filters28

1.3 Aim of the study and conceptual framework 29

References 30

Chapter 2: 39

Toxicity of organic UV-filters to the aquatic midge C*hironomus riparius*. 41

Abstract 41

1. Introduction 42

2. Material & methods 43

2.1 Chemicals 43

2.2 Test Organisms 44

2.3 Chemical analysis 46

2.4 Statistical analysis 48

3. Results 48

3.1 Concentrations of UV-filters in sediments 48

3.2 Effects of UV-filters on *C. riparius* growth and emergence 49

3. 3 Biochemical responses of *C. riparius* exposed to UV-filters 51

4. Discussion 53

References 56

Supplementary data 60

Chapter 3: 63

Ecotoxicity of two organic UV-filters to the freshwater caddisfly *Sericostoma vittatum* 65

Abstract 65

1. Introduction 66
2. Material & methods 68
2.1 Caddisflies collection and maintenance 68
2.2 Chemicals.. 68
2.3 Sediment dosing 69
2.4 Chemical analysis.. 69
2.5 *S. vittatum* exposure trials.. 71
2.6 Determination of biochemical biomarkers.. 71
2.7 Statistical analysis... 73
3. Results 74
4. Discussion 78
References... 81

Chapter 4: 87
Toxicity assessment of binary mixtures of BP3 with 4-MBC (UV-filters), and BP3 with DEET (insect repellent) using the aquatic midge *Chironomus riparius* 89
1. Introduction... 90
2. Material and Methods.. 91
2.1 Test Organisms 91
2.2 Chemical compounds and sediment composition 91
2.3 Chemical dosing and experimental design 92
2.4 Statistical analysis.. 93
3. Results 94
3.1 Effects of BP3, 4-MBC and of their mixture on *C. riparius* 94
3.2 Effects of BP3, DEET and of their mixture on *C. riparius* 99
4. Discussion 104
5. Conclusion 108
References... 108

Chapter 5: 115
Two-generational effects of Benzophenone-3 on the aquatic midge *Chironomus riparius*.......................... 117
1. Introduction... 118
2. Materials & methods.. 119
2.1 Model organism and culture conditions 119
2.2 Experimental design 119
2.3 Statistical analyses... 121
3. Results 122

3.1 Effects of BP3 on *C. riparius* P generation 122
3.2 Effects of BP3 on *C. riparius* F1 generation 122
4. Discussion 125
5. Conclusion 127
References 128

Chapter 6: 133
Effects of the organic UV-filter 4-MBC on freshwater benthic invertebrate communities and ecosystem function 135
1. Introduction 136
2. Materials and methods 137
2.1 Chemical compound and sediment spiking 137
2.2 Leaf collection and macroinvertebrate community characterization 137
2.3 Experimental design 138
2.4 Statistical analysis 139
3. Results and discussion 140
References 143

Chapter 7: 147
General Discussion 149
References 153

Annex I 155
Annex II 163

Chapter 1:
General Introduction

General Introduction

1.1 Personal Care Products: Organic Ultraviolet filters and their presence in aquatic ecosystems

In the last decades, numerous synthetic organic compounds have been produced for agricultural, aquaculture, industrial and domestic use. These products reach the aquatic environment due to sewage discharges, inputs from industrial and agricultural activities, runoff and leaching. Among these compounds, Personal Care Products (PCPs) that are used every day in large quantities have received much attention in the last decades due to their increased detection in ecosystems around the world (Brausch and Rand, 2011; Ramos et al., 2015). PCP's found in aquatic lotions, cosmetics, sunscreens, gels, makeup products, deodorants, moisturizers, toothpastes, etc., are generally applied externally on the human body (beauty and hygiene). PCPs are bioactive and many are persistent in the environment having the potential to bioaccumulate and also show endocrine disruptive effects (Brausch and Rand, 2011; Peck, 2006; Pedrouzo et al., 2011). Taking into account these characteristics, studies aiming at evaluating the toxicity of PCPs in freshwaters ecosystems are of extreme importance (Brausch and Rand, 2011; Chisvert and Salvador, 2007; Pedrouzo et al., 2011). Different classes of PCP's are defined, including fragrances, preservatives, disinfectants, siloxanes, insect repellents and Ultraviolet filters (UV-filters) (Pedrouzo et al., 2011).

1.1.1 Organic Ultraviolet-filters

Solar radiation reaches to the planet earth through visible radiation (44.3% - 400-760 nm), infrared radiation (49.5% - 760 – $1x10^6$ nm) and ultraviolet radiation (6.2 % - 100 – 400 nm). Ultraviolet radiation is prejudicial to human health, can cause sunburn, skin inflammation, allergic reaction and even cancer (Chisvert and Salvador, 2007). Thus, UV-filters compounds are formulated to protect against damage caused by ultraviolet (UV) radiation [UVA (320-400 nm) and UVB (290-320)]. According to their mechanism of action, UV-filters can absorb ultraviolet radiation by absorbing photons and immediately return to ground state by thermally emitting energy (Giokas et al., 2007) and are called organic (or chemical) UV-filters or can reflect and scatter the ultraviolet radiation and are called inorganic (or physical) UV-filters (e.g. nanoparticles: titanium dioxide and zinc oxide) (Brausch and Rand, 2011; Gago-Ferrero et al., 2012; Ramos et al., 2015). Organic UV-filters contain one or more aromatic structures in their composition that can be conjugated with carbon-carbon double bounds and/or carbonyl moieties (Giokas et al., 2007). Organic UV-filters are present in a widely variety of PCPs such as cosmetics, lipsticks, makeup,

shampoos, hair sprays, sunscreens but also in other materials such as furniture, sun glasses, food packaging, adhesives, paints and textiles (Díaz-Cruz et al., 2008). Nevertheless, little is known about their effects in aquatic organisms/aquatic ecosystems.

Organic UV-filters can be categorized according with their chemical structure in different groups such as benzophenones, cinnamates, camphor derivates, triazines, crylenes, among others (Gago-Ferrero et al., 2012). In this work, three different UV-filters belonging to three different classes in use by industry were selected according with their occurrence in aquatic ecosystems (see section below) and their physic-chemical properties. The selected compounds were: Benzophenone 3 (BP3); 3-(4-methylbenzylidene) camphor (4-MBC) and Octocrylene (OC).

BP3 is an UV-filter belonging to the family of benzophenones derivates. BP3 protects the skin and materials against UVA and UVB radiation and can be used in PCP according to current legislation in Europe, United States and Japan (Chisvert and Salvador, 2007; Wahie et al., 2007). BP3 is used in cosmetics and sunbathing but also as a photostabiliser in packaging materials (Environment Agency, 2008; Vione et al., 2013). 4-MBC belongs to the camphor family and protects only against UV-B, it is used in cosmetics (Chisvert and Salvador, 2007) and as photostabiliser of avobenzone. 4-MBC also provides a good combination of photostability and high protection efficacy (Environment Agency, 2008) and its use is allowed in Europe and Australia (Chisvert and Salvador, 2007). Octocrylene belongs to the crylene family and is a UV-filter that protects against UVB radiation and with good protection potential. OC is used in a range of cosmetic products and is allowed in Europe, United States and Japan (Chisvert and Salvador, 2007; Environment Agency, 2008). Table 1 presents the physic-chemical properties of the selected organic UV-filters.

Table 1 - Physic-chemical properties of BP3, 4-MBC and OC.

Chemical name; INCI	Chemical structure	Cas No	Molecular weight (mol/g)	log K_{ow}	log K_{oc}	Solubility in water (g/L)
2-hydroxy-4-methoxybenzophenone; Benzophenone 3	OH O C CH_3O	131-57-7	228.24	3.79	3.10	0.106
3-(4-methylbenzylidene) camphor); 4-methylbenzylidene camphor	CH_3 CH_3 CH_3 O CH_3	36861-47-9	240.35	4.95	3.89	0.017
2-ethylhexyl 2-cyano-3,3-diphenylacrylate; Octocrylene	O O CH_3 CN CH_3	6197-30-4	361.49	6.88	-	$3.6x10^{-4}$

INCI - international nomenclature for cosmetic ingredients
K_{ow} - Octanol-water partition coefficient
K_{oc} - Organic carbon distribution coefficient

1.1.2 Fate and behaviour of organic UV-filters in aquatic ecosystems

As stated above, the daily use of UV-filters by the human population leads to an inevitably release of these compounds into the aquatic environment (Pedrouzo et al., 2011; Ramos et al., 2015). There are two main pathways by which organic UV-filters reach the aquatic environment: directly by washing of the skin and clothes during recreational activities or by discharges of swimming pool waters (Brausch and Rand, 2011; Rodil et al., 2009) and indirectly due to discharges of wastewater treatment plants (WWTPs). In fact, the major source of UV-filters in the aquatic ecosystems is the inefficient removal of UV-filters during the WWTPs processes (Ramos et al., 2016). The figure below (figure 1) illustrates different sources of UV-filters in the environment.

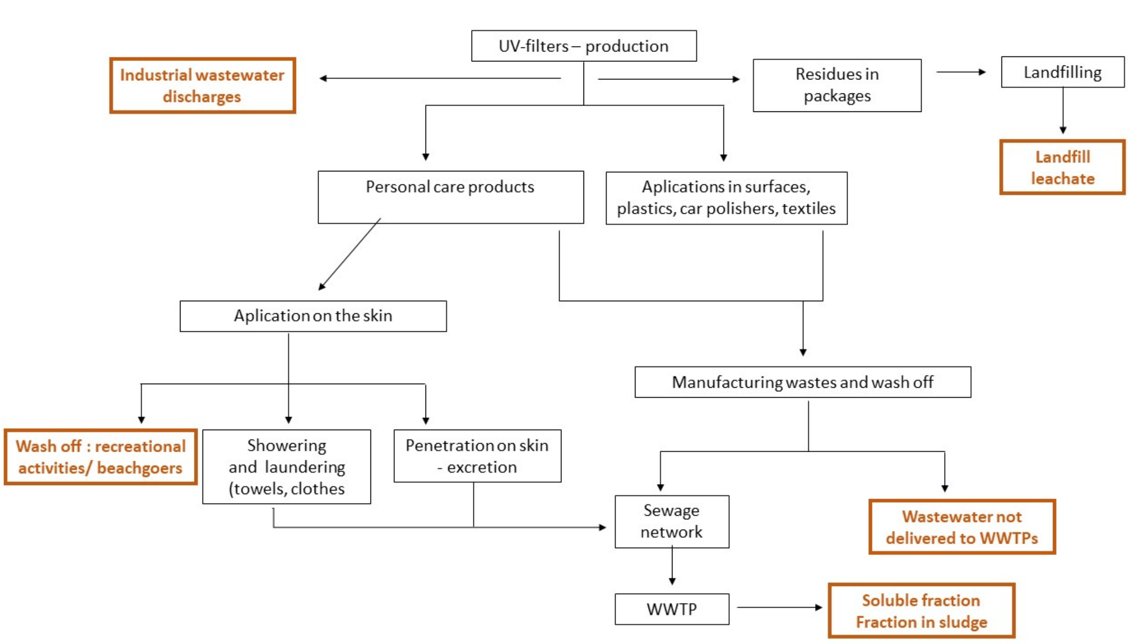


Figure 1 - Main sources of UV-filters in the environment (adapted from Giokas et al., 2007).

In Europe, the use of organic UV-filters and their quantities in PCPs and cosmetics is regulated by the European Union Cosmetics Directive (Chisvert and Salvador, 2007; Environmentt Agency, 2008). However, the environmental presence of UV-filters has been detected around the entire world in different countries such as Thailand, Spain, Czech Republic, China, Taiwan, Japan, Switzerland, Germany, United Kingdom, South Korea, USA, Italy, Greece, Colombia, Lebanon and Chile (Ramos et al., 2015). The presence of these compounds has been observed in different compartments of the aquatic environment namely river and lake waters, groundwater, seawater and even in tap water, generally at ng/L to µg/L levels (Ramos et al., 2015).

Organic UV-filters have low solubility in water and a high Octanol-water partition (log K_{ow}) and organic matter-water partition (log K_{OM}) coefficients. Due to these physic-chemical properties, these UV-filters tend to accumulate in sediments and biota. In fact, UV-filters have been shown to reach concentrations of up to 2.4 mg/Kg in river sediments (Gago-Ferrero et al., 2011), 0.6 mg/Kg in stream sediments (Kameda et al., 2011), 0.09 mg/Kg in lake sediments (Rodil and Moeder, 2008), 0.079 mg/Kg in costal sediments (Amine et al., 2012) and 27 mg/Kg in sewage sludge (Plagellat et al., 2006).

UV-filters can also be found accumulated in organisms' tissues and have been detected in several fish tissues (Buser et al., 2006; Gago-Ferrero et al., 2015; Langford et al., 2015) and also in

clams, mussels, prawn and urchin (Emnet et al., 2015; Langford et al., 2015; Picot Groz et al., 2014).

1.2 Organic Ultraviolet filters in freshwaters: the need for tiered stepwise ecotoxicological approach

The concern about the presence of UV-filters in the aquatic environment and their potential ecological effects is recent in comparison with other stressors like metals and pesticides. Several studies assessing the presence of UV-filters and their ecotoxicity in the aquatic environment have already been performed. However, significant knowledge gaps concerning the ecotoxicological effects of UV-filters are obvious if we consider that: 1) most studies in the literature only address organismal level effects of UV-filters in standard model species; 2) the mode(s) of action of UV-filters is currently unknown and consequently also the target of these chemicals inside organisms; 3) UV-filters are expected to be persistent in the environment and accumulate in sediments and biota. As such, in order to reduce uncertainties and provide data for an improved environmental risk assessment of UV-filters in the aquatic environment it is clear that a more integrated approach is needed. This approach should be focused on ecotoxicity data from sediment dwelling organisms including effects at organismal and sub-organismal levels (Fent et al., 2010; Tsui et al., 2015; Tsui et al., 2014). Moreover, higher tier ecotoxicity studies are needed to address ecological effects of these compounds under relevant exposure scenarios encompassing longer multigenerational exposure periods, the effects of these compounds when in mixtures and finally with studies aiming to evaluate community and ecosystem level effects (Fent et al., 2010; Tsui et al., 2014).

1.2.1 Addressing effects of organic UV-filters towards benthic invertebrates

The majority of studies addressing effects of UV-filters in the aquatic environment are based in standard ecotoxicological tests using sub-lethal endpoints such as growth, reproduction, or feeding rates which allow to assess the status of the organisms/ individual performance. Studies found in the literature showed that UV-filters have estrogenic activity (Blüthgen et al., 2014; Blüthgen et al., 2012; Kim et al., 2014; Wang et al., 2016), alter the expression of genes related with ecdysone (Ozáez et al., 2013, Ozáez et al., 2014, Ozáez et al., 2016b), inhibit the growth and decrease the reproduction of aquatic organisms (Paredes et al., 2014; Schmitt et al., 2008; Sieratowicz et al., 2011). However, although UV-filters are persistent, due to their low photodegradation and adsorption, these substances are eliminated from the aquatic phase (Tolls

et al., 2009) and tend to accumulate in sediments. Therefore, invertebrates living in close contact with sediments are most suitable for evaluation of UV-filters effects. In this work, two species of aquatic benthic detritivores were used: the dipteran *Chironomus riparius* Meigen, 1804 and the tricopteran *Sericostoma vittatum* Rambur, 1842.

Chironomids (Diptera, Chironomidae) are widely distributed in the northern hemisphere where they dominate the benthic communities of lotic and lentic ecosystems both in number and in biomass (Merrit and Cummins, 1996). They can adapt to extreme conditions such as higher variances of pH, depth, temperature, and salinity and also limited oxygen conditions due to their high contents of haemoglobin (Armitage et al. 1995). Chironomids as collectors feed on fine particulate organic matter and play an important role in nutrient cycling (Ferrington, 2008). Chironomid larvae are benthic and live closely associated to the sediments where UV-filters accumulate showing thus to be a good candidate to assess the effects of these contaminants in the ecosystems. Moreover, chironomids are prey of many invertebrates and vertebrates.

The non-biting midge *Chironomus riparius* Meigen (Diptera: Chironomidae) is a multivoltine species with a short life-cycle and includes a most enduring aquatic phase (eggs, four larval stages and pupae) and a short non-feeding aerial phase (adult/imago) (figure 2). Moreover, this species is easy to maintain in laboratorial conditions, being used as a model organism for standardized ecotoxicological assays (OECD, 2004), including multigenerational tests (Lilley et al., 2012; Vogt et al., 2007). Endpoints such as larval behaviour, growth, survival, emergence and biochemical responses have been evaluated as responses to different stressors (Domingues et al., 2007; Pérez et al., 2013; Pestana et al., 2009a; Rodrigues et al., 2015a).

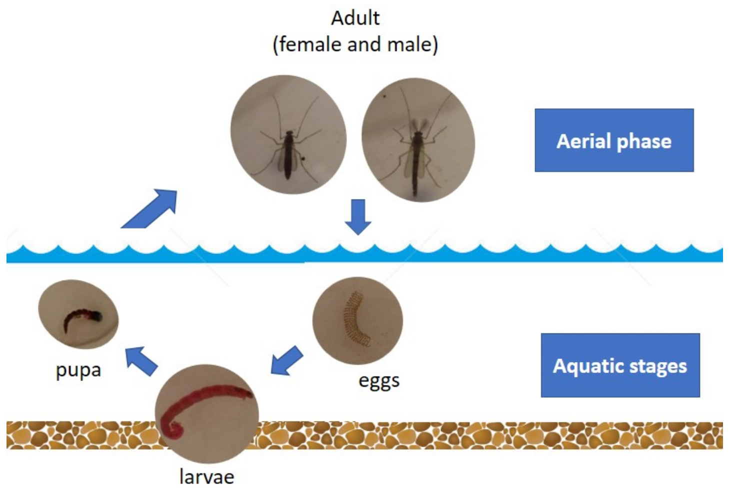


Figure 2 - Life-cycle of *Chironomus riparius*.

Sericostoma vittatum Rambur (Trichoptera: Sericostomatidae) is a cased caddisfly species that plays an important role in the fragmentation of allochthonous leaf litter in streams (Campos et al., 2014; González and Graça, 2003; Feio and Graça, 2000). *S vittatum* is endemic from the Iberian Peninsula. *S vitattum* has a relatively long life-cycle (annual) with several aquatic stages (eggs, larvae and pupae) and also an aerial phase (adult). These organisms live buried in the sediment, feeding on coarse particulate organic matter and are present in streams throughout the year, features that make this species a suitable organism to assess the effects of UV-filters. Although not a model organism, caddisflies have been previously used in ecotoxicological studies (Berra et al., 2006; Pestana et al., 2009a; Schulz and Liess, 2000). Particularly, *S. vittatum* has been previously used in ecotoxicological laboratory bioassays, using feeding behaviour, respiration rates and biochemical parameters to assess the effects of different contaminants and natural stressors (Campos et al., 2014; Campos et al., 2016; Pestana et al., 2009a; Rodrigues et al., 2017).

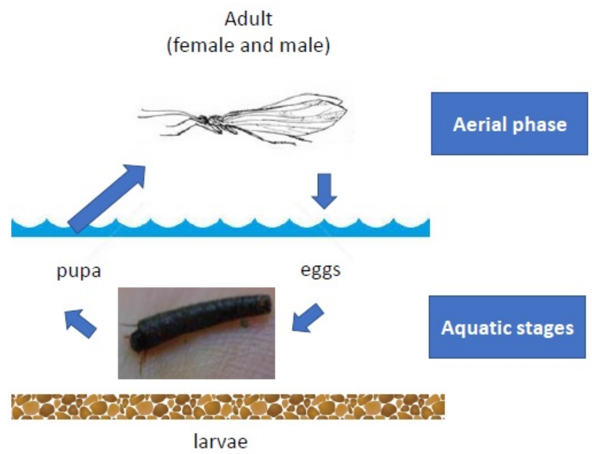


Figure 3 - Life-cycle of *Sericostoma sp.*

1.2.2 Infra-organismal effects of organic UV-filters

The use of organismal endpoints usually fails on providing information regarding the underlying processes responsible for observed effects of determined substance (Hyne and Maher, 2003). In the last decades, biomarkers have been extensively used in ecotoxicological assays as a complement to the individual endpoints. Biomarkers can be defined as "any *biological response to an environmental chemical at the below-individual level, measured inside an organism or in its products (urine, faeces, hairs, feathers, etc.), indicating a departure from the normal status, that cannot be detected from the intact organism"* (Van Gestel and Van Brummelen, 1996). So, the use

of an infra-organismal approach can constitute a benefit as additional information about the mechanisms of action of UV-filters at sub-cellular levels can be gained, which in turn if sensitive and robust can be helpful in providing early warning signs of effects at higher levels of biological organization.

To assess the sub-organismal effects of UV-filters a set of biomarkers should be chosen considering the uncertainties relatively to the mode of action of UV-filters, although it is known that these compounds can induce alterations in antioxidant defences and/or cause oxidative damage (Gao et al., 2013; Liu et al., 2015).

Reactive oxygen species (ROS) are a consequence of aerobic processes and when the equilibrium between their production and elimination is disrupted (for example due to exposure to chemicals) the accumulation of ROS can occur, leading to oxidative stress, i.e. to damage in the cellular constituents (Lushchak, 2011). However, to fight ROS and prevent injuries in cells, organisms have enzymatic (i.e.: superoxide dismutase, catalase, glutathione reductase, and peroxidase) and non-enzymatic (i.e.: total glutathione, vitamin E and C) detoxification mechanisms (Valavanidis et al., 2006). Together with other enzymes, catalase is one of the first lines of defence against ROS and is responsible to convert hydrogen peroxide (H_2O_2), which is toxic to cells, into water and oxygen. Non-enzymatic antioxidant defences, such as reduced glutathione (GSH) can also play an important role in fighting oxidative stress by interacting directly with ROS and also operate as co-factor to several enzymes such as glutathione peroxidase (Doyotte et al., 1997; Lushchak, 2012). Glutathione-*S*-transferase (GST) is also an enzyme acting in the phase II of biotransformation catalysing the conjugation of GSH with several compounds to facilitate excretion by cells (Enayati et al., 2005). If all these defence mechanisms fail, oxidative damage will occur which is easily measured through the lipid peroxidation (LPO) since aquatic organisms have in their constitution high contents of lipids with polyunsaturated fatty acid residues, a preferential substrate for oxidation (Lushchak, 2011).

On the other hand, energy-related biomarkers (energy reserves and energy consumption) are also an important measurement at the sub-organismal level that has been increasingly used as an ecotoxicological parameter (De Coen and Janssen, 2003; Servia et al., 2006; Sokolova et al., 2012). Energy reserves (i.e.: carbohydrates, lipids and proteins contents) are dependent of the energy input (i.e. feeding) and metabolic expenditure of organisms while energy consumption, measured at the cellular level through the electron transport system (ETS) activity, gives an insight into the metabolic activity of organisms that can be used to assess energy requirements and expenditure arising from the metabolic response to stress conditions (Sokolova et al., 2012). As such,

alteration in energetic homeostasis induced by exposure to UV-filters can also be evaluated and be used to predict changes in growth, reproduction and survival of organisms under stressful conditions which is important when dealing with species with a long life-cycle (Calow and Forbes, 1998; Rodrigues et al., 2015b; Sokolova et al., 2012; Sokolova, 2013).

Finally, and despite scarce investigation concerning organic UV-filters, it is important to evaluate their neurotoxicity towards aquatic invertebrates. The inhibition of Acetylcholinesterase (AChE) activity has been observed in many ecotoxicological studies with different contaminants (Campos et al., 2016; Domingues et al., 2010; Mesquita et al., 2011; Pérez et al., 2013; Pestana et al., 2014; Siebel et al., 2010; Xuereb et al., 2009) and has been widely used as an indication of neurotoxic effects, but to our knowledge evidences of neurotoxicity due to UV-filters exposure were only observed to vertebrates (Ruszkiewicz et al., 2017). Moreover, the activity of AChE is related with behavioural endpoints such as feeding rates, and locomotion (Mesquita et al., 2011; Xuereb et al., 2009). AChE is responsible for hydrolysis of acetylcholine to choline and acetate ion in the synaptic cleft. The inhibition of its activity can thus lead to an over-accumulation of the acetylcholine in the nerve terminals, prolonging the electrical activity (Grisaru et al., 1999; Purves et al., 2008).

1.2.3 Effects of mixtures containing organic UV-filters

Environmental risk assessment is mostly based on laboratory assays where organisms are exposed to gradients of single chemicals tested at optimal conditions. However, these standard tests might not be representative of conditions that organisms face in natural ecosystems, since natural freshwaters are constantly suffering inputs of diverse contaminants due to anthropogenic activities (e.g. industrial and urban activities) and consequently aquatic organisms are frequently exposed via water and sediments to a cocktail of contaminants. It is therefore recognized that assessment of effects of binary or complex chemical mixtures should be integrated as part of environmental risk assessment and in the last years, different strategies and approaches have been developed (Backhaus and Faust, 2012; Iwasaki and Brinkman, 2015; Jonker et al., 2005).

Two conceptual models have been mainly used to assess the combined effects of chemicals: the Concentration Addiction (CA) and the Independent Action (IA). These models are based in the premise that the chemicals in a mixture do not interact (physically, chemically or biologically) one with each other (Cedergreen et al., 2008; Hewlett and Plackett, 1959). The CA model have been used mostly when the chemicals of the mixture have similar modes of action (MoA) and "*assumes that 1 chemical can be replaced totally or in part by an equal fraction of an equi-effective*

concentration of another, without diminishing the overall combination effect" (Kortenkamp & Altenburger, 2010). On the other hand, IA model is used for compounds with different modes of action and "*assumes that the joint effects of a combination of agents can be calculated from the response of individual mixtures components*" (Kortenkamp & Altenburger, 2010). Also and since the application of these two conceptual models to experimental data is difficult to accomplish, other approaches such as Generalized linear models have been used to analyse mixture toxicity (Iwasaki and Brinkman, 2015; Morgado et al., 2016; Nieto et al., 2016).

Independently of the approach used, observed results concerning toxicity of chemical mixtures can be categorized as i) additive, when the components of the mixture do not influence one each other and no deviations to the reference models (non-interaction) is observed; ii) synergistic representing the worst scenario since it occurs when there is a significant deviation from the reference model and the toxicity of the mixture is higher than expected and finally iii) antagonistic when there is also a deviation from the reference model but effects of the mixture are smaller than expected (Kortenkamp & Altenburger, 2010).

Occurrence of chemical mixtures is especially relevant in the case of organic UV-filters. First, UV-filters are only one of the many ingredients that constitute the PCPs, which also may have in their constitution more than one UV-filter to guarantee the adequate protection (Ozáez et al., 2016a). On the other hand, several studies have showed the simultaneous presence of different UV-filters in the aquatic environment (Ramos et al., 2015).

To our knowledge few studies have been performed to assess the effects of mixture of different organic UV-filters in aquatic organisms (Kunz and Fent, 2009; Molins-Delgado et al., 2016; Ozáez et al., 2016b; Park et al., 2017) and in general, the results of aqueous exposures indicate a reduction of the toxicity of the mixture. Moreover, it is imperative to understand the effects of sediments contaminated with theses mixtures in organisms if we consider the physic-chemical properties of these compounds.

1.2.4 Relevance of long-term, multigenerational exposures to organic UV-filters

Natural populations are sometimes exposed to persistent contaminants over several generations. However, standard ecotoxicological tests are usually based on exposure of a single generation and thus inefficient to detect effects at population levels (Tassou and Schulz, 2011). Multigenerational ecotoxicity studies are of extreme importance to understand how continuous exposure to contaminants can compromise fitness of populations. Indeed, the toxicity of chemicals can be altered over the generations, i. e.: the toxicity might decrease or increase

throughout the generations but also can be maintained. Moreover, "*the toxicity can also "emerge" across the generations*" (Barata et al., 2017; Bona et al. 2016, Hochmuth et al. 2015, Kafel et al., 2012; Waissi et al., 2017). This is because a previous exposure to a stressor may lead to changes in organism response, which may be only triggered after exposure to a different stressor/chemical and appear latter or even only in subsequent generations (*e.g.* carry-over effects). For example, exposure of parents to chemicals can lead to alterations in the mechanisms of gene expression without changes in the DNA sequence (epigenetic effects) (Curley et al., 2011; Feil and Fraga, 2012) and these alterations might also be transferred to the subsequent generations. Furthermore, exposure to chemicals can lead not only to epigenetic alterations but also to stable genetic alterations (ex: mutations) in the parents that are then transferred (through germ cells) during multiple subsequent generations (transgenerational effects) (Anway et al., 2005). Therefore, transgenerational effects are observed in organisms without direct chemical exposure, but due to exposure of parental generations (Bhandari et al., 2015). Moreover, the alterations of toxicity across generations might also be due to phenotypic plasticity of organisms or even due to parental effects at reproduction level (e.g. decrease investment in eggs; less sperm production) which might compromise offspring (Marinković et al., 2012; Tassou and Schulz, 2013).

Several multigenerational studies using different aquatic organisms have shown that bioassays covering only one generation can mask effects of different classes of contaminants such metals, pesticides and pharmaceuticals in real exposure scenarios (Borgmann et al., 2007; Coimbra et al., 2015; Heye et al., 2016; Silva et al., 2017; Vogt et al., 2007). Multigenerational assays are pivotal to address the ecological effects of organic UV-filters since these compounds have high lipophilicity, low degradability, adsorb to organic matter and accumulate in sediments. Moreover, although with higher concentrations in the summer months, UV-filters are expected to reach aquatic ecosystems throughout the year and consequently organisms might be exposed during several generations to these contaminants. Additionally, UV-filters have shown to be endocrine disruptors (Wang et al., 2016) which can lead to hormonal disorders and consequently to reproductive impairments that need to be evaluated. Exposure to BP3, for instance, has been shown to elicit effects on the reproduction of vertebrate and invertebrate species (Blüthgen et al., 2012; Coronado et al., 2008; Ozáez et al., 2014), alter induction of vitellogenin in fishes and decreases the success hatching of the fish *Oryzias latipes* (Coronado et al., 2008). Moreover, exposure to BP3 affects the expression of ecdysone responsive genes and delay hatching of *C. riparius* embryos (Ozáez et al., 2014; Ozáez et al., 2016b).

Several multigenerational tests using *C. riparius* have been previously performed to evaluate the effects of different chemical stressors such as metals, organometals, pharmaceuticals and also others endocrine disruptors (Heye et al., 2016; Lilley et al., 2012; Marinković et al., 2012; Tassou and Schulz, 2009; Vogt et al., 2007). *C. riparius* is a good model organism for multigenerational ecotoxicity testing and to address the long-term effects of organic UV-filters present in sediments given its short-life cycle with 10 or more generations during a year in laboratory conditions and its sexual mode of reproduction.

1.2.5 High-tier ecotoxicity testing to address Community and ecosystem level effects of organic UV- filters

In the last years, community ecotoxicology testing has increased in an attempt to better assess effects of different stressors to freshwaters (Abelho et al., 2016; Cañedo-Argüelles et al., 2017; Rogers et al., 2016). In fact, the use of such approaches including mesocosms systems where natural communities are exposed to a given stressors eases extrapolation of effects to natural ecosystems comparatively to single species laboratorial standard tests. This is because responses of several species belonging to different trophic guilds are assessed but also because they include wider range of habitat conditions and biological interactions that can modify toxicity (Pestana et al., 2009b; Relyea and Hoverman, 2006; Stewart et al., 2013; Woodward, 2009). Moreover, the aim of community ecotoxicology testing is usually assessing direct and indirect effects of contaminants by focusing on community and ecosystem level responses simulating field conditions thus providing ecological sensitive reliable and relevant data for environmental risk assessment (Lizotte et al., 2013)

The health of an ecosystem can be evaluated using functional (leaf decomposition and primary production) and structural parameters (macroinvertebrates communities structure) (Abelho et al., 2016; Cañedo-Argüelles et al., 2017; Hooper et al., 2012; Lizotte et al., 2013; Rogers et al., 2016; Woodward et al., 2012). Primary production is a key process to the functioning of the ecosystem, contributing to nutrients cycling and influencing carbon storage (Hooper et al., 2012). In small and moderate size streams, periphyton is the major responsible for primary production and plays an important role as a basis of the food web (Tlili et al., 2017). Periphyton is a matrix composed by microorganisms such as algae, bacteria, fungi and protozoa and organic and inorganic detritus (Battin et al., 2016; Martyniuk et al., 2016). Moreover, periphyton is an important biological indicator to classify water bodies (Battin et al., 2016; Sabater et al., 2007) and can be used to assess the effects of stressors in streams (Guasch et al 2016; Elias et al 2017;

Sabater et al., 2007). Another key process in freshwaters ecosystems is leaf litter degradation, especially in low and medium order streams where detritivore macroinvertebrates are dominant in terms of number and biomass and the input of detritus providing from surrounding vegetation is the main source of matter and energy to the stream (Abelho, 2001; Atkinson et al. 2017; Gessner et al 1999; Graça 2001; Wallace et al., 1997). The transfer of energy and matter through organic matter decomposition across trophic levels also contributes to the cycle of nutrients. This process is mediated by microorganisms and detritivores shredders (Gessner et al., 1999; Seena et al., 2017) and it is influenced by abundance of shredders, temperature and also by the quality of litter among other factors (Dangles and Malmqvist, 2004; Friberg et al., 2009; Graça, 2001; Leroy and Marks, 2006). This process has been used as indicator of the ecological status of the ecosystems (Woodard et al 2012; Young et al., 2008) and anthropogenic pressure showed to indirectly affect leaf litter decomposition due to direct effects on organisms that mediate this process (Rasmussen et al., 2012).

Thus, macroinvertebrates play an important role in the ecosystem due to their feeding activity, contributing to the nutrients cycle and providing food to the higher trophic levels (Schmera et al., 2017) and are used as a proxy for determining the ecological status of aquatic ecosystem (Dalu et al., 2017; Vidal et al., 2014). However, an evaluation based only in the structure of macroinvertebrates might be insufficient to understand all direct and indirect, density and trait mediated effects on key ecological processes of the ecosystems and should be complemented with functional parameters (Dalu et al., 2017). It is thus clear that environmental risk assessment of organic UV-filters has much to gain from community ecotoxicity testing focused on simultaneous evaluation of structural and functional responses of benthic invertebrate communities.

1.3 Aim of the study and conceptual framework

This thesis is focused on the ecotoxicological evaluation of organic UV-filters in freshwater ecosystems. In detail, it aimed to answer the following questions:

- Are environmentally relevant concentrations of different organic UV-filters toxic to aquatic insects?
- Are binary mixtures of these compounds more toxic?
- Can prolonged exposure to UV-filters induce multigenerational effects?
- Can relevant environmentally concentrations of UV-filters affect the structure and function of freshwater benthic invertebrate communities?

In order to answer these questions different approaches were pursued. Firstly, individual exposures to different UV-filters (BP3, 4-MBC and OC) were carried out using laboratory toxicity tests with the midge *C. riparius* and the caddisfly *S. vittatum*. Sub-lethal responses at the organism level (feeding rates, growth and emergence) were evaluated. Additionally, biomarkers related with oxidative stress (LPO, CAT, GST, tGSH), neurophysiology (AChE), energy reserves (lipids, carbohydrates and proteins contents) and energy metabolism (electron transport system activity) were used to provide information about the effects of these chemicals at sub-cellular levels (**chapter 2 and 3**). In **chapter 4,** IA model and Generalized Linear Model (GLM) were used to investigate the possible combined effects of simultaneous exposure to BP3 and 4-MBC. Moreover and knowing that insect repellents are used together with sunscreens, we have also used the same approach to address the combined effects of BP3 and a widely used insect repellent (N,N-diethyl-3-methylbenzamide (DEET)) (Aronson et al., 2012; Costanzo et al., 2007). *C. riparius* were exposed to these binary mixtures and endpoints like emergence, development time and imagoes weight were evaluated. For this, a parallel study was conducted to acquire the necessary ecotoxicity data concerning organismal and sub-organismal effects of DEET on *C. riparius* (**Annex I)** and *S. vittatum* **(Annex II**). The **chapter 5** reports on the long-term effects of BP3 in *C. riparius* evaluated using a multigenerational assay. For that, *C. riparius* were exposed during one generation to a gradient of BP3 and the second generation was exposed to the same condition that parents and also to control conditions. The emergence and the development time of *C. riparius* together with fecundity and fertility as well were assessed. Lastly, in **chapter 6** a mesocosms study was conducted, where effects of two environmentally relevant concentrations of 4-MBC were evaluated in terms of leaf decomposition, primary production and benthic invertebrate community structure. Finally, on **chapter 7** a summary of the main findings and prospective research concerning ecotoxicity of UV-filters is presented.

References

Abelho, M., 2001. From Litterfall to Breakdown in Streams: A Review. The Scientific World Journal 17;1:656-80.

Abelho, M., Martins, T.F., Shinn, C., Moreira-Santos, M., Ribeiro, R., 2016. Effects of the fungicide pyrimethanil on biofilm and organic matter processing in outdoor lentic mesocosms. Ecotoxicology 25, 121-131.

Amine, H., Gomez, E., Halwani, J., Casellas, C., Fenet, H., 2012. UV filters, ethylhexyl methoxycinnamate, octocrylene and ethylhexyl dimethyl PABA from untreated wastewater in sediment from eastern Mediterranean river transition and coastal zones. Marine Pollution Bulletin 64, 2435-2442.

Anway, M.D., Cupp, A.S., Uzumcu, M., Skinner, M.K., 2005. Epigenetic Transgenerational Actions of Endocrine Disruptors and Male Fertility. Science 308, 1466-1469.

Armitage, P.D., Pinder, L., Cranston, P., 1995. The Chironomidae: biology and ecology of non-biting midges. Springer Science & Business Media.

Aronson, D., Weeks, J., Meylan, B., Guiney, P.D., Howard, P.H., 2012. Environmental release, environmental concentrations, and ecological risk of N,N-Diethyl-m-toluamide (DEET). Integr Environ Assess Manag 8, 135-166.

Atkinson, C.L., Capps, K.A., Rugenski, A.T., Vanni, M.J., 2017. Consumer-driven nutrient dynamics in freshwater ecosystems: from individuals to ecosystems. Biological Reviews 92, 2003-2023.

Backhaus, T., Faust, M., 2012. Predictive Environmental Risk Assessment of Chemical Mixtures: A Conceptual Framework. Environmental Science & Technology 46, 2564-2573.

Barata, C., Campos, B., Rivetti, C., LeBlanc, G. A., Eytcheson, S., McKnight, S., Tobor-Kaplon, M., de Vries Buitenweg, S., Choi, S., Choi, J., Sarapultseva, E. I., Coutellec, M. A., Coke, M., Pandard, P., Chaumot, A., Queau, H., Delorme, N., Geffard, O., Martinez-Jeronimo, F., Watanabe, H., Tatarazako, N., Lopes, I., Pestana, J. L., Soares, A. M., Pereira, C. M., De Schamphelaere, K., 2017. Validation of a two-generational reproduction test in *Daphnia magna*: An interlaboratory exercise. Science of The Total Environment. 579, 1073-1083

Battin, T.J., Besemer, K., Bengtsson, M.M., Romani, A.M., Packmann, A.I., 2016. The ecology and biogeochemistry of stream biofilms. Nature Reviews Microbiology 14, 251.

Berra, E., Forcella, M., Giacchini, R., Rossaro, B., Parenti, P., 2006. Biomarkers in Caddisfly Larvae of the Species *Hydropsyche pellucidula* (Curtis, 1834) (Trichoptera: Hydropsychidae) Measured in Natural Populations and after Short Term Exposure to Fenitrothion. Bulletin of Environmental Contamination and Toxicology 76, 863-870.

Bhandari, R.K., vom Saal, F.S., Tillitt, D.E., 2015. Transgenerational effects from early developmental exposures to bisphenol A or 17α-ethinylestradiol in medaka, *Oryzias latipes*. Scientific Reports 5, 9303.

Blüthgen, N., Meili, N., Chew, G., Odermatt, A., Fent, K., 2014. Accumulation and effects of the UV-filter octocrylene in adult and embryonic zebrafish (*Danio rerio*). Science of The Total Environment 476–477, 207-217.

Blüthgen, N., Zucchi, S., Fent, K., 2012. Effects of the UV filter benzophenone-3 (oxybenzone) at low concentrations in zebrafish (*Danio rerio*). Toxicology and Applied Pharmacology 263, 184-194.

Bona, M. Dalla, Lizzi, F., Borgato, A., De Liguoro, M., 2016. Increasing toxicity of enro- floxacin over four generations of *Daphnia magna*, Ecotoxicology Environmental Safety 132, 397–402.

Borgmann, U., Bennie, D.T., Ball, A.L., Palabrica, V., 2007. Effect of a mixture of seven pharmaceuticals on *Hyalella azteca* over multiple generations. Chemosphere 66, 1278-1283.

Brausch, J.M., Rand, G.M., 2011. A review of personal care products in the aquatic environment: Environmental concentrations and toxicity. Chemosphere 82, 1518-1532.

Buser, H.R., Balmer, M.E., Schmid, P., Kohler, M., 2006. Occurrence of UV filters 4-methylbenzylidene camphor and octocrylene in fish from various Swiss rivers with inputs from wastewater treatment plants. Environ Sci Technol 40, 1427-1431.

Calow, P., Forbes, V.E., 1998. How do physiological responses to stress translate into ecological and evolutionary processes? Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 120, 11-16.

Campos, D., Alves, A., Lemos, M.L., Correia, A., Soares, A.V.M., Pestana, J.T., 2014. Effects of cadmium and resource quality on freshwater detritus processing chains: a microcosm approach with two insect species. Ecotoxicology 23, 830-839.

Campos, D., Gravato, C., Quintaneiro, C., Koba, O., Randak, T., Soares, A.M., Pestana, J.L., 2016. Are insect repellents toxic to freshwater insects? A case study using caddisflies exposed to DEET. Chemosphere 149, 177-182.

Cañedo-Argüelles, M., Brucet, S., Carrasco, S., Flor-Arnau, N., Ordeix, M., Ponsá, S., Coring, E., 2017. Effects of potash mining on river ecosystems: An experimental study. Environmental Pollution 224, 759-770.

Cedergreen, N., Christensen, A.M., Kamper, A., Kudsk, P., Mathiassen, S.K., Streibig, J.C., Sørensen, H., 2008. A review of independent action compared to concentration addition as reference models for mixtures of compounds with different molecular target sites. Environmental Toxicology and Chemistry 27, 1621-1632.

Chisvert, A., Salvador, A., 2007. 3.1 - UV Filters in Sunscreens and other Cosmetics. Regulatory Aspects and Analytical Methods, Analysis of Cosmetic Products. Elsevier, Amsterdam, pp. 83-120.

Coimbra, A.M., Peixoto, M.J., Coelho, I., Lacerda, R., Carvalho, A.P., Gesto, M., Lyssimachou, A., Lima, D., Soares, J., André, A., Capitão, A., Castro, L.F.C., Santos, M.M., 2015. Chronic effects of clofibric acid in zebrafish (*Danio rerio*): A multigenerational study. Aquatic Toxicology 160, 76-86.

Coronado, M., De Haro, H., Deng, X., Rempel, M.A., Lavado, R., Schlenk, D., 2008. Estrogenic activity and reproductive effects of the UV-filter oxybenzone (2-hydroxy-4-methoxyphenyl-methanone) in fish. Aquatic Toxicology 90, 182-187.

Costanzo, S.D., Watkinson, A.J., Murby, E.J., Kolpin, D.W., Sandstrom, M.W., 2007. Is there a risk associated with the insect repellent DEET (N,N-diethyl-m-toluamide) commonly found in aquatic environments? Science of The Total Environment 384, 214-220.

Curley, J.P., Mashoodh, R., Champagne, F.A., 2011. Epigenetics and the Origins of Paternal Effects. Hormones and behavior 59, 306-314.

Dalu, T., Wasserman, R.J., Tonkin, J.D., Alexander, M.E., Dalu, M.T.B., Motitsoe, S.N., Manungo, K.I., Bepe, O., Dube, T., 2017. Assessing drivers of benthic macroinvertebrate community structure in African highland streams: An exploration using multivariate analysis. Science of The Total Environment 601-602, 1340-1348.

Dangles, O., Malmqvist, B., 2004. Species richness–decomposition relationships depend on species dominance. Ecology Letters 7, 395-402.

De Coen, W.M., Janssen, C.R., 2003. The missing biomarker link: Relationships between effects on the cellular energy allocation biomarker of toxicant-stressed *Daphnia magna* and corresponding population characteristics. Environmental Toxicology and Chemistry 22, 1632-1641.

Díaz-Cruz, M., Llorca, M., Barceló, D., Barceló, D., 2008. Organic UV filters and their photodegradates, metabolites and disinfection by-products in the aquatic environment. TrAC Trends in Analytical Chemistry 27, 873-887.

Domingues, I., Agra, A.R., Monaghan, K., Soares, A.M.V.M., Nogueira, A.J.A., 2010. Cholinesterase and glutathione-*S*-transferase activities in freshwater invertebrates as biomarkers to assess pesticide contamination. Environmental Toxicology and Chemistry 29, 5-18.

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

Doyotte, A., Cossu, C., Jacquin, M.-C., Babut, M., Vasseur, P., 1997. Antioxidant enzymes, glutathione and lipid peroxidation as relevant biomarkers of experimental or field exposure in the gills and the digestive gland of the freshwater bivalve *Unio tumidus*. Aquatic Toxicology 39, 93-110.

Elias, C.L., Rocha, R.J.M., Feio, M.J., Figueira, E., Almeida, S.F.P., 2017. Influence of the colonizing substrate on diatom assemblages and implications for bioassessment: a mesocosm experiment. Aquatic Ecology 51, 145-158.

Emnet, P., Gaw, S., Northcott, G., Storey, B., Graham, L., 2015. Personal care products and steroid hormones in the Antarctic coastal environment associated with two Antarctic research stations, McMurdo Station and Scott Base. Environ. Res. 136, 331–342.

Enayati, A.A., Ranson, H., Hemingway, J., 2005. Insect glutathione transferases and insecticide resistance. Insect Mol Biol 14, 3-8.

Environment Agency, 2008. UV-filters in cosmetics – prioritisation for environmental assessment. Environment Agency, Bristol, BS32 4UD

Feil, R., Fraga, M.F., 2012. Epigenetics and the environment: emerging patterns and implications. Nature Reviews Genetics 13, 97-109.

Feio, M.J., Graça, M.A.S., 2000. Food consumption by the larvae of *Sericostoma vittatum* (Trichoptera), an endemic species from the Iberian Peninsula. Hydrobiologia 439, 7-11.

Fent, K., Kunz, P.Y., Zenker, A., Rapp, M., 2010. A tentative environmental risk assessment of the UV-filters 3-(4-methylbenzylidene-camphor), 2-ethyl-hexyl-4-trimethoxycinnamate, benzophenone-3, benzophenone-4 and 3-benzylidene camphor. Marine Environmental Research 69, Supplement 1, S4-S6.

Ferrington, L.C., 2008. Global diversity of non-biting midges (Chironomidae; Insecta-Diptera) in freshwater, in: Balian, E.V., Lévêque, C., Segers, H., Martens, K. (Eds.), Freshwater Animal Diversity Assessment. Springer Netherlands, Dordrecht, pp. 447-455.

Friberg, N., DybkjÆR, J.B., Olafsson, J.S., Gislason, G.M., Larsen, S.E., Lauridsen, T.L., 2009. Relationships between structure and function in streams contrasting in temperature. Freshwater Biology 54, 2051-2068.

Gago-Ferrero, P., Díaz-Cruz, M.S., Barceló, D., 2011. Fast pressurized liquid extraction with in-cell purification and analysis by liquid chromatography tandem mass spectrometry for the determination of UV filters and their degradation products in sediments. Analytical and Bioanalytical Chemistry 400, 2195-2204.

Gago-Ferrero, P., Díaz-Cruz, M.S., Barceló, D., 2012. An overview of UV-absorbing compounds (organic UV filters) in aquatic biota. Analytical and Bioanalytical Chemistry 404, 2597-2610.

Gago-Ferrero, P., Díaz-Cruz, M.S., Barceló, D., 2015. UV filters bioaccumulation in fish from Iberian river basins. Science of The Total Environment 518, 518-525.

Gao, L., Yuan, T., Zhou, C., Cheng, P., Bai, Q., Ao, J., Wang, W., Zhang, H., 2013. Effects of four commonly used UV filters on the growth, cell viability and oxidative stress responses of the *Tetrahymena thermophila*. Chemosphere 93, 2507-2513.

Gessner, M.O., Chauvet, E., Dobson, M., 1999. A Perspective on Leaf Litter Breakdown in Streams. Oikos 85, 377-384.

Giokas, D.L., Salvador, A., Chisvert, A., 2007. UV filters: From sunscreens to human body and the environment. TrAC Trends in Analytical Chemistry 26, 360-374.

González, J.M., Graça, M.A.S., 2003. Conversion of leaf litter to secondary production by a shredding caddis-fly. Freshwater Biology 48, 1578-1592.

Graça, M.A.S., 2001. The Role of Invertebrates on Leaf Litter Decomposition in Streams – a Review. International Review of Hydrobiology 86, 383-393.

Grisaru, D., Sternfeld, M., Eldor, A., Glick, D., Soreq, H., 1999. Structural roles of acetylcholinesterase variants in biology and pathology. European Journal of Biochemistry 264, 672-686.

Guasch, H., Ricart, M., López-Doval, J., Bonnineau, C., Proia, L., Morin, S., Muñoz, I., Romaní, A.M., Sabater, S., 2016. Influence of grazing on triclosan toxicity to stream periphyton. Freshwater Biology 61, 2002-2012.

Hewlett, P.S., Plackett, R.L., 1959. A Unified Theory for Quantal Responses to Mixtures of Drugs: Non-Interactive Action. Biometrics 15, 591-610.

Heye, K., Becker, D., Lütke Eversloh, C., Durmaz, V., Ternes, T.A., Oetken, M., Oehlmann, J., 2016. Effects of carbamazepine and two of its metabolites on the non-biting midge *Chironomus riparius* in a sediment full life cycle toxicity test. Water Research 98, 19-27.

Hochmuth, J.D., De Meester, L., Pereira, C.M.S., Janssen, C.R., De Schamphelaere, K. A. C., 2015. Rapid adaptation of a *Daphnia magna* population to metal stress is associated with heterozygote excess, Environmental Science & Technology's, 49, 9298-9307.

Hooper, D.U., Adair, E.C., Cardinale, B.J., Byrnes, J.E.K., Hungate, B.A., Matulich, K.L., Gonzalez, A., Duffy, J.E., Gamfeldt, L., O/'Connor, M.I., 2012. A global synthesis reveals biodiversity loss as a major driver of ecosystem change. Nature 486, 105-108.

Hyne, R.V., Maher, W.A., 2003. Invertebrate biomarkers: links to toxicosis that predict population decline. Ecotoxicology and Environmental Safety 54, 366-374.

Iwasaki, Y., Brinkman, S.F., 2015. Application of a generalized linear mixed model to analyze mixture toxicity: Survival of brown trout affected by copper and zinc. Environmental Toxicology and Chemistry 34, 816-820.

Jonker, M.J., Svendsen, C., Bedaux, J.J.M., Bongers, M., Kammenga, J.E., 2005. Significance testing of synergistic/antagonistic, dose level-dependent, or dose ratio-dependent effects in mixture dose-response analysis. Environmental Toxicology and Chemistry 24, 2701-2713.

Kafel, A., Zawisza-Raszka, A., Szulińska, E., 2012. Effects of multigenerational cadmium exposure of insects (*Spodoptera exigua* larvae) on anti-oxidant response in haemolymph and developmental parameters. Environmental Pollution 162, 8-16.

Kameda, Y., Kimura, K., Miyazaki, M., 2011. Occurrence and profiles of organic sun-blocking agents in surface waters and sediments in Japanese rivers and lakes. Environmental Pollution 159, 1570-1576.

Kim, S., Jung, D., Kho, Y., Choi, K., 2014. Effects of benzophenone-3 exposure on endocrine disruption and reproduction of Japanese medaka (*Oryzias latipes*)—A two generation exposure study. Aquatic Toxicology 155, 244-252.

Kortenkamp, A., Altenburger, R., 2010, 3-Toxicity from Combined Exposure to Chemicals, Mixture Toxicity: Linking approaches from Ecological and Human Toxicity, SETAC, pp. 95-119.

Kunz, P.Y., Fent, K., 2009. Estrogenic activity of ternary UV filter mixtures in fish (*Pimephales promelas*) — An analysis with nonlinear isobolograms. Toxicology and Applied Pharmacology 234, 77-88.

Langford, K.H., Reid, M.J., Fjeld, E., Øxnevad, S., Thomas, K.V., 2015. Environmental occurrence and risk of organic UV filters and stabilizers in multiple matrices in Norway. Environment International 80, 1-7.

Leroy, C.J., Marks, J.C., 2006. Litter quality, stream characteristics and litter diversity influence decomposition rates and macroinvertebrates. Freshwater Biology 51, 605-617.

Lilley, T.M., Ruokolainen, L., Pikkarainen, A., Laine, V.N., Kilpimaa, J., Rantala, M.J., Nikinmaa, M., 2012. Impact of tributyltin on immune response and life history traits of *Chironomus riparius*: single and multigeneration effects and recovery from pollution. Environ Sci Technol 46, 7382-7389.

Liu, H., Sun, P., Liu, H., Yang, S., Wang, L., Wang, Z., 2015. Hepatic oxidative stress biomarker responses in freshwater fish *Carassius auratus* exposed to four benzophenone UV filters. Ecotoxicology and Environmental Safety 119, 116-122.

Lizotte, R.E., Testa, S., Locke, M.A., Steinriede, R.W., 2013. Responses of Phytoplankton and *Hyalella azteca* to Agrichemical Mixtures in a Constructed Wetland Mesocosm. Archives of Environmental Contamination and Toxicology 65, 474-485.

Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquatic Toxicology 101, 13-30.

Lushchak, V.I., 2012. Glutathione homeostasis and functions: potential targets for medical interventions. J Amino Acids 2012, 736837.

Marinković, M., de Bruijn, K., Asselman, M., Bogaert, M., Jonker, M.J., Kraak, M.H.S., Admiraal, W., 2012. Response of the Nonbiting Midge *Chironomus riparius* to Multigeneration Toxicant Exposure. Environmental Science & Technology 46, 12105-12111.

Martyniuk, N., Modenutti, B., Balseiro, E., 2016. Forest Structure Affects the Stoichiometry of Periphyton Primary Producers in Mountain Streams of Northern Patagonia. Ecosystems 19, 1225-1239.

Merrit, R. W., Cummins, K. W., 1996. Aquatic Insects of North America. 3rd eds. Kendall / Hunt
Publishing Company, Dubuque

Mesquita, S.R., Guilhermino, L., Guimarães, L., 2011. Biochemical and locomotor responses of *Carcinus maenas* exposed to the serotonin reuptake inhibitor fluoxetine. Chemosphere 85, 967-976.tej

Molins-Delgado, D., Gago-Ferrero, P., Díaz-Cruz, M.S., Barceló, D., 2016. Single and joint ecotoxicity data estimation of organic UV filters and nanomaterials toward selected aquatic organisms. Urban groundwater risk assessment. Environmental Research 145, 126-134.

Morgado, R.G., Gomes, P.A.D., Ferreira, N.G.C., Cardoso, D.N., Santos, M.J.G., Soares, A.M.V.M., Loureiro, S., 2016. Toxicity interaction between chlorpyrifos, mancozeb and soil moisture to the terrestrial isopod *Porcellionides pruinosus*. Chemosphere 144, 1845-1853.

Nieto, E., Hampel, M., González-Ortegón, E., Drake, P., Blasco, J., 2016. Influence of temperature on toxicity of single pharmaceuticals and mixtures, in the crustacean *A. desmarestii*. Journal of Hazardous Materials 313, 159-169.

OECD, 2004. Test no. 219: sediment–water chironomid toxicity using spiked water, in: Publishing, O. (Ed.), OECD Guidelines for the testing of chemicals

Ozáez, I., Martínez-Guitarte, J.L., Morcillo, G., 2013. Effects of in vivo exposure to UV filters (4-MBC, OMC, BP-3, 4-HB, OC, OD-PABA) on endocrine signaling genes in the insect *Chironomus riparius*. Science of The Total Environment 456–457, 120-126.

Ozáez, I., Martínez-Guitarte, J.L., Morcillo, G., 2014. The UV filter benzophenone 3 (BP-3) activates hormonal genes mimicking the action of ecdysone and alters embryo development in the insect *Chironomus riparius* (Diptera). Environmental Pollution 192, 19-26.

Ozáez, I., Morcillo, G., Martínez-Guitarte, J.-L., 2016a. The effects of binary UV filter mixtures on the midge *Chironomus riparius*. Science of The Total Environment 556, 154-162.

Ozáez, I., Morcillo, G., Martínez-Guitarte, J.-L., 2016b. Ultraviolet filters differentially impact the expression of key endocrine and stress genes in embryos and larvae of *Chironomus riparius*. Science of The Total Environment 557–558, 240-247.

Paredes, E., Perez, S., Rodil, R., Quintana, J.B., Beiras, R., 2014. Ecotoxicological evaluation of four UV filters using marine organisms from different trophic *levels Isochrysis galbana, Mytilus galloprovincialis, Paracentrotus lividus, and Siriella armata*. Chemosphere 104, 44-50.

Park, C.-B., Jang, J., Kim, S., Kim, Y.J., 2017. Single- and mixture toxicity of three organic UV-filters, ethylhexyl methoxycinnamate, octocrylene, and avobenzone on *Daphnia magna*. Ecotoxicology and Environmental Safety 137, 57-63

Peck, A.M., 2006. Analytical methods for the determination of persistent ingredients of personal care products in environmental matrices. Analytical and Bioanalytical Chemistry 386, 907-939.

Pedrouzo, M., Borrull, F., Marcé, R.M., Pocurull, E., 2011. Analytical methods for personal-care products in environmental waters. TrAC Trends in Analytical Chemistry 30, 749-760.

Pérez, J., Monteiro, M.S., Quintaneiro, C., Soares, A.M.V.M., Loureiro, S., 2013. Characterization of cholinesterases in *Chironomus riparius* and the effects of three herbicides on chlorpyrifos toxicity. Aquatic Toxicology 144–145, 296-302.

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

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

Pestana, J.L.T., Novais, S.C., Lemos, M.F.L., Soares, A.M.V.M., 2014. Cholinesterase activity in the caddisfly *Sericostoma vittatum*: Biochemical enzyme characterization and in vitro effects of insecticides and psychiatric drugs. Ecotoxicology and Environmental Safety 104, 263-268.

Picot Groz, M., Martinez Bueno, M.J., Rosain, D., Fenet, H., Casellas, C., Pereira, C., Maria, V., Bebianno, M.J., Gomez, E., 2014. Detection of emerging contaminants (UV filters, UV stabilizers and musks) in marine mussels from Portuguese coast by QuEChERS extraction and GC-MS/MS. Sci Total Environ 493, 162-169.

Plagellat, C., Kupper, T., Furrer, R., de Alencastro, L.F., Grandjean, D., Tarradellas, J., 2006. Concentrations and specific loads of UV filters in sewage sludge originating from a monitoring network in Switzerland. Chemosphere 62, 915-925.

Purves, D., George, J.A., David, F., Hall, W.C., LaMantia, A.-S., McNamara, J.O., White,L.E., 2008. Neuroscience. 4th ed. Sinauer Associates.

Ramos, S., Homem, V., Alves, A., Santos, L., 2015. Advances in analytical methods and occurrence of organic UV-filters in the environment — A review. Science of The Total Environment 526, 278-311.

Ramos, S., Homem, V., Alves, A., Santos, L., 2016. A review of organic UV-filters in wastewater treatment plants. Environment International 86, 24-44.

Rasmussen, J.J., Wiberg-Larsen, P., Baattrup-Pedersen, A., Monberg, R.J., Kronvang, B., 2012. Impacts of pesticides and natural stressors on leaf litter decomposition in agricultural streams. Science of The Total Environment 416, 148-155.

Relyea, R., Hoverman, J., 2006. Assessing the ecology in ecotoxicology: a review and synthesis in freshwater systems. Ecology Letters 9, 1157-1171.

Rodil, R., Moeder, M., 2008. Development of a simultaneous pressurised-liquid extraction and clean-up procedure for the determination of UV filters in sediments. Analytica Chimica Acta 612, 152-159.

Rodil, R., Moeder, M., Altenburger, R., Schmitt-Jansen, M., 2009. Photostability and phytotoxicity of selected sunscreen agents and their degradation mixtures in water. Analytical and Bioanalytical Chemistry 395, 1513.

Rodrigues, A.C.M., Gravato, C., Quintaneiro, C., Barata, C., Soares, A.M.V.M., Pestana, J.L.T., 2015a. Sub-lethal toxicity of environmentally relevant concentrations of esfenvalerate to *Chironomus riparius*. Environmental Pollution 207, 273-279.

Rodrigues, A.C.M., Gravato, C., Quintaneiro, C., Bordalo, M.D., Golovko, O., Žlábek, V., Barata, C., Soares, A.M.V.M., Pestana, J.L.T., 2017. Exposure to chlorantraniliprole affects the energy metabolism of the caddisfly *Sericostoma vittatum*. Environmental Toxicology and Chemistry 36, 1584-1591.

Rodrigues, A.C.M., Gravato, C., Quintaneiro, C., Golovko, O., Žlábek, V., Barata, C., Soares, A.M.V.M., Pestana, J.L.T., 2015b. Life history and biochemical effects of chlorantraniliprole on *Chironomus riparius*. Science of The Total Environment 508, 506-513.

Rogers, H.A., Schmidt, T.S., Dabney, B.L., Hladik, M.L., Mahler, B.J., Van Metre, P.C., 2016. Bifenthrin Causes Trophic Cascade and Altered Insect Emergence in Mesocosms: Implications for Small Streams. Environmental Science & Technology 50, 11974-11983.

Ruszkiewicz, J.A., Pinkas, A., Ferrer, B., Peres, T.V., Tsatsakis, A., Aschner, M., 2017. Neurotoxic effect of active ingredients in sunscreen products, a contemporary review. Toxicology Reports 4, 245-259.

Sabater, S., Guasch, H., Ricart, M., Romaní, A., Vidal, G., Klünder, C., Schmitt-Jansen, M., 2007. Monitoring the effect of chemicals on biological communities. The biofilm as an interface. Analytical and Bioanalytical Chemistry 387, 1425-1434.

Schmera, D., Heino, J., Podani, J., Erős, T., Dolédec, S., 2017. Functional diversity: a review of methodology and current knowledge in freshwater macroinvertebrate research. Hydrobiologia 787, 27-44.

Schmitt, C., Oetken, M., Dittberner, O., Wagner, M., Oehlmann, J., 2008. Endocrine modulation and toxic effects of two commonly used UV screens on the aquatic invertebrates *Potamopyrgus antipodarum* and *Lumbriculus variegatus*. Environmental Pollution 152, 322-329.

Schulz, R., Liess, M., 2000. Toxicity of fenvalerate to caddisfly larvae: chronic effects of 1- vs 10-h pulse-exposure with constant doses. Chemosphere 41, 1511-1517.

Seena, S., Carvalho, F., Cássio, F., Pascoal, C., 2017. Does the developmental stage and composition of riparian forest stand affect ecosystem functioning in streams? Science of The Total Environment 609, 1500-1511.

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

Siebel, A.M., Rico, E.P., Capiotti, K.M., Piato, A.L., Cusinato, C.T., Franco, T.M.A., Bogo, M.R., Bonan, C.D., 2010. In vitro effects of antiepileptic drugs on acetylcholinesterase and ectonucleotidase activities in zebrafish (*Danio rerio*) brain. Toxicology in Vitro 24, 1279-1284.

Sieratowicz, A., Kaiser, D., Behr, M., Oetken, M., Oehlmann, J., 2011. Acute and chronic toxicity of four frequently used UV filter substances for *Desmodesmus subspicatus* and *Daphnia magna*. J Environ Sci Health A Tox Hazard Subst Environ Eng 46, 1311-1319.

Silva, A.R.R., Cardoso, D.N., Cruz, A., Pestana, J.L.T., Mendo, S., Soares, A.M.V.M., Loureiro, S., 2017. Multigenerational effects of carbendazim in *Daphnia magna*. Environmental Toxicology and Chemistry 36, 383-394.

Sokolova, I.M., 2013. Energy-limited tolerance to stress as a conceptual framework to integrate the effects of multiple stressors. Integr Comp Biol 53, 597-608.

Sokolova, I.M., Frederich, M., Bagwe, R., Lannig, G., Sukhotin, A.A., 2012. Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. Mar Environ Res 79, 1-15.

Stewart, R. I. A., M. Dossena, D. A. Bohan, E. Jeppesen, R. L. Kordas, M. E. Ledger, M. Meerhoff, B. Moss, C. Mulder, J. B. Shurin, B. Suttle, R. Thompson, M. Trimmer & G. Woodward, 2013. Mesocosm experiments as a tool for ecological climate change research. Advances in Ecological Research 48, 71–181.

Tassou, K.T., Schulz, R., 2009. Effects of the insect growth regulator pyriproxyfen in a two-generation test with *Chironomus riparius*. Ecotoxicology and Environmental Safety 72, 1058-1062.

Tassou, K.T., Schulz, R., 2011. Two-generation effects of the chitin synthesis inhibitor, teflubenzuron, on the aquatic midge *Chironomus riparius*. Ecotoxicology and Environmental Safety 74, 1203-1209.

Tassou, K.T., Schulz, R., 2013. Low field-relevant tebufenozide concentrations affect reproduction in *Chironomus riparius* (Diptera: Chironomidae) in a long-term toxicity test. Environmental Science and Pollution Research 20, 3735-3742.

Tlili, A., Hollender, J., Kienle, C., Behra, R., 2017. Micropollutant-induced tolerance of in situ periphyton: Establishing causality in wastewater-impacted streams. Water Research 111, 185-194.

Tolls, J., Berger, H., Klenk, A., Meyberg, M., Beiersdorf, A.G., Müller, R., Rettinger, K., Steber, J., 2009. Environmental safety aspects of personal care products—A European perspective. Environmental Toxicology and Chemistry 28, 2485-2489.

Tsui, M.M.P., Leung, H.W., Kwan, B.K.Y., Ng, K.-Y., Yamashita, N., Taniyasu, S., Lam, P.K.S., Murphy, M.B., 2015. Occurrence, distribution and ecological risk assessment of multiple classes of UV filters in marine sediments in Hong Kong and Japan. Journal of Hazardous Materials 292, 180-187.

Tsui, M.M.P., Leung, H.W., Wai, T.-C., Yamashita, N., Taniyasu, S., Liu, W., Lam, P.K.S., Murphy, M.B., 2014. Occurrence, distribution and ecological risk assessment of multiple classes of UV filters in surface waters from different countries. Water Research 67, 55-65.

Valavanidis, A., Vlahogianni, T., Dassenakis, M., Scoullos, M., 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. Ecotoxicology and Environmental Safety 64, 178-189.

Van Gestel, C.A.M., Van Brummelen, T.C., 1996. Incorporation of the biomarker concept in ecotoxicology calls for a redefinition of terms. Ecotoxicology 5, 217-225.

Vidal, T., Santos, J.I., Marques, C.R., Pereira, J.L., Claro, M.T., Pereira, R., Castro, B.B., Soares, A., Gonçalves, F., 2014. Resilience of the macroinvertebrate community of a small mountain river (Mau River, Portugal) subject to multiple stresses. Marine and Freshwater Research 65, 633-644.

Vione, D., Caringella, R., De Laurentiis, E., Pazzi, M., Minero, C., 2013. Phototransformation of the sunlight filter benzophenone-3 (2-hydroxy-4-methoxybenzophenone) under conditions relevant to surface waters. Science of The Total Environment 463–464, 243-251.

Vogt, C., Belz, D., Galluba, S., Nowak, C., Oetken, M., Oehlmann, J., 2007. Effects of cadmium and tributyltin on development and reproduction of the non-biting midge *Chironomus riparius* (Diptera)—baseline experiments for future multi-generation studies. Journal of Environmental Science and Health, Part A 42, 1-9.

Wahie, S., Lloyd, J.J., Farr, P.M., 2007. Sunscreen ingredients and labelling: a survey of products available in the UK. Clinical and Experimental Dermatology 32, 359-364.

Waissi, G. C., Vaananen, K., Nybom, I., Pakarinen, K., Akkanen, J., Leppanen, M. T., Kukkonen, J. V. K., 2017. The chronic effects of fullereneC60-associated sediments in the midge *Chironomus riparius* - Responses in the first and the second generation. Environmental Pollution. 229, 423-430.

Wallace, J.B., Eggert, S.L., Meyer, J.L., Webster, J.R., 1997. Multiple Trophic Levels of a Forest Stream Linked to Terrestrial Litter Inputs. Science 277, 102-104.

Wang, J., Pan, L., Wu, S., Lu, L., Xu, Y., Zhu, Y., Guo, M., Zhuang, S., 2016. Recent Advances on Endocrine Disrupting Effects of UV Filters, International journal of environmental research and public health.

Woodward, G.U.Y., 2009. Biodiversity, ecosystem functioning and food webs in fresh waters: assembling the jigsaw puzzle. Freshwater Biology 54, 2171-2187.

Woodward, G., Gessner, M.O., Giller, P.S., Gulis, V., Hladyz, S., Lecerf, A., Malmqvist, B., McKie, B.G., Tiegs, S.D., Cariss, H., Dobson, M., Elosegi, A., Ferreira, V., Graça, M.A.S., Fleituch, T., Lacoursière, J.O., Nistorescu, M., Pozo, J., Risnoveanu, G., Schindler, M., Vadineanu, A., Vought, L.B.-M., Chauvet, E., 2012. Continental-Scale Effects of Nutrient Pollution on Stream Ecosystem Functioning. Science 336, 1438-1440.

Xuereb, B., Lefèvre, E., Garric, J., Geffard, O., 2009. Acetylcholinesterase activity in *Gammarus fossarum* (Crustacea Amphipoda): Linking AChE inhibition and behavioural alteration. Aquatic Toxicology 94, 114-122.

Young, R.G., Matthaei, C.D., Townsend, C.R., 2008. Organic matter breakdown and ecosystem metabolism: functional indicators for assessing river ecosystem health. Journal of the North American Benthological Society 27, 605-625.

Chapter 2:
Toxicity of organic UV-filters to the aquatic midge *Chironomus riparius*

Published in Ecotoxicology and Environmental Safety
DOI:10.1016/j.ecoenv.2017.05.005

Toxicity of organic UV-filters to the aquatic midge C*hironomus riparius*.

Abstract

Despite the frequent detection of organic ultraviolet filters (UV-filters) in freshwater sediments, there is a lack of ecotoxicological data undermining a correct risk assessment for these emerging contaminants. The present study assessed the effects of three of the most commonly used UV-filters (benzophenone-3 – BP3; 3-(4-methylbenzylidene)camphor – 4-MBC and octocrylene – OC) on *Chironomus riparius* life history and biochemical responses. Standard ecotoxicological assays confirmed that all compounds impaired growth of *C. riparius* larvae and induced developmental effects such as delayed emergence and a reduction of imagoes weight. Concerning the biochemical responses analysed no evidences of oxidative damage in lipids or neurotoxicity (tested assessing acetylcholinesterase activity) were observed for any of the tested compounds. However, 4-MBC exposure induced a decrease in catalase activity and an increase in glutathione-*S*-transferase activity at 14.13 mg/Kg while OC exposure caused an increase in total glutathione levels at 0.23 and 18.23 mg/Kg. Exposure to all UV-filters tested, increased energy consumption on *C. riparius* with significant differences above 1.00 mg/Kg for BP3, 0.09 mg/Kg for 4-MBC and 2.13 mg/Kg for OC. These results suggest that environmental relevant concentrations of UV-filters can cause deleterious effects to aquatic benthic species, such as *C. riparius* and call for further research concerning effects of organic UV-filters on natural invertebrate communities and ecosystem functioning.

Key-words: personal care products; aquatic macroinvertebrates; sublethal effects; biomarkers

1. Introduction

Organic UV-filters absorb and protect against specific wavelengths of ultraviolet radiation (UV-A 320-400 nm; UV-B 280-320 nm) being included in a wide variety of personal care products (PCP's) such as, sunscreens, lipsticks, hair sprays and shampoos, and also in plastic products and textiles (Brausch and Rand, 2011; Díaz-Cruz and Barceló, 2009). Given their wide application, organic UV-filters are reaching aquatic ecosystems due to washing-off from skin or clothes during water recreational activities (direct input) and by discharges of swimming pool waters (Brausch and Rand, 2011; Rodil et al., 2009). It has been also reported that the major sources of UV-filters release in the aquatic environment are effluents and sewage sludge resulting from inefficient removal of UV-filters in wastewater treatment plants (Ramos et al., 2016). Thus, contamination by UV-filters has been frequently and increasingly detected worldwide during the last decades in lakes (Balmer et al., 2005; Kameda et al., 2011), rivers (Kameda et al., 2011; Poiger et al., 2004), effluents and influents (Balmer et al., 2005; Golovko et al., 2014), and coastal areas (Amine et al., 2012), reaching concentrations of up to 5.79 µg/L in rivers (see Ramos et al., 2015). Moreover, due to their physic-chemical properties (Table 1), such as high lipophilicity (Díaz-Cruz and Barceló, 2009; Gago-Ferrero et al., 2012), UV-filters have been also found in solid matrices in concentrations up to 27.7 mg/Kg dry weight in sewage sludge (Plagellat et al., 2006) and up to 2.4 mg/Kg dry weight in river sediments (Gago-Ferrero et al., 2011).

Among this class of compounds, benzophenones (e.g. Benzophenone, 3- BP3), camphor derivatives (e.g. 3-(4-methylbenzylidene) camphor, 4-MBC) and crylenes (e.g. 2-ethylhexyl 2-cyano-3,3-diphenylacrylate - octocrylene, OC), are the most commonly organic UV-filters reported as contaminants of aquatic environments (Balmer et al., 2005; Gago-Ferrero et al., 2012; Kameda et al., 2011; Plagellat et al., 2006; Ramos et al., 2015). Several studies have already showed some negative effects of organic UV-filters in aquatic biota. Examples include growth impairments as observed in the crustacean *Daphnia magna* (Sieratowicz et al., 2011), altered feeding rates in *Sericostoma vittatum* (Campos et al., 2017), reductions of the reproductive output observed in oligochaete *Lumbriculus variegatus* (Schmitt et al., 2008), in the snails *Potamopyrgus antipodarum* and *Melanoides tuberculata* (Kaiser et al., 2012; Schmitt et al., 2008) and in fish (Kunz and Fent, 2006). Also, exposure to organic UV-filters has been shown to cause bleaching of corals (Danovaro et al., 2008).

The main mode of action of UV-filters is unknown but effects on genes related with development and reproduction of arthropods, as observed to *Chironomus riparius* (Ozáez et

al., 2013; Ozáez et al., 2014) suggest that they might act as endocrine disruptors (Wang et al., 2016).

The environmental risk assessment and research on toxicity of UV-filters is a priority especially since it has not been focused on benthic species and on sediments where these compounds tend to accumulate (Kaiser et al., 2012; Sieratowicz et al., 2011). Also, information on sub-lethal effects of these compounds in terms of oxidative stress, antioxidant defences or even neurotoxicity is still scarce despite reports of sub-organismal level effects in both invertebrates and vertebrates (Gao et al., 2013; Liu et al., 2015).

In order to tackle these research gaps, the present study main aim was to assess the organismal and biochemical effects of different organic UV-filters in a benthic insect, the non-biting midge *Chironomus riparius* (Meigen). For that we focused on environmental concentrations of BP3, 4-MBC and OC and on their potential sub-lethal effects in terms of *C. riparius* growth and development as organismal endpoints. In addition, effects on commonly used biochemical biomarkers associated with important physiological functions such as oxidative stress, neurophysiological, antioxidant and phase II biotransformation enzymes, non-enzymatic antioxidant defences and cellular respiration were also determined to understand how and which of these biochemical changes might be associated with organism level toxicity. For that we evaluated the effects of short exposures of the different compounds on lipid peroxidation (LPO), acetylcholinesterase (AChE), catalase (CAT), and glutathione-*S*-transferase (GST) activities, total glutathione (tGSH) and electron transport system (ETS) activity.

2. Material & methods

2.1 Chemicals

The 2-hydroxy-4-methoxybenzophenone (or benzophenone-3) (BP-3; CAS No. 131-57-7; purity ≥ 98%), 3-(4-methylbenzylidene) camphor (4-MBC; CAS No. 36861-47-9, purity ≥ 98%) and 2-ethylhexyl 2-cyano-3,3-diphenylacrylate (or octocrylene) (OC; CAS No. 6197-30-4, purity ≥97%) were obtained from Sigma-Aldrich (Portugal). Other relevant properties of these compounds are presented in Table 1. Stock solutions and subsequent dilutions of UV-filters were prepared in ethanol (96%) for chronic and biomarker exposures due to low water solubility of UV- filters. Solvent controls were also prepared with 96% ethanol.

Table 1 – Physico-chemical properties of UV-filters.

Compounds	Formula	Abbreviation	CAS No	Molecular weight (g/mol)	Log K_{ow}	Water solubility (g/L)
2-hydroxy-4-methoxybenzophenone (Benzophenone-3)	$C_{14}H_{12}O_3$	BP3	131-57-7	228.24	3.79	0.10
3-(4-methylbenzylidene) camphor	$C_{18}H_{22}O$	4-MBC	36861-47-9	254.37	4.95	0.017
2-Ethylhexyl 2-cyano-3,3-diphenylacrylate (Octocrylene)	$C_{12}H_{17}NO$	OC	6197-30-4	361.48	6.88	$3.6x10^{-4}$

Based on Gago-Ferrero et al. (2012).

2.2 Test Organisms

C. riparius were obtained from laboratory cultures established at the University of Aveiro. Organisms were maintained in plastic containers with a layer of inorganic fine sediment (<1 mm, previously burned at 500ºC during 4h) and reconstituted hard water (ASTM) (ASTM, 1980) at constant temperature (20 ± 1ºC) and with a photoperiod of 16:8h (light: dark). Larvae were fed every two days with a suspension of macerated Tetramin® (TetraWerk, Melle, Germany) and the medium was changed every week.

2.2.1 Partial life-cycle test

The chronic 28-days toxicity test was performed according to OECD 218 guideline with minor modifications (OECD, 2004). Briefly, organisms were exposed to three concentrations of UV-filters in glass vials containing artificial sediment and were fed with Tetramin®.

Larvae with less than 48h post-hatching (1st instar) were exposed to a gradient of three concentrations (2.5, 5 and 10 mg/Kg) of UV-filters and to control and solvent control treatments. In these chronic tests 50 g (dry weight) of artificial sediment composed of 75% inorganic fine sediment, 20% kaolin, 5% α-cellulose and 0.1 % calcium carbonate (OECD, 2004) was used in each replicate. The sediment was spiked with 10 mL of respective UV-filter solution and left evaporating for 24h. For the solvent control treatments, 10 mL of 96% ethanol were added to the sediment and allowed to evaporate for the same period. Ten mL of ASTM were then added to sediments and mixed thoroughly until a homogenous sediment paste was formed. Finally, a volume of 150 mL of ASTM was added in each replicate to attain a 4:1 water column: sediment and vials were left for 48h to equilibrate.

To evaluate effects, we used twelve replicates with five larvae each. Organisms were fed every other day with a suspension of macerated Tetramin® (0.5 mg per organism per day) and

maintained under the same conditions as described for laboratory cultures. Using highly nutritive macerated fish food allowed for the measurement of emergence data and imagoes weight within 28 days of exposure. After 10 days, organisms of half of the replicates (30 larvae per concentration) were removed and kept in 70% ethanol for larval measurements. Total length of each larva was measured with a stereo dissecting microscope fitted with a calibrated eyepiece micrometer. Larval growth was estimated measuring the final length of larvae (i.e. after 10 days of exposure) which was compared to the length of an initial batch of organisms (day 0, 1st instar larvae). The remaining replicates in each treatment were used to assess emergence rates until the end of the test (28d). The number of adult midges was recorded daily to assess cumulative percentage of emergence and the mean time to emergence of organisms. Imagoes were collected with the aid of an aspirator, preserved in 70% ethanol and then dried 24h at 50ºC and weighted in a microbalance (Mettler UMT2).

2.2.2 Neurophysiological, oxidative stress and energy-related biomarkers

C riparius larvae (4th instar, 12 days) were exposed during 48h to 0.25, 2.5 and 25 mg/Kg of BP3, 4-MBC and OC in 50 g of artificial sediment and 150 mL of ASTM. Control and solvent control treatments were also set up. Seven replicates with fifteen larvae each were used for each experimental condition. The test was performed using the same conditions of previously described for the chronic test. Organisms were not fed during the entire exposure period of 48h. After exposure, organisms were quickly dried in filter paper, immediately weighted, frozen in liquid nitrogen and stored at -80ºC until further use. Each sample was then homogenized by sonication in 1600 µL of Milli-Q water. Two aliquots of 300 µL for ETS and 200 µL for LPO were set aside. The remaining 900 µL homogenate was diluted in 900 µL of 0.2 M K-phosphate buffer (pH= 7.4), and centrifuged at 10000g for 2 min at 4ºC. The resulting post-mitochondrial supernatant (PMS) was separated in aliquots for CAT, GST, tGSH, AChE (Pérez et al., 2013) and protein quantification.

ETS activity was determined following the method described by De Coen and Janssen (1997). To measure ETS activity, 150 µL of homogenization buffer (0.3 M Tris; 150% (w/v) Poly Vinyl Pyrrolidone; 8 mM $MgSO_4$; 0.6% (v/v) Triton X-100) were added to the 300 µL of homogenized samples. After centrifugation (1000g, 10 min, 4ºC), the resulting supernatant was removed for ETS activity measurement. Fifty µL of each sample was put in a multi-well plate in quadruplicated and 150 µL of a buffered solution B [2% (v/v) solution A (6.67M Tris; 0.27% (v/v) Triton X-100); 1.8 mM NADH; 280 µM NADPH] were added. Reaction at 25ºC was started by adding 100 µL of INT solution (p-iodonitrotetrazolium; 8 mM) and the absorbance

was immediately measured at 490 nm over a period of 3 min. Results were expressed as mJ/mg organism/h.

Lipid peroxidation was measured on 200 µL of homogenized samples plus 4 µL of 4% 2,6-Di-tert-butyl-4-methylphenol in methanol using the thiobarbituric acid-reactive substances (TBARS) assay (Bird and Draper, 1984; Ohkawa et al., 1979). The absorbance was read at 535 nm and the results were expressed as nmol TBARS per mg of wet weight.

Glutathione-*S*-transferase activity was measured using the method described by Habig et al. (1974). The enzymatic activity was measured in 50 µL of PMS following conjugation of reduced L-glutathione with 1-chloro-2,4-dinitrobenzene at 340 nm. The enzymatic activity was expressed in nmol per min per mg of protein.

Catalase activity was determined on 10 µL of PMS by measuring the decomposition of the substrate hydrogen peroxide (H_2O_2) at 240 nm (Clairborne, 1985). The results were expressed as µmol per min per mg of protein.

Total glutathione (Tgsh) level was determined in 50 µL PMS using the method described by Baker et al. (1990). The absorbance was read at 412 nm during 3 min following the recycling reaction of reduced glutathione in the presence of an excess of glutathione reductase. The total glutathione levels were expressed as µM per mg of protein, using L-GSH as a standard.

Finally, AChE activity was determined in 50 µL of PMS following the Ellman's method (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996) using acetylthiocholine as subtract. The absorbance was read at 414 nm and the enzymatic activity was expressed in nmol per min per mg of protein. Protein was quantified in 10 µL of PMS by Bradford method (Bradford, 1976) adapted from Biorad's Bradford micro-assay. The absorbance was read at 590 nm and γ-globulin was used as a standard. For a more detailed protocol see Campos et al., (2016) and Rodrigues et al. (2015).

2.3 Chemical analysis

2.3.1 Chemicals

Liquid chromatography–mass spectrometry (LC-MS) grade methanol and acetonitrile (Li Chrosolv Hypergrade) were purchased from Merck (Darmstadt, Germany). Formic acid for the mobile phases acidification was purchased from Labicom (Olomouc, Czechia). Ultra-pure water was produced using an Aqua-MAX-Ultra System (Younglin, Kyounggi-do, Korea). All analytical standards used were of high purity (> 98%). All UV-filters and diclofenac were purchased from Sigma Aldrich (UK). Diclofenac was used as internal standard. Stock solutions of compounds were prepared in methanol at a concentration of 1 mg/mL and stored at -20°C. For each UV-

filters, a spiking solution was prepared by diluting stock in methanol to a final concentration of 1 µg/mL and stored at -20°C.

2.3.2 Instrumentation

A triple stage quadrupole MS/MS TSQ Quantum Ultra mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) coupled with an Accela 1250 LC pump (Thermo Fisher Scientific) and an HTS XT-CTC autosampler (CTC Analytics AG, Zwingen, Switzerland) was used for analysis of BP3 and 4-MBC in sediment samples.

Cogent Bidentate C18 column (50 mm × 2.1 mm i.d., 4 µm particle size from MicroSolv Technology Corporation Eatontown, NJ, USA) was used as analytical column for chromatographic separation of BP3 and 4-MBC. Heated electrospray ionization (HESI) was used in order to ionize target compounds.

A hybrid quadrupole/orbital trap Q-Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) coupled with an Accela 1250 LC pump (Thermo Fisher Scientific) and an HTS XT-CTC autosampler (CTC Analytics AG, Zwingen, Switzerland) was used for analysis of OC.

Hypersil GOLD Phenyl column (50 mm × 2.1 mm i.d., 3 µm, Thermo Fisher Scientific) was used as an analytical column for chromatographic separation of the OC. An atmospheric pressure chemical ionization coupled with atmospheric pressure photoionization (APCI/APPI) in negative mode was used to ionize target compounds.

MS/MS parameters are reported in Table SD1, A and B. LC gradient for the elution of target compounds is reported in Table SD2, A and B.

2.3.3 Sample preparation

An ultrasonic based solvent approach was used to extract the UV-filters from the sediments (Golovko et al., 2016). Briefly, around 2 grams of sediment were weighted into 10-mL vials and 20 ng of IS was added to each sample. Four milliliters of extraction solvent (acetonitrile and water (1/1 v/v with 0.1% formic acid)) were added and the samples were sonicated (DT 255, Bandelin electronic, Sonorex digitec, Berlin, Germany) for 15 min. The supernatant was filtered through a syringe filter (0.45 µm, regenerated cellulose, Labicom, Olomouc, Czech Republic) into 10-mL vials. The same step was repeated with 4 mL of acetonitrile, 2-propanol, and water (3/3/4 v/v/v with 0.1% formic acid). Two supernatants were mixed after extraction procedure. Each sample was prepared and analysed in duplicate.

This method was validated in the range of tested concentrations and exhibited good linearity in the concentration range between 0.005 and 0.5 mg/kg BP3, 4-MBC and OC; R^2 = 0.999 and

average recovery of BP3 and 4-MBC was 108 % (±8) and for OC was 97% (±10), respectively. Average limit of quantification was 0.09 mg/kg for BP3, 0.07 mg/kg for 4-MBC and 0.006 mg/kg for OC.

Internal standard method was used for quantification of target compounds. The matrix effect was assessed for each compound, and corrections for ion suppression or enhancement were accomplished using matrix-matched standards for sediment samples. Matrix-matched standards were prepared from tested sediment extract by spiking with both IS and target compounds at 0.01 mg/kg and 0.1 mg/kg, respectively.

2.4 Statistical analysis

The effects of UV-filters on *C. riparius* biochemical and life-history responses were evaluated using one-way analysis of variance (ANOVA). Since t-tests did not find significant differences between control and solvent control for any of the endpoints analysed, multiple comparisons between UV-filters treatments and the solvent control were examined. For all statistical tests the significance level was set at $p < 0.05$. All variables were assessed for normality using residual probability plots while Levene's and Bartlett's tests verified the homoscedascity of data ($p > 0.05$). *C. riparius* growth data for the 4-MBC exposure was Log-transformed to fulfil normality of data. Effects on *C. riparius* development time data for all compounds tested and ETS activity data for the OC exposure were analysed with Kruskal-Wallis test followed by Dunn's *post-hoc* test while all other responses were analysed by Dunnett's *post-hoc* tests. All statistical analyses were performed in prism 6.0. (GraphPad Software, La Jolla California USA).

3. Results

3.1 Concentrations of UV-filters in sediments

Concentrations of the different organic UV-filters were measured in the sediments 5 days after spiking (Table 2). In the chronic toxicity test measured concentrations in sediments were up to 70, 68 and 79% lower than nominal concentrations of BP3, 4-MBC and OC, respectively.

Concerning biochemical biomarkers exposure, the measured concentrations were up to 74%, 64% and 27% lower than nominal concentrations to BP3, 4-MBC and OC respectively.

Table 2 – UV-filters concentrations measured in sediments in partial life-cycle and biomarkers bioassays (mean ±SD).

	Nominal concentration (mg/Kg)	BP3	4-MBC	OC
Chronic exposure	2.5	0.75 ± 0.10	0.80 ± 0.04	0.53 ±0.04
	5	1.55 ± 0.39	2.05 ± 0.38	1.27 ± 0.10
	10	3.41 ± 0.16	4.17 ± 0.08	2.33 ± 0.29
Biochemical exposure	0.25	0.23 ± 0.01	0.09 ± 0.02	0.23 ± 0.05
	2.5	1.00 ± 0.15	1.12 ± 0.09	2.13 ± 0.45
	25	6.49 ± 2.99	14.13 ± 0.24	18.23± 1.02

3.2 Effects of UV-filters on *C. riparius* growth and emergence

The validity criteria of the test was met. The pH ranged between 7.83 and 8.28, the dissolved oxygen was above 7.0 and the temperature did not vary more than ± 1ºC in all treatments of UV-filters. *C. riparius* larval growth was significantly reduced after exposure to 0.75, 2.05 and 2.33 mg/Kg of BP3, 4-MBC and OC, respectively (Figure 1a, b, c; Table 3). The emergence in control treatments was above 93% to all tested compounds and no significant effects of any compound tested were observed for percentage of emergence (data not shown). However, exposure to UV-filters contaminated sediments affected other life cycle endpoints, particularly development time (days until emergence) and imagoes' weight. BP3 and 4-MBC delayed the development time of *C. riparius* females exposed to 3.41 mg/Kg and 2.05 and 4.17 mg/Kg, respectively (Figure 1d, e; Table 3). Nonetheless, no significant effects have been observed for weight of female imagoes exposed to BP3 and 4-MBC, when compared to the solvent control treatment (Figure 1 g, h; Table 3). In contrast, development time of *C. riparius* males was not affected by exposure to BP3 and 4-MBC (Figure 1d, e; Table 3), whereas their average weight was significantly reduced after exposure to 3.41 mg BP3/Kg and 4.17 mg 4-MBC/Kg (Figure 1g, h; Table 3). OC did not significantly affect development time of female nor male' *C. riparius* (Figure 1f; Table 3) although a non-significant reduction of 8.47% and 18.63% in the weight of male and female imagoes, respectively, has been observed (Figure 1i; Table 3).

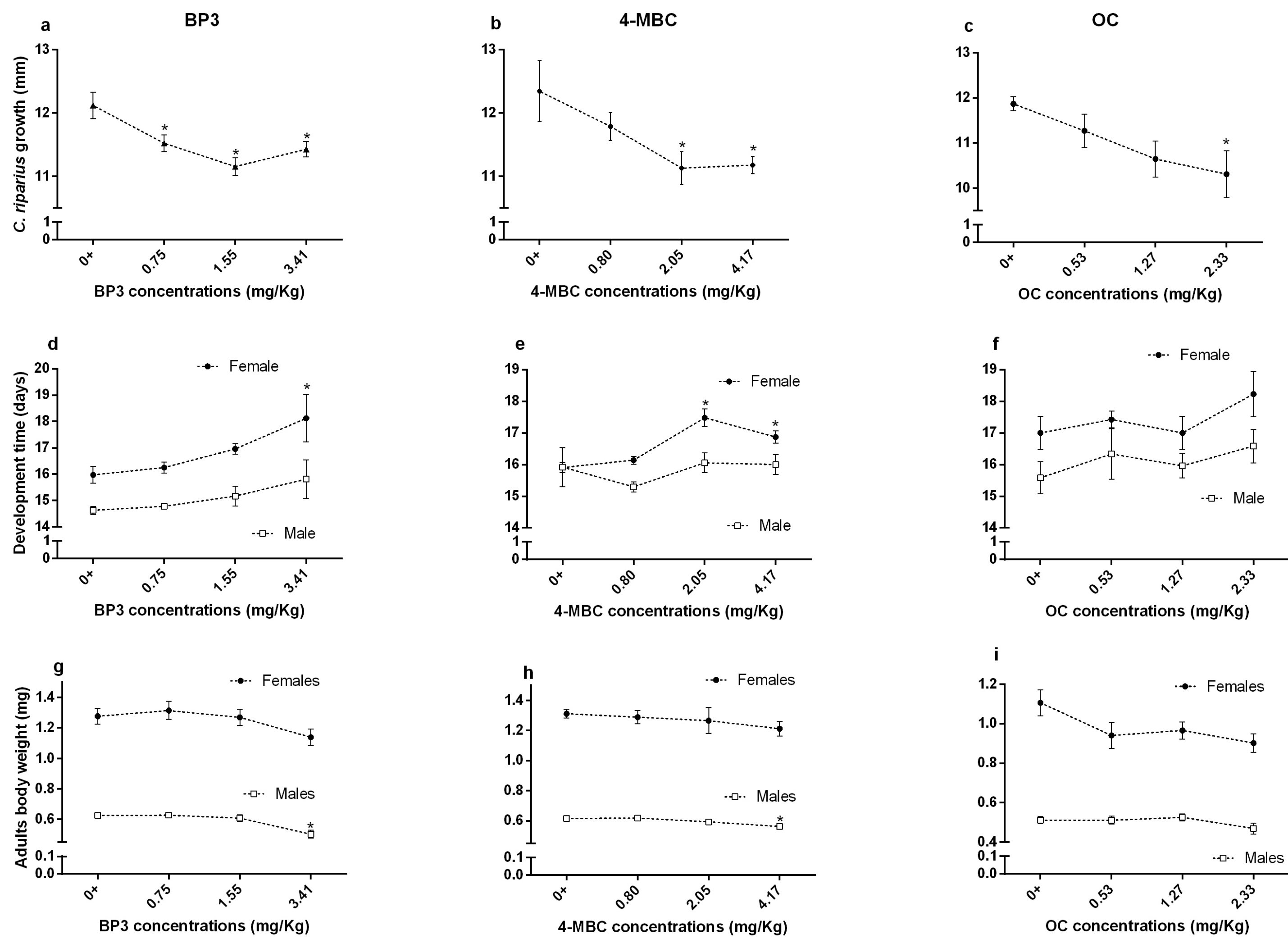


Figure 1 – Chronic effects of BP3, 4-MBC and OC on *Chironomus riparius* growth, development time and adult size. a,b,c: Growth (mm; mean ± SEM) after 10 days of exposure. d,e,f: development time (days; mean ± SEM). g,h,i: adult body weight (mg; mean ± SEM). Asterisks (*) denote statistically significant differences compared to solvent control (0+) (Dunnet's *post hoc* test, $p < 0.05$ (growth and adult body weight) and Dunn´s *post hoc* test (development time)).

Table 3 – One-way ANOVA results testing for effects on growth and imagoes weight (males and females) and Kruskal- Wallis results testing for effects on development time (male and female) with degrees of freedom (Df), F/H-values and significance levels (*p*).

	BP3			4-MBC			OC		
	Df	**F/H**	***p***	**Df**	**F/H**	***p***	**Df**	**F/H**	***p***
Growth	3	7.00	**0.002**	3	3.76	**0.027**	3	3.24	**0.044**
Development time									
male	3	6.51	0.089	3	4.88	0.181	3	1.92	0.590
female	3	9.39	**0.025**	3	14.48	**0.002**	3	1.05	0.789
Imagoes weight									
Male	3	6.17	**0.005**	3	3.17	**0.048**	3	1.33	0.293
Female	3	1.60	0.224	3	0.77	0.526	3	2.21	0.083

3. 3 Biochemical responses of *C. riparius* exposed to UV-filters

Electron transport system activity followed a dose-response relationship with significant increases on *C. riparius* larvae exposed to 1.00 and 6.49 mg/kg of BP3 ($F_{(3,24)}$ = 83.60: p <0.0001) and in all tested concentrations of 4-MBC ($F_{(3,24)}$ = 117.10: p <0.0001) and OC (H= 22.21; Df=3; p < 0.0001) (Figure 2). The increase in ETS activity in the highest tested concentrations of BP3, 4-MBC and OC was 56.68, 61.85 and 88.53%, respectively. Concerning CAT activity, a slightly decrease was observed in the highest concentrations of all tested compounds with a reduction of 20.90, 22.84 and 6.95% for BP3, 4-MBC and OC respectively. However, significant differences were only observed when larvae were exposed to 14.13 mg/Kg of 4-MBC ($F_{(3,24)}$ = 3.52; p <0.05; Table 4).

A clear dose-dependent response in terms of GST activity was observed when *C. riparius* were exposed to 4-MBC but significant differences were only observed for 14.13 mg/Kg 4-MBC ($F_{(3,23)}$ = 5.43; p <0.01; Table 4). No significant effects of BP3 and of OC were observed in *C. riparius'* GST activity ($F_{(3,24)}$ = 0.02; p>0.05 and $F_{(3,24)}$ = 2.47; p>0.05, respectively) (Table 4).

OC also induced a significant increase of tGSH content on *C. riparius* exposed to 0.23 and 18.23 mg/Kg ($F_{(3,24)}$ =7.41: p <0.01; Table 4).

Table 4 – Biomarker responses of *C. riparius* after exposure to different concentrations of BP3, 4-MBC and OC during 48h. GST and AChE activity are expressed in nmol/min/mg protein, CAT activity is expressed in µmol/min/mg protein, TG is expressed in µM/mg of protein and LPO is expressed in TBARS nmol/g wet weight. Values corresponding to mean ± standard deviation. Asterisks (*) denote statistically significant differences compared to solvent control (0+) (Dunnet's *post hoc* test, $p < 0.05$).

		BP3 concentrations (mg/Kg)			**4-MBC concentrations (mg/Kg)**			**OC concentrations (mg/Kg)**		
	0+	**0.23**	**1.00**	**6.49**	**0.09**	**1.12**	**14.13**	**0.23**	**2.13**	**18.23**
GST	9.93 ±0.91	9.85 ±0.69	9.86 ±0.63	9.90 ±0.71	10.00 ±1.28	11.10 ±1.28	12.04 ±1.05*	10.79 ±1.28	11.38 ±1.26	10.80 ±0.87
CAT	46.45 ±8.00	42.77 ±5.66	49.50 ±8.45	36.74 ±12.22	43.21 ±9.10	47.91 ±4.57	35.84 ±7.90*	45.10 ±3.41	49.01 ±4.96	43.22 ±9.88
TG	16.10 ±2.97	17.52 ±3.43	12.07 ±4.80	12.13 ±2.61	13.36 ±2.99	15.48 ±2.31	19.27 ±3.69	21.62 ±3.16*	18.21 ±5.02	24.15 ±2.00*
LPO	37.07 ±5.41	28.49 ±1.92*	41.07 ±5.85	38.81 ±5.15	37.75 ±4.02	38.10 ±6.91	39.07 ±5.91	40.01 ±6.64	46.11 ±10.77	38.83 ±7.19
AChE	11.30 ±2.42	11.06 ±1.58	10.48 ±2.27	10.35 ±1.86	9.26 ±0.55*	10.75 ±1.47	12.61 ±0.90	12.13 ±2.20	12.95 ±1.64	13.30 ±1.88

None of the UV-filters tested, elicited monotonic or dose dependent responses in terms of oxidative damage (LPO) and neurotoxicity (AChE). However, a significant decrease in LPO levels was observed in *C. riparius* larvae exposed to 0.23 mg/Kg of BP3 ($F_{(3,24)}$ = 5.91: $p < 0.001$) (Table 4). *C. riparius* larvae exposed to 0.09 mg/Kg 4-MBC showed a decrease in AChE activity of ($F_{(3,24)}$ = 9.02; $p < 0.01$) (Table 4).

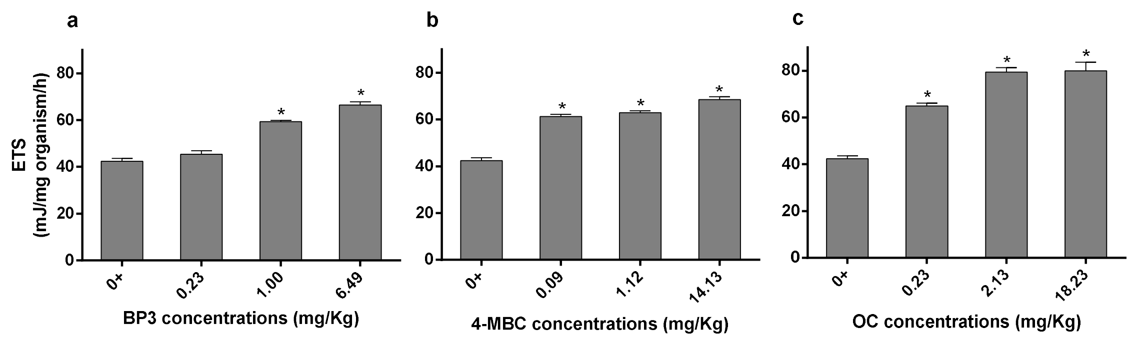


Figure 2 - Short-terms effects of BP3, 4-MBC and OC on ETS (mJ/ mg organism/ h; mean ± SE). Asterisks (*) denote statistically significant differences compared to solvent control (0+) (Dunnet's *post hoc* test, p < 0.05 to BP3 and 4-MBC and Dunn´s *post hoc* test to OC).

4. Discussion

Given their highly lipophilic nature and their slow degradation in natural ecosystems (Díaz-Cruz and Barceló, 2009; Gago-Ferrero et al., 2012), ecotoxicological studies using freshwater benthic invertebrate species are critical for a correct evaluation of the potential ecological effects and for the risk assessment of organic UV-filters.

The present study shows that sediments spiked with sub-lethal and environmental relevant concentrations of BP3, 4-MBC and OC significantly impaired *C. riparius* larval growth and development. These results are in good agreement with previous studies where decreased growth of the larvae of sea urchin *Paracentrotus lividus* (EC_{50}= 3.28 mg BP3/L and 0.85 mg 4-MBC/L) (Paredes et al., 2014) and a reduction of the length of the crustacean *D. magna* at 0.2 mg 4-MBC/L (Sieratowicz et al., 2011) were observed as a results of exposure to UV-filters. Effects in somatic growth was also observed in *D. magna* exposed to other UV-filters such as 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC (LOEC= 0.08 mg/L) and 3-benzylidene-camphor (3-BC) (0.2 mg/L) (Sieratowicz et al., 2011). Furthermore, reductions in the cell density of *Desmodesmus subspicatus* algae and *Isochrysis galbana* microalgae have been also shown in response to UV-filters exposure (Paredes et al., 2014; Sieratowicz et al., 2011). The effects on growth and development could be

related to feeding inhibition as suggested by lower feeding rates of the aquatic caddisfly *S. vittatum* exposed to concentrations of 3.55 mg/Kg BP3 and 2.57 mg/Kg 4-MBC (Campos et al., 2017). In our current study, reproductive effects of selected UV-filters on *C. riparius* were not directly assessed. Nevertheless, together with the effects on larval growth and development, the concomitant effect on weight of adult midges suggests a reproductive impairment caused by UV-filters exposure.

BP3 and 4-MBC significantly increased time to emergence of *C. riparius* female imagoes without any significant effect on female imagoes' weight. In contrast, time to emergence of *C. riparius* males was not significantly affected by BP3 or 4-MBC, but a decrease in weight of male imagoes was clear. This decreased body weight of male imagoes induced by BP3 and 4-MBC suggests that reproduction of *C. riparius* might be affected since dipterans male adult body weight is related to flying performance and total number of gametes produced, and thus reproductive success (Lilley et al., 2012; Ponlawat and Harrington, 2007). Thus, the weight reduction observed for male imagoes has been considered as an indication of potential reproductive effects in *C. riparius* and can be used as a sensitive endpoint for a better evaluation of population level effects of contaminants (Campos et al., 2016; Rodrigues et al., 2015). Although not significantly, OC also decreased the body weight of male imagoes at concentrations lower than BP3 and 4-MBC tested in this study. Therefore, effects of OC on development and reproduction might also be expected when testing higher concentrations. Moreover, OC also increased time to emergence for males and females, although not significantly. This lack of effects of OC has been observed in several invertebrates including *C. riparius* (Kaiser et al., 2012; Ozáez et al., 2013), *L. variegatus*, *M. tuberculata* or *P. antipodarum* (Kaiser et al., 2012) and *T. thermophila* (Gao et al., 2013). However, most studies report nominal concentrations only (see for example (Gao et al., 2013; Kaiser et al., 2012)) and that can be misleading given OC's high lipophilicity that might reduce its bioavailability. Interestingly, we also observed that, BP3, 4-MBC and especially OC induce a reduction (albeit not significantly) in the size of female imagoes in the highest concentrations tested. Again, weight of female imagoes is related to fecundity and fertility through effects on viability and size eggs mass (Ponlawat and Harrington, 2007).

The observed effects on development induced by BP3 and 4-MBC on *C. riparius* larvae might be related to alterations in the ecdysteroid hormone levels mediated by the ecdysone receptor, since this hormone is involved in the regulation of growth, development and reproduction of arthropods (Riddiford et al., 2000). In fact previous studies have demonstrated that 4-MBC stimulated the transcription of the ecdysone receptor gene and BP3 activated the expression of

the ecdysone receptor gene in salivary gland cells of *C. riparius* larvae (Ozáez et al., 2013; Ozáez et al., 2014). In line with our results, 4-MBC has been shown to decrease the reproduction of *L. variegatus*; while increasing the embryo production in *P. antipodarum* (Schmitt et al., 2008) and exposure to BP3 caused a reduction in the number of eggs produced by *Oryzias latipes* (Coronado et al., 2008). Moreover, reproductive toxicity of other UV-filters such as EHMC has been reported for the snail species *P. antipodarum* (400 µg/Kg) and *M. tuberculata* (10 mg/Kg) (Kaiser et al., 2012; Schmitt et al., 2008), and also observed in *L. variegatus* and *D. magna* exposed to 3-BC (Schmitt et al., 2008; Sieratowicz et al., 2011).

Relatively to the biochemical responses evaluated, our results showed different responses of *C. riparius* under UV-filters exposure. The inhibition of CAT activity suggests 4-MBC might lead to oxidative stress as alterations on antioxidant defences occur. However, oxidative damage might be prevented by an induction of GST (phase II detoxification enzyme) as previously suggested by Enayati et al. (2005), whereas no significant effects were observed for BP3 or OC. On the other hand, in the present study, the OC induced responses suggest that oxidative stress was prevented by an increased level of total glutathione given its primordial role in maintaining the cellular redox status of the cell (Doyotte et al., 1997; Rikans and Hornbrook, 1997) interacting directly with reactive oxigen species or as cofactor of other enzymes (Lushchak, 2012). In fact, it has been shown that concentrations of up to 1.0 mg/L OC do not alter the activity of enzymes such as CAT and GSH in *T. thermophila* (Gao et al., 2013), demonstrating the controversial results when determining those biomarkers on organisms exposed to organic UV-filters. For instance, exposure to BP3 and 4-MBC has been shown to increase the activity of CAT on the protozoan *T. thermophila* (Gao et al., 2013), while reductions of CAT activity and increased GST activity have been observed in the fish *Carassius auratus* (Liu et al., 2015).

Also, the absence of effects in terms of AChE is in accordance with the responses of other aquatic organisms, namely dipterans and frogs (Campos et al., 2017; Martins et al., 2017) and suggests that UV-filters are not neurotoxic.

Finally, an increase in the energy consumption of organisms was consistently observed for organisms exposed to all compounds tested. This increased ETS activity is usually related to an increase of energy requirements for defence mechanisms (Choi et al., 2001; Sokolova et al., 2012) and migth compromise the allocation of energy for growth and reproduction (Rodrigues et al., 2015; Servia et al., 2006; Sokolova et al., 2012). These metabolic costs of the stress response were probably responsible, together with other behavioural and physiological responses (e.g. feeding

and assimilation), for the reduced growth and development of *C. riparius* exposed to BP3, 4-MBC and OC.

In summary, the results obtained in this study suggest that sediments contaminated with low and environmentally relevant concentrations of UV-filters can impair several life-history traits in *C. riparius*. The results also point to potential reproductive effects that can compromise natural population dynamics under UV-filters exposure. Since the endocrine activity of UV-filters has been also suggested (Ozáez et al., 2013; Wang et al., 2016), an obvious extension of this work is certainly to address the transgenerational effects of such compounds. Chironomids which are model organisms for sediment ecotoxicity testing are ideal for those long-term multigenerational assays. More so if we consider that they are a preferential prey item for several invertebrate and vertebrate species and thus also extremely relevant for studies addressing bioaccumulation and transport of UV-filters along natural food webs.

References

Amine H, Gomez E, Halwani J, Casellas C, Fenet H. UV filters, ethylhexyl methoxycinnamate, octocrylene and ethylhexyl dimethyl PABA from untreated wastewater in sediment from eastern Mediterranean river transition and coastal zones. Marine Pollution Bulletin 2012; 64: 2435-2442.

ASTM. Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates and amphibians. American Standards for Testing and Materials, Philadelphia, P.A 1980.

Baker MA, Cerniglia GJ, Zaman A. Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. Analytical Biochemistry 1990; 190: 360-365.

Balmer ME, Buser H-R, Müller MD, Poiger T. Occurrence of some organic UV Filters in wastewater, in surface waters, and in fish from Swiss lakes. Environmental Science & Technology 2005; 39: 953-962.

Bird RP, Draper HH. Comparative studies on different methods of malonaldehyde determination. Methods Enzymol 1984; 105: 299-305.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 1976; 72: 248-254.

Brausch JM, Rand GM. A review of personal care products in the aquatic environment: Environmental concentrations and toxicity. Chemosphere 2011; 82: 1518-1532.

Campos D, Gravato C, Quintaneiro C, Soares AMVM, Pestana JLT. Responses of the aquatic midge *Chironomus riparius* to DEET exposure. Aquatic Toxicology 2016; 172: 80-85.

Campos D, Gravato C, Fedorova G, Burkina V, Soares AMVM, Pestana JLT. Ecotoxicity of two organic UV-filters to the freshwater caddisfly *Sericostoma vittatum*.Envpoll 2017; 228, 370-377.

Choi J, Roche H, Caquet T. Hypoxia, hyperoxia and exposure to potassium dichromate or fenitrothion alter the energy metabolism in *Chironomus riparius* Mg. (Diptera: Chironomidae) larvae. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 2001; 130: 11-17.

Clairborne A. Catalase activity In: Greenwald RAE, editor. Handbook of methods for oxygen radical research. CRC Press, Boca Raton, 1985, pp. 283-284.

Coronado M, De Haro H, Deng X, Rempel MA, Lavado R, Schlenk D. Estrogenic activity and reproductive effects of the UV-filter oxybenzone (2-hydroxy-4-methoxyphenyl-methanone) in fish. Aquatic Toxicology 2008; 90: 182-187.

Danovaro R, Bongiorni L, Corinaldesi C, Giovannelli D, Damiani E, Astolfi P, et al. Sunscreens cause coral bleaching by promoting viral infections. Environ Health Perspect 2008; 116: 441-7.

De Coen WM, Janssen CR. The use of biomarkers in *Daphnia magna* toxicity testing. IV. Cellular Energy Allocation: a new methodology to assess the energy budget of toxicant-stressed *Daphnia* populations. Journal of Aquatic Ecosystem Stress and Recovery 1997; 6: 43-55.

Díaz-Cruz MS, Barceló D. Chemical analysis and ecotoxicological effects of organic UV-absorbing compounds in aquatic ecosystems. TrAC Trends in Analytical Chemistry 2009; 28: 708-717.

Doyotte A, Cossu C, Jacquin M-C, Babut M, Vasseur P. Antioxidant enzymes, glutathione and lipid peroxidation as relevant biomarkers of experimental or field exposure in the gills and the digestive gland of the freshwater bivalve *Unio tumidus*. Aquatic Toxicology 1997; 39: 93-110.

Ellman GL, Courtney KD, Andres jr V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology 1961; 7: 88-95.

Enayati AA, Ranson H, Hemingway J. Insect glutathione transferases and insecticide resistance. Insect Mol Biol 2005; 14: 3-8.

Gago-Ferrero P, Díaz-Cruz MS, Barceló D. Fast pressurized liquid extraction with in-cell purification and analysis by liquid chromatography tandem mass spectrometry for the determination of UV filters and their degradation products in sediments. Analytical and Bioanalytical Chemistry 2011; 400: 2195-2204.

Gago-Ferrero P, Díaz-Cruz MS, Barceló D. An overview of UV-absorbing compounds (organic UV filters) in aquatic biota. Analytical and Bioanalytical Chemistry 2012; 404: 2597-2610.

Gao L, Yuan T, Zhou C, Cheng P, Bai Q, Ao J, et al. Effects of four commonly used UV filters on the growth, cell viability and oxidative stress responses of the *Tetrahymena thermophila*. Chemosphere 2013; 93: 2507-2513.

Golovko O, Koba O, Kodesova R, Fedorova G, Kumar V, Grabic R. Development of fast and robust multiresidual LC-MS/MS method for determination of pharmaceuticals in soils. Environmental Science and Pollution Research 2016; 23: 14068-14077.

Golovko O, Kumar V, Fedorova G, Randak T, Grabic R. Removal and seasonal variability of selected analgesics/anti-inflammatory, anti-hypertensive/cardiovascular pharmaceuticals and UV filters in wastewater treatment plant. Environmental Science and Pollution Research 2014; 21: 7578-7585.

Guilhermino L, Lopes MC, Carvalho AP, Soares AMVM. Acetylcholinesterase Activity in Juveniles of *Daphnia magna* Straus. Bulletin of Environmental Contamination and Toxicology 1996; 57: 979-985.

Habig WH, Pabst MJ, Jakoby WB. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 1974; 249: 7130-9.

Kaiser D, Sieratowicz A, Zielke H, Oetken M, Hollert H, Oehlmann J. Ecotoxicological effect characterisation of widely used organic UV filters. Environmental Pollution 2012; 163: 84-90.

Kameda Y, Kimura K, Miyazaki M. Occurrence and profiles of organic sun-blocking agents in surface waters and sediments in Japanese rivers and lakes. Environmental Pollution 2011; 159: 1570-1576.

Kunz PY, Fent K. Multiple hormonal activities of UV filters and comparison of *in vivo* and *in vitro* estrogenic activity of ethyl-4-aminobenzoate in fish. Aquatic Toxicology 2006; 79: 305-324.

Lilley TM, Ruokolainen L, Pikkarainen A, Laine VN, Kilpimaa J, Rantala MJ, et al. Impact of tributyltin on immune response and life history traits of *Chironomus riparius*: single and multigeneration effects and recovery from pollution. Environ Sci Technol 2012; 46: 7382-9.

Liu H, Sun P, Liu H, Yang S, Wang L, Wang Z. Hepatic oxidative stress biomarker responses in freshwater fish *Carassius auratus* exposed to four benzophenone UV filters. Ecotoxicology and Environmental Safety 2015; 119: 116-122.

Lushchak VI. Glutathione homeostasis and functions: potential targets for medical interventions. J Amino Acids 2012; 2012: 736837.

Martins D, Monteiro MS, Soares AMVM, Quintaneiro C. Effects of 4-MBC and triclosan in embryos of the frog *Pelophylax perezi*. Chemosphere 2017; 178: 325-332.

OECD. Test no. 218: Sediment-water chironomid toxicity test using spiked sediment. In: Publishing O, editor. OECD Guidelines for the Testing of Chemicals, 2004.

Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry 1979; 95: 351-358.

Ozáez I, Martínez-Guitarte JL, Morcillo G. Effects of in vivo exposure to UV filters (4-MBC, OMC, BP-3, 4-HB, OC, OD-PABA) on endocrine signaling genes in the insect *Chironomus riparius*. Science of The Total Environment 2013; 456–457: 120-126.

Ozáez I, Martínez-Guitarte JL, Morcillo G. The UV filter benzophenone 3 (BP-3) activates hormonal genes mimicking the action of ecdysone and alters embryo development in the insect *Chironomus riparius* (Diptera). Environmental Pollution 2014; 192: 19-26.

Paredes E, Perez S, Rodil R, Quintana JB, Beiras R. Ecotoxicological evaluation of four UV filters using marine organisms from different trophic levels *Isochrysis galbana*, *Mytilus galloprovincialis*, *Paracentrotus lividus*, and *Siriella armata*. Chemosphere 2014; 104: 44-50.

Pérez J, Monteiro MS, Quintaneiro C, Soares AMVM, Loureiro S. Characterization of cholinesterases in *Chironomus riparius* and the effects of three herbicides on chlorpyrifos toxicity. Aquatic Toxicology 2013; 144–145: 296-302.

Plagellat C, Kupper T, Furrer R, de Alencastro LF, Grandjean D, Tarradellas J. Concentrations and specific loads of UV filters in sewage sludge originating from a monitoring network in Switzerland. Chemosphere 2006; 62: 915-925.

Poiger T, Buser H-R, Balmer ME, Bergqvist P-A, Müller MD. Occurrence of UV filter compounds from sunscreens in surface waters: regional mass balance in two Swiss lakes. Chemosphere 2004; 55: 951-963.

Ponlawat A, Harrington LC. Age and body size influence male sperm capacity of the dengue vector *Aedes aegypti* (Diptera: Culicidae). J Med Entomol 2007; 44: 422-6.

Ramos S, Homem V, Alves A, Santos L. Advances in analytical methods and occurrence of organic UV-filters in the environment — A review. Science of The Total Environment 2015; 526: 278-311.

Ramos S, Homem V, Alves A, Santos L. A review of organic UV-filters in wastewater treatment plants. Environment International 2016; 86: 24-44.

Riddiford LM, Cherbas P, Truman JW. Ecdysone receptors and their biological actions. Vitam Horm 2000; 60: 1-73.

Rikans LE, Hornbrook KR. Lipid peroxidation, antioxidant protection and aging. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease 1997; 1362: 116-127.

Rodil R, Moeder M, Altenburger R, Schmitt-Jansen M. Photostability and phytotoxicity of selected sunscreen agents and their degradation mixtures in water. Analytical and Bioanalytical Chemistry 2009; 395: 1513-1524.

Rodrigues ACM, Gravato C, Quintaneiro C, Golovko O, Žlábek V, Barata C, et al. Life history and biochemical effects of chlorantraniliprole on *Chironomus riparius*. Science of The Total Environment 2015; 508: 506-513.

Schmitt C, Oetken M, Dittberner O, Wagner M, Oehlmann J. Endocrine modulation and toxic effects of two commonly used UV screens on the aquatic invertebrates *Potamopyrgus antipodarum* and *Lumbriculus variegatus*. Environmental Pollution 2008; 152: 322-329.

Servia MJ, Pery AR, Heydorff M, Garric J, Lagadic L. Effects of copper on energy metabolism and larval development in the midge *Chironomus riparius*. Ecotoxicology 2006; 15: 229-40.

Sieratowicz A, Kaiser D, Behr M, Oetken M, Oehlmann J. Acute and chronic toxicity of four frequently used UV filter substances for *Desmodesmus subspicatus* and *Daphnia magn*a. J Environ Sci Health A Tox Hazard Subst Environ Eng 2011; 46: 1311-9.

Sokolova IM, Frederich M, Bagwe R, Lannig G, Sukhotin AA. Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. Mar Environ Res 2012; 79: 1-15.

Wang J, Pan L, Wu S, Lu L, Xu Y, Zhu Y, et al. Recent Advances on Endocrine Disrupting Effects of UV Filters. International journal of environmental research and public health. 13, 2016.

Supplementary data

Table SD 1- MS/MS parameters for triple quadrupole detection of targeted compounds:

A) MS/MS parameters for BP3 and 4-MBC, (TSQ Quantum Ultra mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA))

Compound	*Mode*	*Quantification transition*	*Confirmation transition*	*Tube Lens voltage (V)*	*Collision energy*	*Retention time (min)*
BP3	+	229.062→ 150.670	229.062→ 105.200	91	18	9.44
4-MBC	+	255.124→ 104.780	255.124→ 164.810	94	30	10.12
Diclofenac	+	296.000→ 214.010	*	86	33	9.13

B) MS/MS parameters for OC, (Q-Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA))

Compound	*Mode*	*Quantification transition*	*Confirmation transition*	*NCE*	*Retention time (min)*
Octocrylene	-	361.2047→ 204.0815	361.2047→ 203.0739	25	9.80
Diclofenac	-	294.0094→ 250.0185	*	20	8.10

*No confirmation ion.

NCE - normalized collision energy

Table SD 2- LC gradient for the elution of targeted compounds.

A) LC gradient for the elution of BP3 and 4-MBC, Cogent Bidentate C18 column.

Time, min	*Mobile phase composition*		*Flow rate, µL/min*
	Water (0.1 % FA)	Acetonitrile (0.1 % FA)	
0.00	100	0	300
1.00	100	0	300
7.00	60	40	350
9.00	0	100	400
10.00	0	100	400
10.01	100	0	300
13.00	100	0	300

B) LC gradient for the elution of OC, Hypersil Gold Phenyl column

Time, min	*Mobile phase composition*		*Flow rate, µL/min*
	Water	Methanol	
0.00	90	10	300
1.00	90	10	350
3.00	60	40	400
10.00	0	100	400
12.00	0	100	350
12.01	90	10	300
15.00	90	10	300

Chapter 3:
Ecotoxicity of two organic UV-filters to the freshwater caddisfly *Sericostoma vittatum*

Published in Environmental Pollution
DOI: 10.1016/j.envpol.2017.05.021

Ecotoxicity of two organic UV-filters to the freshwater caddisfly *Sericostoma vittatum*

Abstract

Organic ultraviolet filters (UV-filters) used for protection against radiation in personal care products and other materials (e.g. textiles, plastic products) are considered emerging contaminants of aquatic ecosystem. Benzophenone-3 (BP3) and 3-(4-methylbenzylidene)camphor (4-MBC) are the most commonly used organic UV-filters and have been reported in freshwater environments due to contamination through discharges from wastewater treatment plants and swimming pools or by direct contamination from recreational activities. Our aim was to evaluate the ecotoxicological effects of these UV-filters using the freshwater caddisfly S*ericostoma vittatum'* biochemical biomarkers and energy processing related endpoints (feeding behaviour, energy reserves and cellular metabolism). In laboratory trials, both compounds induced feeding inhibition of *S. vittatum* at 3.55 mg/Kg of BP3 and at concentrations ≥ 2.57 mg/Kg of 4-MBC, decreased carbohydrates content at 3.55 and 6.95 mg/Kg of BP3 and 4-MBC respectively, and increased total glutathione levels at concentrations ≥ 1.45 and 1.35 mg/Kg of BP3 and 4-MBC respectively. No significant effects were observed on endpoints associated with oxidative stress, antioxidant defences, phase II biotransformation or neurotoxicity after exposure to the two UV-filters. Our results show that environmental relevant concentrations of BP3 and 4-MBC, can negatively impact freshwater insects and demonstrate the importance of monitoring the ecological effects of organic UV-filters using non-model invertebrate species.

Keywords: BP3; 4-MBC; feeding behaviour; freshwater insects; personal care products; sunscreens

1. Introduction

Organic ultraviolet filters (UV-filters) absorb ultraviolet radiation (UVA-UVB) and are commonly used to protect skin from sun radiation and to protect a variety of materials from degradation. They are present in textiles, plastic materials, and in several personal care products such as sunscreens, lotions, shampoos and makeup products (Díaz-Cruz et al., 2008; Pedrouzo et al., 2011). After being used, these compounds can reach the aquatic ecosystems, namely streams and rivers by washing-off from skin and clothes of beachgoers during recreational activities, by discharges of swimming pools and sewage or due to insufficient removal of wastewater treatment plants (WWTP) (Brausch and Rand, 2011; Díaz-Cruz et al., 2008; Giokas et al., 2005; Golovko et al., 2014).

UV-filters have been detected worldwide both in surface and ground waters, as observed by several studies in Spain (Rodil et al., 2008; Román et al., 2011), Korea (Jeon et al., 2006), Switzerland (Giokas et al., 2005), Australia (Liu et al., 2012), and Japan (Kameda et al., 2011). UV-filters concentrations of up to 1040 ng/L were found in river waters (Kameda et al., 2011) and 4381 ng/L in lake waters (Rodil et al., 2009). Also, concentrations of up to 6812 ng/L (Tsui et al., 2014) and 621 ng/L (Román et al., 2011) were found in seawater and tap water, respectively. In solid matrices, UV-filters have been detected in concentrations of up to 0.128 mg/Kg dry weight (dw) in sediments from coastal areas (Amine et al., 2012), 0.9 mg/Kg (dw) in lake sediments (Rodil and Moeder, 2008), 27.7 mg/Kg (dw) in sewage sludge (Plagellat et al., 2006), 0.635 mg/Kg (dw) and 2.4 mg/Kg (dw) in sediments from lotic ecosystems (Kameda et al., 2011, Gago-Ferrero et al., 2011).

Given its continuous application and consequent ubiquity in the environment, concerns of deleterious effects of UV- filters to aquatic biota have been raised and an evaluation of their ecotoxicity is vital (Sánchez-Quiles and Tovar-Sánchez, 2015; Tsui et al., 2014). It was demonstrated that UV-filters affected reproduction of several invertebrate species. As examples, reproduction of the oligochaete *Lumbriculus variegatus* was reduced after 28 days of exposure to 3-benzylidene-camphor (3-BC) and 3-(4-methylbenzylidene)camphor (4-MBC) (Schmitt et al., 2008), in the crustacean *Daphnia magna,* exposure to 3-BC during 21 days caused the reduction of number of neonates per adult (Sieratowicz et al., 2011), in the snail *Potamopyrgus antipodarum,* the number of unshelled embryos per snail increased after 56 days of exposure to 3-BC and 4-MBC (Schmitt et al., 2008) and in the snail *Melanoides tuberculata* the number of embryos per snail decreased after 28 days of exposure to ethylhexyl-methoxycinnamate (EHMC) (Kaiser et al., 2012). Delayed development was also observed in the dipteran *Chironomus riparius* exposed to Benzophenone-3 (BP3) and 4-MBC

(Campos et al., 2017) while decreases of *D.magna* somatic growth were observed after 21 days of exposure to 4-MBC, 3-BC and EHMC (Sieratowicz et al., 2011). However, absence of effects during exposure to UV-filters were also reported. Exposure to octocrilene and butyl-methoxydibenzoylmethane (B-MDM) during 28 days did not affect the mean emergence time of *C. riparius* or the number of embryos per snail of *M. tuberculata* (Kaiser at al., 2012). Also, 21 days exposure to 4-MBC and BP3 has been shown to cause no effects on the number of neonates produced by *D. magna* (Sieratowicz et al., 2011).

Nevertheless, due to their physico-chemical properties, namely an elevated K_{ow} and low water solubility, organic UV-filters tend to accumulate in biota and sediments (Brausch and Rand, 2011; Rodil and Moeder, 2008) especially near fluvial beaches, coastal areas and effluents, which calls for an ecotoxicological evaluation of these compounds using non-model benthic species. This assessment should comprise responses at different levels of biological organization in order to provide clues to organismal and population level effects.

Thus, the main objective in this study was to address the potential effects of organic UV-filters present in sediments, using the freshwater caddisfly *Sericostoma vittatum* (Rambur) as a test species. *S. vittatum* is widely distributed in streams and rivers across the Iberian Peninsula with an important role in the fragmentation of allochthonous organic matter in streams (Campos et al., 2014; Feio and Graça, 2000). Additionally, *S. vittatum* has been used previously in ecotoxicity studies (Campos et al., 2014; Campos et al., 2016; Pestana et al., 2009b) and given their close contact to sediments and detritivore feeding habits is a potential indicator of sediment contamination by lipophilic compounds such as organic UV-filters. This ecotoxicological evaluation is focused on feeding rate as an organismal endpoint, and on sub-cellular endpoints including energy reserves, energy consumption and biochemical parameters measured on *S. vittatum* larvae exposed to a gradient of environmentally relevant concentrations of organic UV-filters.

Feeding inhibition has been recognized as a general stress response to different contaminants, used as a early warning indicator and a complement to traditional parameters such as growth and reproduction especially important when dealing with organisms with a long life-cycle (Maltby et al., 2002; Pestana et al., 2007). Feeding behaviour has thus been used as an ecotoxicological relevant endpoint in a variety of aquatic invertebrate detritivores organisms including caddisflies (Campos et al., 2016; Pestana et al., 2009b; Rodrigues et al., 2016) Furthermore, energy parameters can be also used to better understand the long-term effects of altered energy intake and expenditure (feeding and respiration/ detoxification). This is because detoxification processes, decrease food intake and assimilation and also, metabolic changes contribute for altered energy homeostasis in organisms under stressful conditions (De

Coen and Janssen, 2003; Moolman et al., 2007; Sokolova et al., 2012; Sokolova, 2013). In this sense, we have also assessed effects of UV-filters exposure on energy reserves in terms of sugars, lipids and protein contents, as well as, energy consumption by measuring the activity of ETS (electron transport system) as a proxy for cellular metabolism.

Moreover and since it is known that UV-filters can increase the production of Reactive Oxygen Species (ROS) and induce alterations in antioxidant defences and/or cause oxidative damage (Gao et al., 2013; Liu et al., 2015) biochemical biomarkers chosen as sub-organismal endpoints in the present study included lipid peroxidation (LPO); antioxidant enzymes (catalase - CAT), phase II biotransformation enzyme (glutathione-*S*-transferase - GST) and non-enzymatic antioxidant defences (total glutathione - tGSH).

Finally, we also assessed the effects of UV-filters exposure on Acetylcholinesterase activity (AChE) as indicator of neurotoxicity. Despite no information on effects of UV-filters on AChE, this enzyme activity has been linked with behavioural parameters such as feeding and locomotion (Mesquita el al., 2011; Xuereb et al., 2009) and effects using AChE activity as indicator of neurotoxic have been reported to emergent contaminants (Mesquita et al., 2011), namely in caddisflies species (Pestana et al., 2014; Pradhan et al., 2016).

For this ecotoxicological assessment we selected BP3 and 4-MBC, that correspond to different classes of UV-filters (benzophenones and camphor derivatives, respectively) which are two of the most frequently detected UV-filters in the aquatic environment (Balmer et al., 2005; Gago-Ferrero et al., 2011; Kameda et al., 2011; Ramos et al., 2015).

2. Material & methods

2.1 Caddisflies collection and maintenance

S. vittatum larvae (size: 18.99 (± 3.87(SD)) mg wet weight) were collected from a low order stream in central Portugal (40°06’N, 8°14’W) using a hand net. Organisms were maintained under laboratory conditions (20 ± 1ºC and light-dark cycle of 16:8h) during one week, in American Society for Testing Materials hard water medium (ASTM) (ASTM, 1980), previously burnt (500ºC for 4 hours) inorganic fine sediment (< 1 mm) and alder leaves (*Alnus glutinosa*) as food which were previously conditioned during one week in 1500 mL of local river water with aeration and in laboratory conditions (20 ± 1ºC, 16:8 h light: dark photoperiod).

2.2 Chemicals

2-hydroxy-4-methoxybenzophenone (or benzophenone-3, BP3; CAS No. 131-57-7; purity ≥ 98%) and 3-(4-methylbenzylidene) camphor (4-MBC; CAS No. 36861-47-9, purity ≥ 98%) were

obtained from Sigma-Aldrich (Portugal). Physico-chemical properties are presented in table 1. Stock solutions and subsequent gradient of UV-filters experimental solutions of both compounds were prepared in ethanol (96%) due to low water solubility (table 1).

Table 1 - Physico-chemical properties of UV-filters.

Compounds	Formula	Abbreviation	CAS N	Molecular weight g/mol	Log K_{ow}	Water solubility (g/L)
2-hydroxy-4-methoxybenzophenone (Benzophenone-3)	$C_{14}H_{12}O_3$	BP3	131-57-7	228.24	3.79	0.10
3-(4-methylbenzylidene) camphor	$C_{18}H_{22}O$	4-MBC	36861-47-9	254.37	4.95	0.017

Based in Gago-Ferrero et al. (2012)

2.3 Sediment dosing

Artificial sediment was composed of 75% inorganic fine sediment (<1 mm), 20% kaolin, 5% α-cellulose and 0.1 % calcium carbonate. Tests were conducted using 180 mL glass vessels and each replicate contained 50 g dw of sediment. Ten mL of UV-filter solutions (prepared in ethanol) were used to dose the sediment which were mixed thoroughly and then allowed to evaporate during 24h. To prepare the solvent controls 10 mL of 96% ethanol were added to each replicate. Afterwards, 10 mL of ASTM hard water medium (ASTM, 1980) were added to the sediment in all treatments including controls to form the sediment paste. Immediately after, 150 mL of ASTM hard water medium (ASTM, 1980) were added in all treatments and the sediment remained stabilizing during 48h before exposing organisms.

2.4 Chemical analysis

2.4.1 Chemicals

Liquid chromatography–mass spectrometry (LC-MS) grade methanol and acetonitrile (Li Chrosolv Hypergrade) were purchased from Merck (Darmstadt, Germany). Formic acid for the mobile phases acidification was purchased from Labicom (Olomouc, Czech Republic). BP3, 4-MBC and diclofenac (used as internal standard) were purchased from Sigma Aldrich (UK). A spiking solution was prepared for BP3 and 4-MBC by diluting respective stock solutions (1 mg/ mL methanol) in methanol to a final concentration of 1 µg/mL and stored at -20°C.

2.4.2 Instrumentation

To analyse UV-filters in sediment samples, a triple stage quadrupole MS/MS TSQ Quantum Ultra mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) coupled with an Accela 1250 LC pump (Thermo Fisher Scientific) and an HTS XT-CTC autosampler (CTC Analytics AG, Zwingen, Switzerland) was used.

The analytical column for chromatographic separation of BP3 and 4-MBC used was Cogent Bidentate C18 column (50 mm × 2.1 mm i.d., 4 µm particle size from MicroSolv Technology Corporation Eatontown, NJ, USA). Heated electrospray ionization (HESI) was used to ionize target compounds.

MS/MS parameters are reported in Table SD1. LC gradient for the elution of target compounds is reported in Table SD 2.

2.4.3 Sample preparation

An ultrasonic based solvent approach was used to extract the UV-filters from the sediments (Golovko et al., 2016). Briefly, around 2 grams of sediment were weighted into 10 mL vials and 20 ng of IS was added to each sample. Four mL of extraction solvent (acetonitrile and water (1/1 v/v with 0.1% formic acid)) were added and the samples were sonicated (DT 255, Bandelin electronic, Sonorex digitec, Berlin, Germany) during 15 min. The supernatant was filtered through a syringe filter (0.45 µm, regenerated cellulose, Labicom, Olomouc, Czech Republic) into 10-mL vials. The same step was repeated with 4 mL of acetonitrile, 2-propanol, and water (3/3/4 v/v/v with 0.1% formic acid). Two supernatants were mixed after extraction procedure. Each sample was prepared and analysed in duplicate.

This method was validated in the range of tested concentrations and exhibited good linearity in the concentration range between 0.005 and 0.5 mg/kg BP3 and 4-MBC; R^2 = 0.999 and average recovery of BP3 and 4-MBC was 108 % (±8). Average limit of quantification was 0.09 mg/kg for BP3 and 0.07 mg/kg for 4-MBC.

Internal standard method was used for quantification of target compounds. The matrix effect was assessed for each compound, and corrections for ion suppression or enhancement were accomplished using matrix-matched standards for sediment samples. Matrix-matched standards were prepared from tested sediment extract by spiking with both IS and target compounds at 0.01 mg/kg and 0.1 mg/kg, respectively.

2.5 *S. vittatum* exposure trials

Alnus glutinosa leaves were collected, air dried and stored in the darkness until use as food in bioassays. Leaf discs with app. 12 mm diameter were prepared after soaking leaves in distilled water. Leaf discs were then autoclaved and conditioned during one week in 1500 mL of local river water with aeration and in laboratory conditions (20 ± 1ºC, 16:8 h light: dark photoperiod). After the conditioning period, the leaf discs were dried at 50ºC for four days and then weighed. Just before use in the feeding test, leaf discs were soaked in ASTM hard water medium (ASTM, 1980) during 96h.

Feeding bioassays were conducted according to Campos et al. (2016). *S. vittatum* larvae were individually exposed to a gradient of UV-filters concentrations (2.5, 5, 10 mg/Kg) plus control (ASTM only) and solvent control treatment in laboratory conditions (20ºC ± 1ºC and light-dark cycle of 16:8h), with aeration. Ten replicates were used in each treatment in which six alder leaf discs were added as food. After the exposure period of 6 days, organisms were collected, removed from their mineral cases, quickly dried on filter paper, immediately weighed, frozen in liquid nitrogen and stored at -80ºC for determination of biochemical responses. Alder leaves discs were also collected and rinsed in distilled water and dried at 50ºC during 96h. The differences in initial and final leaf disc dry mass (mg) together with wet mass of organism (mg) allowed us to calculate *S. vittatum*' feeding rate (Pestana et al., 2009b). Replicate vials with leaf discs and no larvae were used to determine correction factors for leaf weight change.

2.6 Determination of biochemical biomarkers

To evaluate sub-cellular and biochemical effects induced by UV-filters exposure on *S. vittatum*, each organism previously frozen was homogenized in 1600 µL of milli-Q water by sonication. After homogenization, 3 aliquots of 300 µL were used to analyse carbohydrates and proteins, lipids, and ETS. Another aliquot of 200 µL of homogenized sample containing 4 µl of 4% 2,6-Di-tert-butyl-4-methylphenol in methanol was used for lipid peroxidation determination. Five hundred µL of K-phosphate buffer (0.2 M; pH = 7.4) was added to the remaining volume (500 µL) of homogenized sample, followed by centrifugation at 10000 xg for 20 min at 4ºC. The resulting post-mitochondrial supernatant (PMS) was separated in aliquots for catalase, glutathione-*S*-transferase, total glutathione, acetylcholinesterase, and protein quantification.

2.6.1 Biomarkers related with oxidative stress and neurotransmission

LPO was determined in the homogenate by measuring thiobarbituric acid-reactive substances (TBARS) at 535 nm using $1.56x10^5$ $M^{-1}cm^{-1}$ as molar extinction coefficient (Bird and Draper, 1984; Ohkawa et al., 1979). The results were expressed as nmol TBARS per g of wet weight. CAT activity was assessed with PMS fraction by measuring decomposition of hydrogen peroxide at 240 nm using 40 $M^{-1}cm^{-1}$ as molar extinction coefficient (Clairborne, 1985). Results were expressed as µmol per min per mg of protein. Glutathione-*S*-transferase activity was measured with PMS through the conjugation of L-glutathione reduced with 1-chloro-2,4-dinitrobenzene at 340 nm for 5 min using 9.6 x $10^3M^{-1}cm^{-1}$ as molar extinction coefficient (Habig et al., 1974). The enzymatic activity was expressed in nmol per min per mg of protein. Total glutathione content was measured with PMS fraction using the recycling reaction of reduced glutathione with 5,5′-dithiobis-(2-nitrobenzoic acid) in the presence of an excess of glutathione reductase (Baker et al., 1990). The absorbance was read at 412 nm during 3 min. Total glutathione levels were expressed as µM per mg of protein, using a standard curve with known concentrations of L-GSH. Considering that cholinesterases present in *S. vittatum* have been previously characterized as AChE (Pestana et al., 2014), enzymatic activity was measured, in the present study, with PMS fraction using acetylthiocholine as substrate and Ellman's reagent (Ellman et al., 1961), addapted to microplate (Guilhermino et al., 1996). The reaction was followed at 414 nm and the enzymatic activity was expressed in nmol per min per mg of protein using $13.6x10^3$ $M^{-1}cm^{-1}$ as molar extinction coefficient. The protein concentration was determined with PMS fraction according to Bradford method (Bradford, 1976) adapted from BioRad´s Bradford micro-assay, using γ-globuline as a standard.

2.6.2 Energy reserves and energy consumption

Lipids, carbohydrates and proteins contents and energy consumption (ETS activity) were measured according to protocols by De Coen and Janssen (1997) with slight modifications (Rodrigues et al., 2015).

Homogenate for lipids measurement (300 µL) were pre-treated with 500 µL of chloroform (119.38 M; ACS spectrophotometric grade, ≥99.8%), 500 µL of methanol (32.04 M; ACS reagent, ≥99.8%) and then centrifuged at 1000g during 5 min. After centrifugation 100 µL of each sample was transferred to a glass tube, 500 µL of sulfuric acid was added and the samples were incubated at 200 ºC during 15 min. After that, the samples were cooled down at room temperature and 1500 µL of ultra-pure water was added. The absorbance was read at 375 nm using tripalmitine as a standard.

For the measurement of carbohydrates and protein content, to 300 µL of homogenate was added 100 µL of 15% (w/v) trichloroacetic acid, following an incubation at -20ºC during 10 min. After that, the samples were centrifuged and the supernatant was collected to measure the carbohydrates contents. The pellet was suspended with 500µL of NaOH, incubated at 60ºC during 30 min and lastly 280µL of HCl was added. Total proteins content was measured following Bradford's method (Bradford, 1976). The absorbance was read at 592 nm after 30 min of incubation using γ-globuline as a standard. To measure carbohydrates content, 200 µL of supernatant of each sample were transferred to a glass tube and 200 µL of 5% phenol and 800 µL of sulfuric acid were added. The mixture and standard curve with known concentrations of glucose were incubated for 30 min at room temperature. The absorbance was read at 492 nm. Energetic values were calculated based on the energy of combustion of the different fractions (De Coen and Janssen, 1997).

ETS activity was measured with 300 µL of homogenate, 150 µL of homogenization buffer (0.3 M Tris base; 0.45% (w/v) Poly Vinyl Pyrrolidone; 459 µM $MgSO_4$; 0.6% (v/v) Triton X-100 at a pH of 8.5) and centrifuged at 4ºC during 10 min (1000g). To 50 µL of supernatant was added 150 µL of buffered solution (0.13 M Tris base containing 0.27% (v/v) Triton X-100; 1.7 mM NADH; 274 µM NADPH) and 100 µL of INT solution (p-iodonitrotetrazolium; 8 mM). The absorbance was read at 490 nm over 3 min. The cellular oxygen consumption rate was calculated using the stoichiometric relationship (2 µmol of INT-formazan formed 1 µmol of oxygen consumed) and using formula of Lambert–Beer using an $\varepsilon = 15,900\ M^{-1}cm^{-1}$ for INT-formzan. Caloric values were calculated from ETS activity using specific oxyenthalpic equivalent for an average lipid, protein and carbohydrate mixture of 480 kJ/mol.

2.7 Statistical analysis

Effects of UV-filters in *S. vittatum* on biochemical endpoints and in feeding rate were evaluated using analysis of variances (ANOVA) with multiple comparisons examined by Dunnett's *post hoc* test. Since no significant differences were found between control and solvent control (evaluated by t-test) all comparisons were made against solvent control treatment. All variables were previously assessed for normality using Shapiro-Wilk test while Brown-Forsythe test verified the homoscedasticity of data. Carbohydrates data were log transformed and Kruskal-Wallis test followed by Dunn's *post hoc* test was used for analysis of proteins and ETS data. All data were analysed using Prism 6.0. (GraphPad Software, La Jolla California USA) with significance level set at $p < 0.05$.

3. Results

Nominal and measured concentrations of BP3 and 4-MBC in the sediment are presented in Table 2. Since chemical analyses showed that concentrations of BP3 and 4-MBC in sediments at the end of the exposure period (6 days) were up to 71% and 48.6 % lower than nominal concentrations, measured concentrations are presented in all figures and tables. The differences observed between nominal and measured concentrations of UV-filters might reflect some microbial degradation, bioaccumulation of UV-filters in organisms and adsorption to leaf material although we cannot exclude patchy contamination of sediments.

Table 2 - UV-filters concentrations measured in sediment (mg/Kg) in the end of exposure period (after 6 days) (mean ±SD).

	Nominal concentrations (mg/Kg)	Measured concentrations (mg/Kg)	
		BP3	**4-MBC**
Six-day exposure	2.5	0.89 (±0.04)	1.35 (±0.21)
	5	1.45 (±0.09)	2.57 (±0.35)
	10	3.55 (±0.59)	6.95 (±1.83)

After six days of exposure, BP3 and 4-MBC caused feeding inhibition in *S. vittatum* larvae. A significant decrease of 54% was observed in feeding rates of *S. vittatum* exposed to 3.55 mg/Kg of BP3 (figure 1a; $F_{(3,36)} = 5.71$; $p < 0.01$) when compared to solvent control. The feeding rate upon exposure to 4-MBC followed a clear dose-response relationship with reductions of 44 and 69% in *S. vittatum* larvae exposed to 2.57 and 6.95 mg/Kg of 4-MBC, respectively (figure 1b; $F_{(3,36)} = 12.28$; $p < 0.001$).

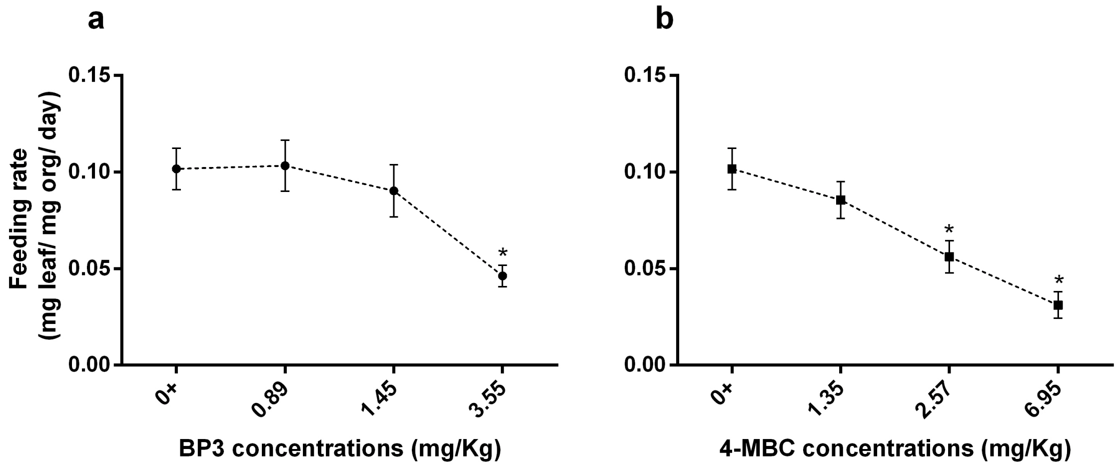


Figure 1 - Feeding rates of *S. vittatum* (mg leaf/mg organism/day; mean ± SE) exposed to BP3 (a) and 4-MBC (b). Asterisks (*) denote statistically significant differences compared to solvent control (0+) (Dunnet's *post hoc* test, p < 0.05).

Concerning biochemical responses, total glutathione level was significantly increased in larvae of *S. vittatum* exposed to UV-filters during 6 days in comparison to larvae under control conditions A dose-response relationship was observed on total glutathione levels when *S. vittatum* larvae were exposed to BP3 showing significant effects at concentrations ≥ 1.45 mg/Kg (figure 2a; $F_{(3,35)} = 22.70$; $p < 0.001$). Also, a significant increase on total glutathione levels was observed on larvae exposed at concentrations ≥1.35 mg/Kg 4-MBC (figure 2b; $F_{(3,36)} = 20.86$; $p < 0.001$;). BP3 did not elicited a monotonic or dose-dependent response in terms of LPO, despite the significant effects detected by ANOVA (table 3; BP3: $F_{(3,33)} = 3.43$; $p < 0.05$) with no significant differences found by *post-hoc* test for any of the concentrations in comparison with the solvent control treatment. 4-MBC did not significantly affected LPO levels either (table 3; 4-MBC: $F_{(3,34)} = 2.67$; $p > 0.05$). Both UV-filters tested did not significantly affected the activity of CAT (table 3; BP3: $F_{(3,36)} = 0.46$; $p > 0.05$; 4-MBC: $F_{(3,36)} = 2.34$; $p > 0.05$) nor GST (table 3; BP3:$F_{(3,35)} = 0.91$; $p > 0.05$; 4-MBC: $F_{(3,36)} = 0.51$; $p > 0.05$) of exposed *S. vittatum* larvae in comparison with the solvent control treatment. Concerning neurotoxic effects, AChE activity of *S. vittatum* was not significantly inhibited by BP3 ($F_{(3,36)} = 3.97$; $p < 0.05$) or 4-MBC ($F_{(3,36)} = 0.48$; $p > 0.05$) compared to solvent control treatment (table 3).

Table 3 – Effect of different concentrations of BP3 and 4-MBC on LPO levels, CAT, GST and AChE activity (mean ± SE) in *S. vittatum* larvae exposed for 6 days.

		BP3 concentrations (mg/Kg)			**4-MBC concentrations (mg/Kg)**		
	0+	**0.89**	**1.45**	**3.55**	**1.35**	**2.57**	**6.95**
LPO (TBARS nmol/g wet weight)	36.04 (±2.99)	43.92 (±4.01)	46.72 (±3.26)	33.47 (±2.95)	47.61 (±4.32)	48.35 (±4.40)	38.76 (±4.35)
CAT (µmol/min/mg protein)	86.77 (±4.93)	85.02 (±4.98)	83.13 (±7.86)	93.12 (±7.31)	92.41 (±2.63)	97.95 (±3.54)	85.52 (±3.47)
GST (nmol/min/mg protein)	1.81 (±0.25)	2.01 (±0.19)	1.83 (±0.30)	1.82 (±0.24)	1.86 (±0.37)	1.76 (±0.25)	2.23 (±0.29)
AChE (nmol/min/mg protein)	6.67 (±0.51)	5.30 (±0.78)	4.86 (±0.56)	7.62 (±0.66)	7.31 (±0.79)	6.33 (±0.46)	7.32 (±0.94)

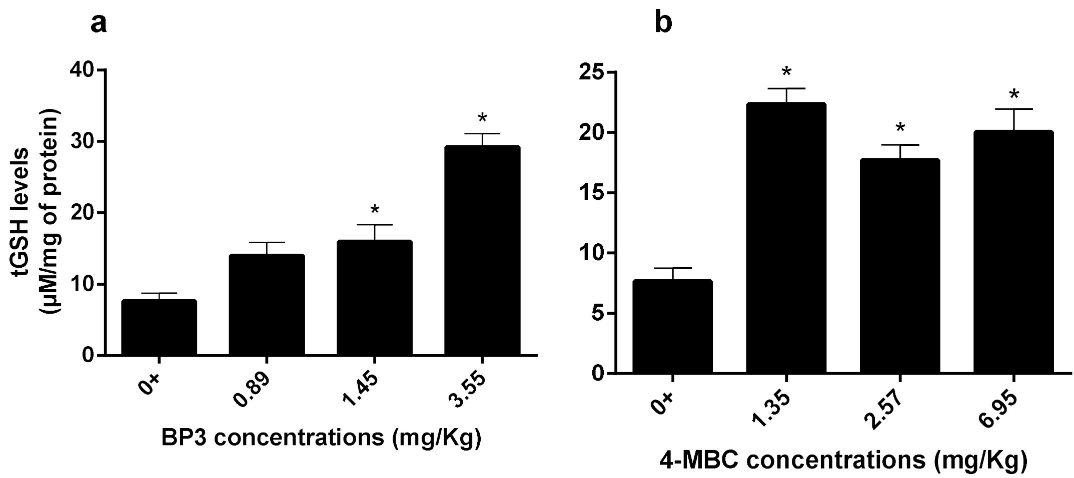


Figure 2 - Effects of BP3 (a) and 4-MBC (b) exposure on total glutathione contents (µM/mg of protein; mean ± SE). Asterisks (*) denote statistically significant differences compared to solvent control (0+) (Dunnett's *post hoc* test, $p < 0.05$).

Carbohydrates content was significantly decreased on larvae exposed at 3.55 mg/Kg of BP3 (figure 3a; $F_{(3,35)} = 6.80$; $p = 0.001$) and 6.95 mg/Kg of 4-MBC (figure 3b; $F_{(3,35)} = 8.38$; $p < 0.001$). On the other hand, no significant effects were observed on lipid (BP3: $F_{(3,36)} = 1.21$; $p > 0.05$; 4-MBC: $F_{(3,36)} = 0.76$; $p > 0.05$) and protein contents (BP3: H= 0.64, Df= 3; $p > 0.05$; 4-MBC: H = 4.50, Df= 3; $p > 0.05$) (figure 3a and b) when compared to the solvent control treatment. Concerning cellular respiration, ETS activity in *S. vittatum* larvae was not significantly changed after exposure to BP3 (H=5.7, Df= 3; $p > 0.05$) or 4-MBC (H=0.27, Df= 3; $p > 0.05$) (figure 3c and d respectively).

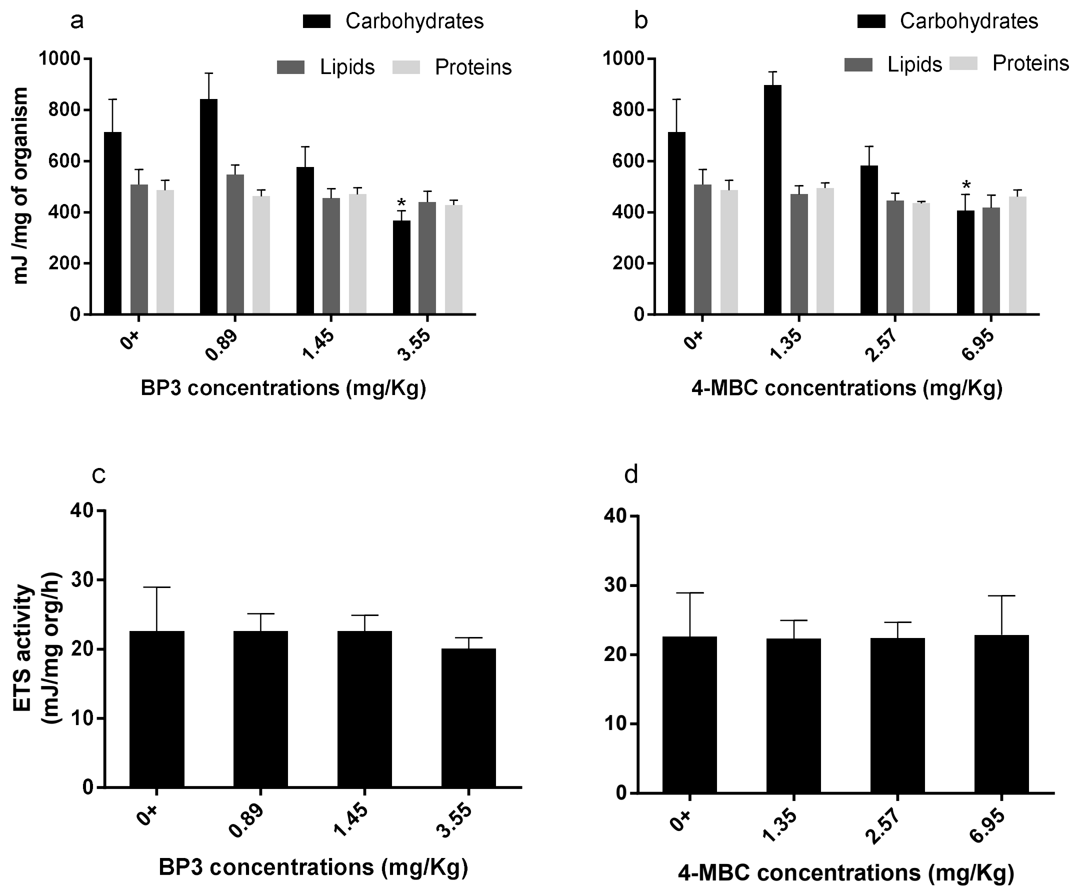


Figure 3 - Energy budget of *S. vittatum* after 6 days of exposure to BP3 (a,c) and 4-MBC (b,d). a and b correspond to fractions of energy reserves (E_{lipids}, $E_{carbohydrates}$ and $E_{proteins}$; mJ/mg organism; mean ± SE) and c and d correspond to energy consumption (ETS activity; mJ/mg organism/h; mean ± SE). Asterisks (*) denotes significant differences compared to the solvent control (0+) treatment at $p < 0.05$ (Dunnett's *post hoc* test (carbohydrates and lipids) and Dunn´s *post hoc* test (proteins and ETS)).

4. Discussion

UV-filters are emerging contaminants of many aquatic ecosystems and their effects in the environment should be closely monitored. To date only scarce information exists, being mostly based in toxicity data for a limited number of model aquatic species.

In the present study, we have shown that organic UV-filters such as BP3 and 4-MBC can cause feeding inhibition in aquatic insects. Exposure to dosed sediments reflecting environmentally relevant concentrations of BP3 and 4-MBC induced a 50% inhibition of *S. vittatum* larvae feeding rates, which may lead to deleterious consequences in terms of growth and development of organisms, and therefore alter population dynamics, compromise organic matter processing in streams and reduce performance of other detritivores species (Campos et al., 2014). Moreover,

there are also evidences that both, BP3 and 4-MBC can directly affect genes related with the development of arthropods. The organic UV-filter 4-MBC induced overexpression of the ecdysone receptor gene and BP3 activated the expression of a set of ecdysone responsive genes in *C. riparius* (Ozáez et al., 2013, 2014), thus showing the potential for endocrine disruption caused by this type of compounds in invertebrates. Furthermore, it was previously shown that UV-filters can alter estrogenic and hormonal activity in fish (Kunz and Fent, 2006; Kunz et al., 2006; Wang et al., 2016).

These results are also in good agreement with previous studies showing that exposure to UV-filters, can compromise growth of different species, such as, the dipteran *C. riparius* (Campos et al., 2017), the sea urchin *Paracentrotus lividus* (Paredes et al., 2014) or the crustacean *D. magna* (Sieratowicz et al., 2011). Chronic effects of UV-filters have also been reported for the algae *Desmodesmus subspicatus* (Sieratowicz et al., 2011), the protozoan *Tetrahymena thermophila* (Gao et al., 2013), and marine microalgae *Isochrysis galbana* (Paredes et al., 2014).

Our results also show a reduction of sugars content without any significant changes in lipid and protein contents nor ETS activity, reflecting only minor changes in the energy budget of *S. vittatum* larvae exposed to BP3 and 4-MBC. However, although we cannot exclude the possibility of a reduction of sugar content due to reduced feeding rates or altered assimilation, carbohydrates are most likely being used as easily metabolizable resources to fuel higher energy requirements of larvae related with detoxification mechanisms (Arrese and Soulages, 2010; Choi et al, 2001; De Coen and Janssen, 2003; Sokolova et al., 2012). In fact, the lack of significant effects in terms of LPO and related enzymes, such as, CAT and GST, could indicate that increase in tGSH might play an important role for the detoxification of UV-filters preventing oxidative stress. The increase of glutathione contributes to maintain the cellular redox status since glutathione protects the cell against reactive oxygen species and free radicals (Doyotte et al., 1997; Lushchak, 2012; Sen, 1997). Synthesis and recycling of tGSH are probably contributing to the decrease of energetic reserves suggested by the reduction in carbohydrates observed in larvae exposed to UV-filters. In line with our study an increase in glutathione levels was observed in *Carassius auratus* exposed to UV-filters (Liu et al., 2015). Also, no evidences of oxidative stress induced by UV-filters exposure could be detected in the aquatic midge *C. riparius* (Campos et al., 2017) and in the fish *C. auratus* (Liu et al., 2015). Interestingly, no effects were observed in terms of ETS activity, which is commonly assumed to reflect the energetic costs of detoxification mechanisms. (Choi et al., 2001; Sokolova et al., 2012). This lack of response was unexpected especially given the increase ETS activity observed before for Chironomids exposed to these organic UV-filters

(Campos et al., 2017). However, reductions in oxygen consumption and in ETS activity have been previously observed in *S. vittatum* larvae in response to other contaminants namely insecticides (Pestana et al., 2009b, Rodrigues et al., 2016). In fact, metabolic depression in cased caddisflies is assumed to be a general response to stress (Kuhara et al., 2001) and can lead to reduced activity and feeding inhibition as observed in the present study. Because foraging and digestion are also energy consuming processes, this reduced activity observed for *S. vittatum* larvae exposed to organic UV-filter for 6 days might have compensate the energetic costs of increased synthesis of tGSH. In addition, exposure to these organic UV-filters did not altered AChE activity which is in accordance with previous results showing no neurotoxicity of these compounds (Campos et al., 2017).

The present study shows that environmental relevant concentrations of BP3 and 4-MBC can alter the energetic metabolism of *S. vittaum* larvae with reductions in feeding activity and decrease in carbohydrate levels probably related with detoxification processes (increase in tGSH levels). These two compounds showed similar toxicity, which has been previously observed with *C. riparius* (Campos et al., 2017). In fact, several studies have shown that exposure to environmental relevant concentrations of UV-filters can cause detrimental effects on feeding, growth or reproduction, in different aquatic organisms (Paredes et al., 2014; Schmitt et al., 2008; Sieratowicz et al., 2011).

The characterisation of toxicity of organic UV-filters and their environmental risk assessment should continue and also include relevant exposure scenarios of exposure to better predict their ecological effects in natural systems. Firstly, the suggested role of organic UV-filters as endocrine disruptors in invertebrates, call for research on potential multigenerational effects (Ozaéz et al., 2013, 2014).

Also, it is important to address effects of organic UV-filters when in mixtures with other chemical and abiotic stressors so as to evaluate the potential for synergistic or antagonistic effects (Ozaéz et al.,2016). As an example, it has been demonstrated that UV-filters can generate reactive oxygen species in the aqueous solution when exposed to ultraviolet radiation (Hanson et al., 2006; Inbaraj et al., 2002; Sánchez-Quiles and Tovar-Sánchez, 2014) and this should also be taken into account to better understand the ecological effects of UV-filters.

Furthermore, considering the high lipophilicity and stability of organic UV-filters, their bioaccumulation and potential magnification within food webs should also be considered (Kaiser et al., 2012).

Finally, our results come from laboratory single species assays and should be complemented with multispecies assays to encompass biotic interactions in the stress response (Campos et al., 2014; Pestana et al., 2009a). In this sense mesocosms experiments using invertebrate natural communities can be used to elucidate the direct and indirect effects of these emerging contaminants on ecosystem functioning (Pestana et al., 2009a).

References

Amine, H., Gomez, E., Halwani, J., Casellas, C., Fenet, H., 2012. UV filters, ethylhexyl methoxycinnamate, octocrylene and ethylhexyl dimethyl PABA from untreated wastewater in sediment from eastern Mediterranean river transition and coastal zones. Marine Pollution Bulletin 64, 2435-2442.

Arrese, E.L., Soulages, J.L., 2010. Insect fat body: energy, metabolism, and regulation. Annu Rev Entomol 55, 207-225.

ASTM, 1980. Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates and amphibians. American Standards for Testing and Materials, Philadelphia, P.A.

Baker, M.A., Cerniglia, G.J., Zaman, A., 1990. Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. Analytical Biochemistry 190, 360-365.

Balmer, M.E., Buser, H.-R., Müller, M.D., Poiger, T., 2005. Occurrence of some organic UV Filters in wastewater, in surface waters, and in fish from swiss lakes. Environmental Science & Technology 39, 953-962.

Bird, R.P., Draper, H.H., 1984. Comparative studies on different methods of malonaldehyde determination. Methods Enzymol 105, 299-305.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248-254.

Brausch, J.M., Rand, G.M., 2011. A review of personal care products in the aquatic environment: Environmental concentrations and toxicity. Chemosphere 82, 1518-1532.

Campos, D., Alves, A., Lemos, M.L., Correia, A., Soares, A.V.M., Pestana, J.T., 2014. Effects of cadmium and resource quality on freshwater detritus processing chains: a microcosm approach with two insect species. Ecotoxicology 23, 830-839.

Campos, D., Gravato, C., Quintaneiro, C., Koba, O., Randak, T., Soares, A.M., Pestana, J.L., 2016. Are insect repellents toxic to freshwater insects? A case study using caddisflies exposed to DEET. Chemosphere 149, 177-182.

Campos, D., Gravato, C., Quintaneiro, C., Golovko, O., Žlábek, V., Soares, A.M.V.M., Pestana, J.L.T., 2017. Toxicity of organic UV-filters to the aquatic midge Chironomus riparius. Ecotoxicology and Environmental Safety 143, 210-216.

Choi, J., Roche, H., Caquet, T., 2001. Hypoxia, hyperoxia and exposure to potassium dichromate or fenitrothion alter the energy metabolism in *Chironomus riparius* Mg. (Diptera: Chironomidae) larvae. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 130, 11-17.

Clairborne, A., 1985. Catalase activity in: Greenwald, R.A.E. (Ed.), Handbook of methods for oxygen radical research. CRC Press, Boca Raton, pp. 283-284.

De Coen, W.M., Janssen, C.R., 1997. The use of biomarkers in *Daphnia magna* toxicity testing. IV. Cellular Energy Allocation: a new methodology to assess the energy budget of toxicant-stressed Daphnia populations. Journal of Aquatic Ecosystem Stress and Recovery 6, 43-55.

De Coen, W.M., Janssen, C.R., 2003. The missing biomarker link: Relationships between effects on the cellular energy allocation biomarker of toxicant-stressed *Daphnia magna* and corresponding population characteristics. Environmental Toxicology and Chemistry 22, 1632-1641.

Díaz-Cruz, M.S., Llorca, M., Barceló, D., Barceló, D., 2008. Organic UV filters and their photodegradates, metabolites and disinfection by-products in the aquatic environment. TrAC Trends in Analytical Chemistry 27, 873-887.

Doyotte, A., Cossu, C., Jacquin, M.-C., Babut, M., Vasseur, P., 1997. Antioxidant enzymes, glutathione and lipid peroxidation as relevant biomarkers of experimental or field exposure in the gills and the digestive gland of the freshwater bivalve *Unio tumidus*. Aquatic Toxicology 39, 93-110.

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

Feio, M.J., Graça, M.A.S., 2000. Food consumption by the larvae of *Sericostoma vittatum* (Trichoptera), an endemic species from the Iberian Peninsula. Hydrobiologia 439, 7-11.

Gago-Ferrero, P., Díaz-Cruz, M.S., Barceló, D., 2011. Fast pressurized liquid extraction with in-cell purification and analysis by liquid chromatography tandem mass spectrometry for the determination of UV filters and their degradation products in sediments. Analytical and Bioanalytical Chemistry 400, 2195-2204.

Gago-Ferrero, P., Díaz-Cruz, M.S., Barceló, D., 2012. An overview of UV-absorbing compounds (organic UV filters) in aquatic biota. Analytical and Bioanalytical Chemistry 404, 2597-2610.

Gao, L., Yuan, T., Zhou, C., Cheng, P., Bai, Q., Ao, J., Wang, W., Zhang, H., 2013. Effects of four commonly used UV filters on the growth, cell viability and oxidative stress responses of the *Tetrahymena thermophila*. Chemosphere 93, 2507-2513.

Giokas, D.L., Sakkas, V.A., Albanis, T.A., Lampropoulou, D.A., 2005. Determination of UV-filter residues in bathing waters by liquid chromatography UV-diode array and gas chromatography–mass spectrometry after micelle mediated extraction-solvent back extraction. Journal of Chromatography A 1077, 19-27.

Golovko, O., Koba, O., Kodesova, R., Fedorova, G., Kumar, V., Grabic, R., 2016. Development of fast and robust multiresidual LC-MS/MS method for determination of pharmaceuticals in soils. Environmental Science and Pollution Research 23, 14068-14077.

Golovko, O., Kumar, V., Fedorova, G., Randak, T., Grabic, R., 2014. Removal and seasonal variability of selected analgesics/anti-inflammatory, anti-hypertensive/cardiovascular pharmaceuticals and UV filters in wastewater treatment plant. Environmental Science and Pollution Research 21, 7578-7585.

Guilhermino, L., Lopes, M.C., Carvalho, A.P., Soares, A.M.V.M., 1996. Acetylcholinesterase Activity in Juveniles of *Daphnia magna* Straus. Bulletin of Environmental Contamination and Toxicology 57, 979-985.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione-*S*-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 249, 7130-7139.

Hanson, K.M., Gratton, E., Bardeen, C.J., 2006. Sunscreen enhancement of UV-induced reactive oxygen species in the skin. Free Radical Biology and Medicine 41, 1205-1212.

Inbaraj, J.J., Bilski, P., Chignell, C.F., 2002. Photophysical and Photochemical Studies of 2-phenylbenzimidazole and UVB Sunscreen 2-phenylbenzimidazole-5-sulfonic Acid. Photochemistry and Photobiology 75, 107-116.

Jeon, H.-K., Chung, Y., Ryu, J.-C., 2006. Simultaneous determination of benzophenone-type UV filters in water and soil by gas chromatography–mass spectrometry. Journal of Chromatography A 1131, 192-202.

Kaiser, D., Sieratowicz, A., Zielke, H., Oetken, M., Hollert, H., Oehlmann, J., 2012. Ecotoxicological effect characterisation of widely used organic UV filters. Environmental Pollution 163, 84-90.

Kameda, Y., Kimura, K., Miyazaki, M., 2011. Occurrence and profiles of organic sun-blocking agents in surface waters and sediments in Japanese rivers and lakes. Environmental Pollution 159, 1570-1576.

Kuhara, N., Nakano, S., Miyasaka H., 2001. Alterations in the grazing activities of cased caddisfly larvae in response to variations in predation risk and resource level. Ecological Research 16, 705-714.

Kunz, P.Y., Fent, K., 2006. Multiple hormonal activities of UV filters and comparison of in vivo and in vitro estrogenic activity of ethyl-4-aminobenzoate in fish. Aquatic Toxicology 79, 305-324.

Kunz, P.Y., Gries, T., Fent, K., 2006. The Ultraviolet Filter 3-Benzylidene Camphor adversely affects reproduction in Fathead Minnow (*Pimephales promelas*). Toxicological Sciences 93, 311-321.

Liu, Y.-S., Ying, G.-G., Shareef, A., Kookana, R.S., 2012. Occurrence and removal of benzotriazoles and ultraviolet filters in a municipal wastewater treatment plant. Environmental Pollution 165, 225-232.

Liu, H., Sun, P., Liu, H., Yang, S., Wang, L., Wang, Z., 2015. Hepatic oxidative stress biomarker responses in freshwater fish *Carassius auratus* exposed to four benzophenone UV filters. Ecotoxicology and Environmental Safety 119, 116-122.

Lushchak, V.I., 2012. Glutathione homeostasis and functions: potential targets for medical interventions. J Amino Acids 2012, 736837.

Maltby, L., Clayton, S.A., Wood, R.M., McLoughlin, N. 2002. Evaluation of the *Gammarus pulex* in situ feeding assay as a biomonitor of water quality: Robustness, responsiveness, and relevance. Environ Toxicol Che 21, 361-368.

Mesquita, S.R., Guilhermino, L., Guimarães, L., 2011. Biochemical and locomotor responses of *Carcinus maenas* exposed to the serotonin reuptake inhibitor fluoxetine. Chemosphere 85, 967-976.

Moolman, L., Van Vuren, J.H.J., Wepener, V., 2007. Comparative studies on the uptake and effects of cadmium and zinc on the cellular energy allocation of two freshwater gastropods. Ecotoxicology and Environmental Safety 68, 443-450.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry 95, 351-358.

Ozáez, I., Martínez-Guitarte, J.L., Morcillo, G., 2013. Effects of in vivo exposure to UV filters (4-MBC, OMC, BP-3, 4-HB, OC, OD-PABA) on endocrine signaling genes in the insect *Chironomus riparius*. Science of The Total Environment 456–457, 120-126.

Ozáez, I., Martínez-Guitarte, J.L., Morcillo, G., 2014. The UV filter benzophenone 3 (BP-3) activates hormonal genes mimicking the action of ecdysone and alters embryo development in the insect *Chironomus riparius* (Diptera). Environmental Pollution 192, 19-26.

Ozáez, I., Morcillo, G., Martínez-Guitarte, J.-L., 2016. The effects of binary UV filter mixtures on the midge *Chironomus riparius*. Science of The Total Environment 556, 154-162.

Paredes, E., Perez, S., Rodil, R., Quintana, J.B., Beiras, R., 2014. Ecotoxicological evaluation of four UV filters using marine organisms from different trophic levels *Isochrysis galbana*, *Mytilus galloprovincialis*, *Paracentrotus lividus*, and *Siriella armata*. Chemosphere 104, 44-50.

Pedrouzo, M., Borrull, F., Marcé, R.M., Pocurull, E., 2011. Analytical methods for personal-care products in environmental waters. TrAC Trends in Analytical Chemistry 30, 749-760.

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

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

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

Pestana, J.L.T., Novais, S.C., Lemos, M.F.L., Soares, A.M.V.M., 2014. Cholinesterase activity in the caddisfly *Sericostoma vittatum*: Biochemical enzyme characterization and in vitro effects of insecticides and psychiatric drugs. Ecotoxicology and Environmental Safety 104, 263-268.

Pradhan, A., Silva, C. O., Pascoal, C., Cássio, F., 2016. Enzymatic biomarkers can portray nanoCuO-induced oxidative and neuronal stress in freshwater shredders. Aquatic Toxicology 180, 227-235.

Plagellat, C., Kupper, T., Furrer, R., de Alencastro, L.F., Grandjean, D., Tarradellas, J., 2006. Concentrations and specific loads of UV filters in sewage sludge originating from a monitoring network in Switzerland. Chemosphere 62, 915-925.

Ramos, S., Homem, V., Alves, A., Santos, L., 2015. Advances in analytical methods and occurrence of organic UV-filters in the environment — A review. Science of The Total Environment 526, 278-311.

Rodil, R., Moeder, M., 2008. Development of a simultaneous pressurised-liquid extraction and clean-up procedure for the determination of UV filters in sediments. Analytica Chimica Acta 612, 152-159.

Rodil, R., Quintana, J.B., López-Mahía, P., Muniategui-Lorenzo, S., Prada-Rodríguez, D., 2008. Multiclass Determination of Sunscreen chemicals in water samples by Liquid Chromatography−Tandem Mass Spectrometry. Analytical Chemistry 80, 1307-1315.

Rodil, R., Schrader, S., Moeder, M., 2009. Non-porous membrane-assisted liquid-liquid extraction of UV filter compounds from water samples. Journal of chromatography. A 1216, 4887-4894.

Rodrigues, A.C.M., Gravato, C., Quintaneiro, C., Golovko, O., Žlábek, V., Barata, C., Soares, A.M.V.M., Pestana, J.L.T., 2015. Life history and biochemical effects of chlorantraniliprole on *Chironomus riparius*. Science of The Total Environment 508, 506-513.

Rodrigues A.C.M., Gravato C., Quintaneiro C., Bordalo M.D., Golovko O., Žlábek V., Barata, C., Soares, A.M.V.M., Pestana, J.L.T. 2016. Exposure to chlorantraniliprole affects the energy metabolism of the caddisfly *Sericostoma vittatum*. Environ Toxicol Che 9999, 1-8.

Román, I.P., Chisvert, A., Canals, A., 2011. Dispersive solid-phase extraction based on oleic acid-coated magnetic nanoparticles followed by gas chromatography–mass spectrometry for UV-filter determination in water samples. Journal of Chromatography A 1218, 2467-2475.

Sánchez-Quiles, D., Tovar-Sánchez, A., 2014. Sunscreens as a Source of Hydrogen Peroxide Production in Coastal Waters. Environmental Science & Technology 48, 9037-9042.

Sánchez-Quiles, D., Tovar-Sánchez, A., 2015. Are sunscreens a new environmental risk associated with coastal tourism? Environment International 83, 158-170.

Schmitt, C., Oetken, M., Dittberner, O., Wagner, M., Oehlmann, J., 2008. Endocrine modulation and toxic effects of two commonly used UV screens on the aquatic invertebrates *Potamopyrgus antipodarum* and *Lumbriculus variegatus*. Environmental Pollution 152, 322-329.

Sen, C.K., 1997. Nutritional biochemistry of cellular glutathione. The Journal of Nutritional Biochemistry 8, 660-672.

Sieratowicz, A., Kaiser, D., Behr, M., Oetken, M., Oehlmann, J., 2011. Acute and chronic toxicity of four frequently used UV filter substances for *Desmodesmus subspicatus* and *Daphnia magna*. J Environ Sci Health A Tox Hazard Subst Environ Eng 46, 1311-1319.

Sokolova, I.M., 2013. Energy-limited tolerance to stress as a conceptual framework to integrate the effects of multiple stressors. Integr Comp Biol 53, 597-608.

Sokolova, I.M., Frederich, M., Bagwe, R., Lannig, G., Sukhotin, A.A., 2012. Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. Mar Environ Res 79, 1-15.

Tsui, M.M.P., Leung, H.W., Wai, T.-C., Yamashita, N., Taniyasu, S., Liu, W., Lam, P.K.S., Murphy, M.B., 2014. Occurrence, distribution and ecological risk assessment of multiple classes of UV filters in surface waters from different countries. Water Research 67, 55-65.

Wang, J., Pan, L., Wu, S., Lu, L., Xu, Y., Zhu, Y., Guo, M., Zhuang, S., 2016. Recent Advances on Endocrine Disrupting Effects of UV Filters. International journal of environmental research and public health 13 (8).

Xuereb, B., Lefèvre, E., Garric, J., Geffard, O., 2009. Acetylcholinesterase activity in *Gammarus fossarum* (Crustacea Amphipoda): Linking AChE inhibition and behavioural alteration. Aquatic Toxicology 94, 114-122.

Supplementary data

Table SD 1- MS/MS parameters for triple quadrupole detection of targeted compounds:

Compound	*Mode*	*Quantification transition*	*Confirmation transition*	*Tube Lens voltage (V)*	*Collision energy*	*Retention time (min)*
BP3	+	229.062→ 150.670	229.062→ 105.200	91	18	9.44
4-MBC	+	255.124→ 104.780	255.124→ 164.810	94	30	10.12
Diclofenac	+	296.000→ 214.010	*	86	33	9.13

*No confirmation ion.

Table SD 2- LC gradient for the elution of targeted compounds, Cogent Bidentate C18 column.

Time, min	*Mobile phase composition*		*Flow rate, µL/min*
	Water (0.1 % FA)	Acetonitrile (0.1 % FA)	
0.00	100	0	300
1.00	100	0	300
7.00	60	40	350
9.00	0	100	400
10.00	0	100	400
10.01	100	0	300
13.00	100	0	300

Chapter 4:

Toxicity assessment of binary mixtures of BP3 with 4-MBC (UV-filters), and BP3 with DEET (insect repellent) using the aquatic midge *Chironomus riparius*

Toxicity assessment of binary mixtures of BP3 with 4-MBC (UV-filters), and BP3 with DEET (insect repellent) using the aquatic midge *Chironomus riparius*

Abstract

To increase protection against ultraviolet radiation, personal care products have diverse organic ultraviolet filters (UV-filters) in their composition. Some products also add insect repellents to reduce mosquito's bites. Consequently, these compounds reach freshwaters and the aquatic organisms are exposed to a cocktail of contaminants. In this study, the joint effects of two UV-filters (Benzophenone – 3 (BP3) and 3-(4-methylbenzylidene) camphor) and of BP3 combined with an insect repellent (N, N diethyl- 3-methylbenzamide – DEET) were evaluated using life history traits of the aquatic midge *Chironomus riparius* such as emergence rate, development time and imagoes body weight. A synergistic interaction between BP3 and 4-MBC was only found for *C. riparius* emergence rate while no interaction was observed for the other endpoints. Concerning the effects of BP3 and DEET mixture our analysis suggests synergism in the case of males but antagonism in the case of females' development time. Our results imply that effects of UV-filters present in sediments within chemical mixtures are complex and that the evaluation of effects using different life-history traits can yield different patterns of responses. These results also indicate that individual chemical testing can underestimate toxicity of organic UV-filters.

Key-words: combined effects; personal care products; generalized linear models; aquatic insects

1. Introduction

Organic ultraviolet filters (UV-filters) are present in several personal care products (PCPs) (e.g. sunscreens and cosmetics) and other materials (e.g. plastics and paints) to prevent damage/injuries caused by ultraviolet radiation (Díaz-Cruz et al., 2008). These compounds are being increasingly detected in different environmental matrices around the world, such as surface and marine waters, effluents and influents and also in sediments (Ramos et al., 2015). Their presence in these ecosystems is attributed to direct inputs (recreational activities and beachgoers) but mainly to the inefficient removal during wastewater treatment plants (Brausch and Rand, 2011; Giokas et al., 2005; Golovko et al., 2014).

Previous studies showed that UV-filters can affect cell growth, feeding, development time and reproduction of several aquatic organisms, and also have the potential to bioaccumulate in fish tissues (Campos et al., 2017a; Campos et al., 2017b; Langford et al., 2015; Paredes et al., 2014; Schmitt et al., 2008). Nevertheless, these studies have mainly focused on single chemical exposure using standardized testing protocols whereas commercial sunscreens have in their composition a combination of different UV-filters to improve adequate protection against UV-radiation (Ozáez et al., 2016b; Ramos et al., 2015). Besides, other compounds such as insect repellents are also used simultaneously with UV-filters and thus a cocktail of PCPs may reach the aquatic environment (Kameda et al., 2011; Kasichayanula et al., 2007; Ramos et al., 2016; Tsui et al., 2014). As a consequence, macroinvertebrate communities living in aquatic ecosystems contaminated by such compounds are simultaneously exposed to different xenobiotics, which calls for an assessment of their combined effects to better represent realistic scenarios of exposure and better support environmental risk assessment strategies (Backhaus and Faust, 2012).

In the present study, we evaluated the combined effects of two organic UV-filters and one UV-filter with an insect repellent, on life-history traits of the aquatic midge *Chironomus riparius* (Meigen). BP3 and 4-MBC were chosen as model UV-filters since they are two of the most detected UV-filters in aquatic environment. Additionally, previous studies showed that BP3 and 4-MBC act as endocrine disruptors (Coronado et al., 2008; Ozáez et al., 2013; Wang et al., 2016) and research has shown that both compounds impair the growth and the development of *C. riparius* (Campos et al., 2017b). Considering that BP3 and the insect repellent DEET (N, N diethyl- 3-methylbenzamide) are essential active ingredients used in commercial sunscreens and in

mosquito repellent preparations, they might be used simultaneously by humans (Fediuk et al., 2012; Kasichayanula et al., 2007) and might be present at the same time in many freshwaters.

Taking into consideration the physic-chemical properties of UV-filters, specially their low water solubility and accumulation in sediments, *C. riparius* was chosen as a test species since they spend most part of their short life-cycle in contact with sediment. Moreover, this non-biting midge plays an important role in detritus processing of organic matter and serve as food to other macroinvertebrates and fishes. Thus, the main objective of this study was to assess the toxicity of BP3 - 4-MBC and BP3 - DEET binary mixtures, by measuring three relevant reproductive endpoints in *C. riparius*: emergence rate, development time and imagoes body weight.

2. Material and Methods

2.1 Test Organisms

For all tests, *C. riparius* egg ropes were obtained from a culture established in University of Aveiro. The organisms were cultured in plastic aquariums in American Society for Testing Materials (ASTM) hard water medium (ASTM, 1980), inside plastic aquariums with fine sediment (< 1mm) previously burned at 500 ºC during 4h and with aeration. Organisms were maintained at 20 ± 2 ºC, with a photoperiod of 16:8 h light: dark. Moreover, organisms were fed with a suspension of macerated Tetramin® (Germany), three times a week and the medium changed every week.

2.2 Chemical compounds and sediment composition

Benzophenone 3 (BP3; 2-hydroxy-4-methoxybenzophenone; CAS No. 131-57-7; purity ≥ 98%), and 3-(4-methylbenzylidene) camphor (4-MBC; CAS No. 36861-47-9, purity ≥ 98%) were obtained from Sigma-Aldrich (Portugal) and the insect repellent, N, N diethyl- 3-methylbenzamide (DEET; CAS No. 134-62-3, purity ≥ 97%) were obtained from Sigma-Aldrich (Germany). The physic-chemical properties of each compound are presented in table 1.

Artificial sediment was composed by <1 mm inorganic fine sediment (previously burned during 4h at 500ºC) (75%), kaolin (20%), α-cellulose (5%) and calcium carbonate (0.1%) (OECD, 2004a).

Table 1- Physico-chemical properties of BP3, 4-MBC and DEET.

Compounds	Formula	Abbreviation	CAS No	Molecular weight (g/mol)	Log K_{ow}	Water solubility (g/L)
2-hydroxy-4-methoxybenzophenone (Benzophenone-3)	$C_{14}H_{12}O_3$	BP3	131-57-7	228.24	3.79	0.10
3-(4-methylbenzylidene) camphor	$C_{18}H_{22}O$	4-MBC	36861-47-9	254.37	4.95	0.017
N, N diethyl- 3-methylbenzamide	$C_{12}H_{17}NO$	DEET	134-62-3	191.27	2.20	>1.0

Based on Aronson et al.(2012) and Gago-Ferrero et al. (2012)

2.3 Chemical dosing and experimental design

Different dosing procedures were used according to the Log K_{ow} and solubility of each compound. Considering that BP3 and 4-MBC have a log K_{ow} > 3 and low water solubility, the sediment was dosed. On the other hand, since DEET has a log K_{ow} < 3, the medium (ASTM) was dosed. The treatments were selected according to previous experiments and considering concentrations reported in the literature for the aquatic environment. A full factorial design was used in both binary mixtures (figure 1).

BP3 and 4-MBC stock and experimental solutions (6, 12, 24 mg/Kg) were prepared in ethanol (96%). The artificial sediment (50 g dry weight (dw)) was then contaminated with 10 mL of respective UV-filters solution and their mixtures and were left to evaporate during 72h. Also, 10 mL of 96% ethanol were added to the sediment to perform solvent control. After 72h of evaporation, 10 mL of ASTM (to formulate the final composition of sediment) were added in all treatments including solvent control (0+) and mixed until a homogeneous sediment paste was achieved. Finally, 150 mL of ASTM medium were also added and the sediment was left to equilibrate for 48h.

In the test addressing combined effects of BP3 and DEET, BP3 was spiked into the sediment (see above) whereas DEET stock solution was prepared in ASTM. DEET experimental solutions (40, 200, 1000 µg/L) were prepared by diluting the stock solution in ASTM.

A full factorial design experiment was performed, consisting of the simultaneous testing of three single chemical concentrations, nine mixture ratios and a solvent control (figure 1). The exposure was performed according to the OECD guideline (OECD, 2004b). Five replicates with a pool of five larvae with less than 24 h post hatching (1st instar) were used in all treatments. The

test was conducted at 20 ± 1ºC with a photoperiod of 16:8 light-dark. During 28 days, organisms were fed every two days with a suspension of macerated Tetramin® (0.25 mg per organism per day provided until day 9 and 0.5 mg henceforth, as older organisms consume more). The endpoints assessed were emergence rate, development time of males and females and imagoes body weight of males and females. For that, emergence was checked daily (between 14 and 28 day) and imagoes were collected with an aspirator and kept in 70% ethanol. Imagoes were dried for 24h at 50ºC and weighted in a microbalance (RAWAG MYA 2.3Y).

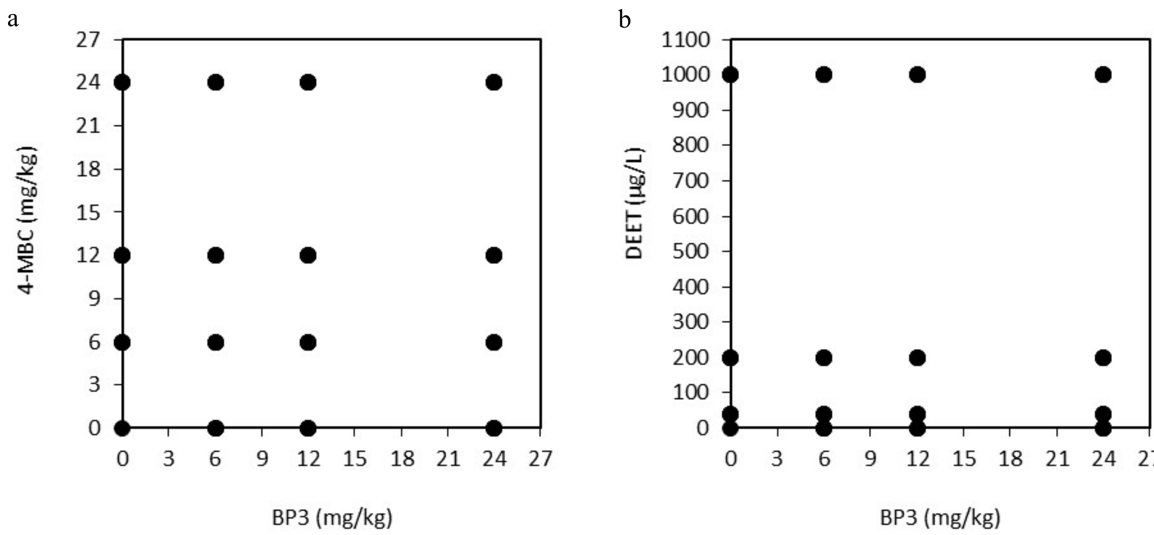


Figure 1 – Full factorial design of the mixture experiments with three concentrations of a) BP3 - 4-MBC and b) BP3 - DEET.

2.4 Statistical analysis

A combined approach, including statistical modelling and the reference mixture toxicity model of independent action (IA), was followed to evaluate the effects of the tested binary mixtures. Whereas statistical modelling was used to detect the significance of chemical interactions, the IA model provided a mixture toxicity theoretical framework to infer the direction of the interaction between predictors (synergism or antagonism).

First, a Generalized Linear Model (GLM) with a binomial distribution and logit link function was applied on emergence rate of organisms and a GLM with a Gaussian distribution and identity link function was applied on development time and imagoes body weight (males and females). Model selection followed a backward stepwise approach based on the Akaike´s Information

Criterion (AIC). We started with the full model and then removed the non-significant terms to test which factor were necessary in the minimal adequate model. The best model (with lower AIC) was calculated by the difference between the AIC (ΔAIC). GLM were previously used to assess the combined toxicity of metals and pesticides (Iwasaki and Brinkman, 2015; Morgado et al., 2016). Development time of females and imagoes body weight in the BP3 - 4-MBC experiment and development time of males in the BP3 - DEET experiment were log transformed since data did not follow a normal distribution. These analyses were performed using R studio (version 1.0.136, 2016).

Whenever the minimal adequate model chosen included an interaction term, deviations from additivity (i.e. synergistic/ antagonistic effects) of mixtures were then evaluated comparing observed responses to predicted responses given by IA reference model considering the unaffected proportion. The IA mathematical model considers the observed responses of single compounds and calculates their predicted effects in mixtures using probability statistics. Since our data are continuous variables (emergence rate, development time and imagoes body weight) the probability of nonresponse to the chemicals was calculated according to the following equation:

$$mixture\ toxicity\ (q1 \ldots\ldots, qn) = max \prod_{i=1}^{n} qi\ (Ci)$$

where *max* is the maximum value observed and *qi*(*Ci*) is the probability of nonresponse at concentration *c* of the toxicant *i* (Martin et al., 2009). The deviations to additivity were evaluated after calculation of the confidence intervals (α= 0.05).

3. Results

3.1 Effects of BP3, 4-MBC and of their mixture on *C. riparius*

Exposure to organic UV-filters had clear effects on *C. riparius* reproductive traits. The exposure to BP3 and 4-MBC reduced the emergence rate of *C. riparius*, particularly BP3 whose highest concentration tested led to total absence of emerged imagoes (figure 2, table 2). Exposure to 4-MBC delayed the development time of both *C. riparius* male and female, (figure 3, table 2). Regarding BP3, sub-lethal endpoints were restricted to two concentrations due to absence of emergence, which eventually limited our ability to detect effects. However, a delay in development time was still observed for *C. riparius* female (figure 3b, table 2). The UV-filter 4-MBC also reduced the weight of *C. riparius* imagoes at emergence with stronger effects observed in the case of females (figure 4, table 2).

Concerning the effects of the combined exposure to both UV-filters our results show that exposure to sediments contaminated with a mixture of BP3 and 4-MBC can induce stronger effects on *C. riparius* emergence rates than anticipated by considering each chemical independently (figure 2; table 2). In fact, the minimal adequate GLM model (i.e. the model with the lowest AIC value) for emergence rate included an interaction term (table 1SM), as it proved to improve the goodness of the fit. The interaction regression coefficient was negative, suggesting a synergistic effect on emergence rate for binary mixtures of BP3 and 4-MBC. This pattern was further confirmed by the IA model, whose predictions for *C. riparius* emergence rates consistently exceeded our observed results considerably (figure 2).

Contrary to the effects on emergence rates, there seems to be no departure from additivity for development time and imagoes weight since none of the most adequate models included the interaction term (table 1 SM). In fact, male development time and male imagoes weight was best explained by 4-MBC alone (figure 3a, 4a; table 2) while the predictors “BP3” and “4-MBC” were presented in the most parsimonious model in the case of female imagoes traits (figure 3b, 4b; table 2).

Table 2 - Summary of the results for Generalized Linear Model for the effect of BP3 and 4-MBC on *C. riparius* emergence rate, development time and imagoes weight. Only best minimal adequate models, i.e. model with lowest AIC, are presented.

Emergence rate	**β**	**Standard error**	**z value**	***p*-value**
BP3	-0.092	0.021	-4.330	1.49×10^{-5}
4-MBC	-0.037	0.016	-2.266	0.023
BP3:4-MBC	-0.004	0.002	-1.698	0.090
Development time (males)				
4-MBC	0.127	0.030	4.270	0.0001
Development time (females)				
BP3	0.005	0.003	1.885	0.066
4-MBC	0.004	0.002	2.411	0.020
Imagoes weight (males)				
4-MBC	-0.003	0.003	-1.312	0.197
Imagoes weight (females)				
BP3	-0.008	0.004	-1.85	0.075
4-MBC	-0.013	0.002	-5.35	3.66×10^{-6}

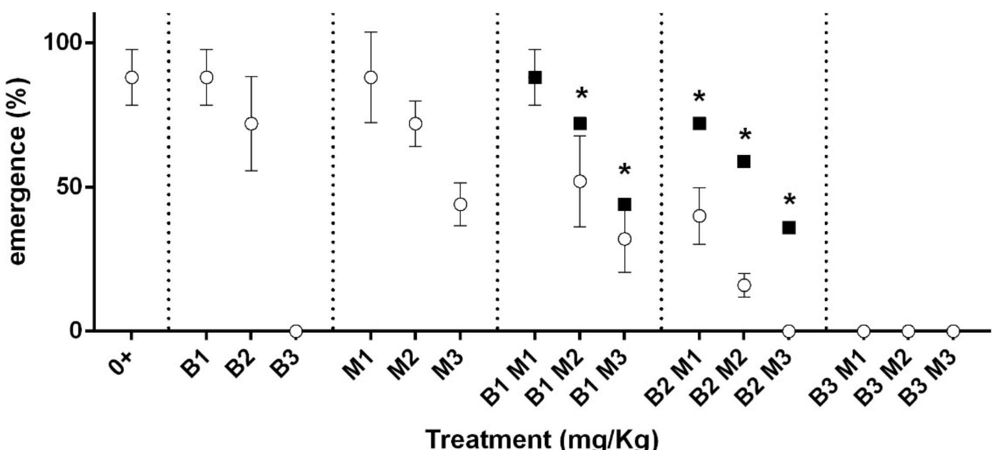
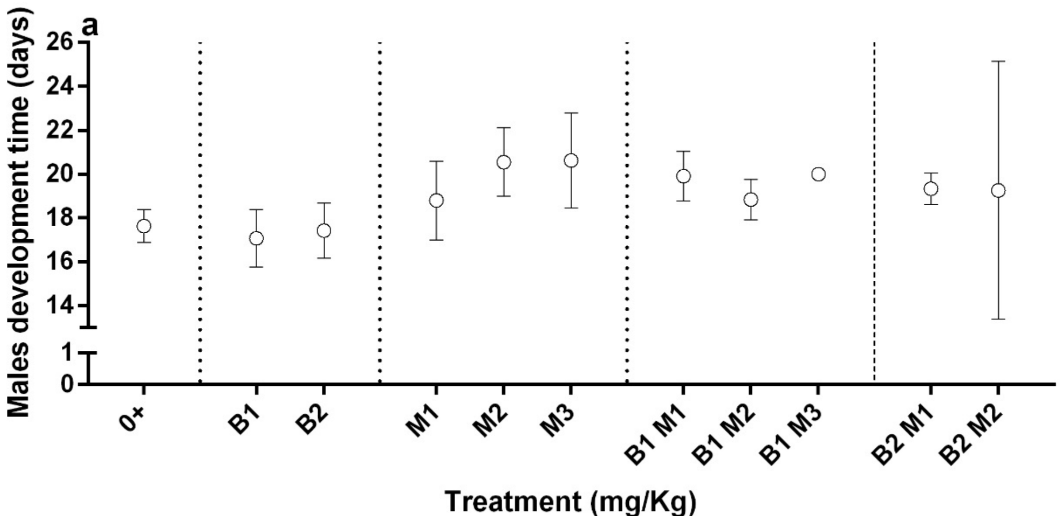


Figure 2 - Effects of BP3 (B), 4-MBC (M) and their binary mixture on *C. riparius* emergence rate. Empty symbols denote observed responses (mean ± CI) and filled symbols represent effects predicted by independent action reference model. * denotes values predicted by the independent action model that were significantly different from the observed values (i. e. were outside the confidence intervals (95%)).



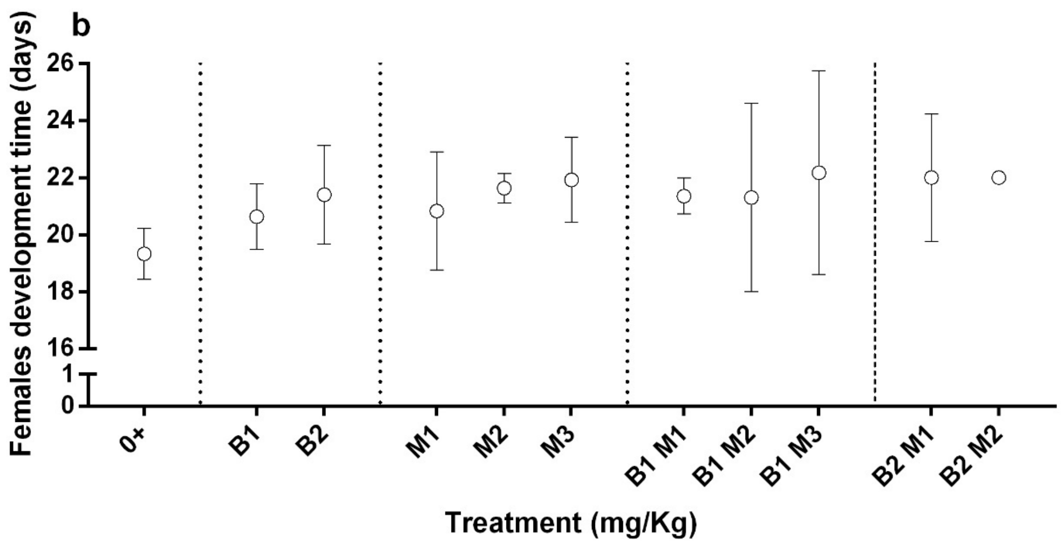
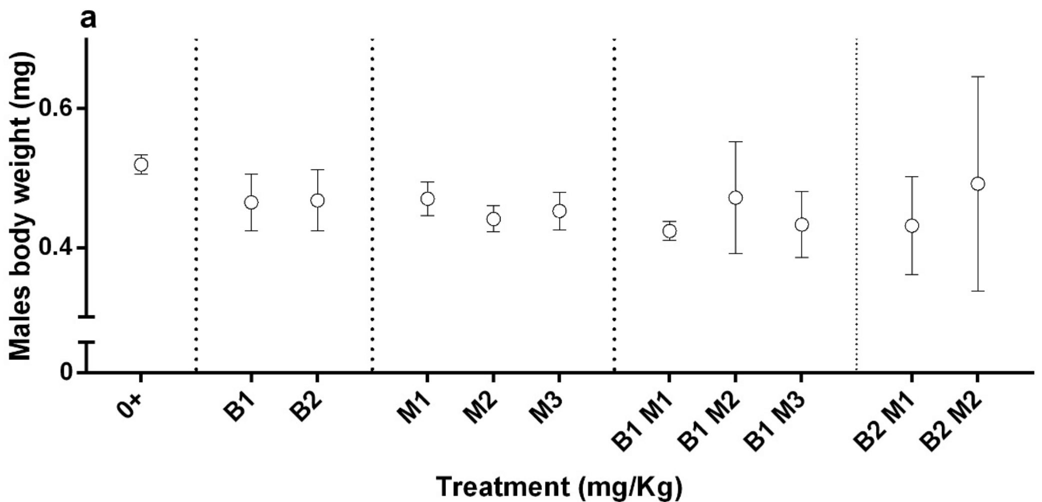


Figure 3 - Effects of BP3 (B), 4-MBC (M) and their binary mixture on *C. riparius* development time of a) males and b) females, (mean ± CI).



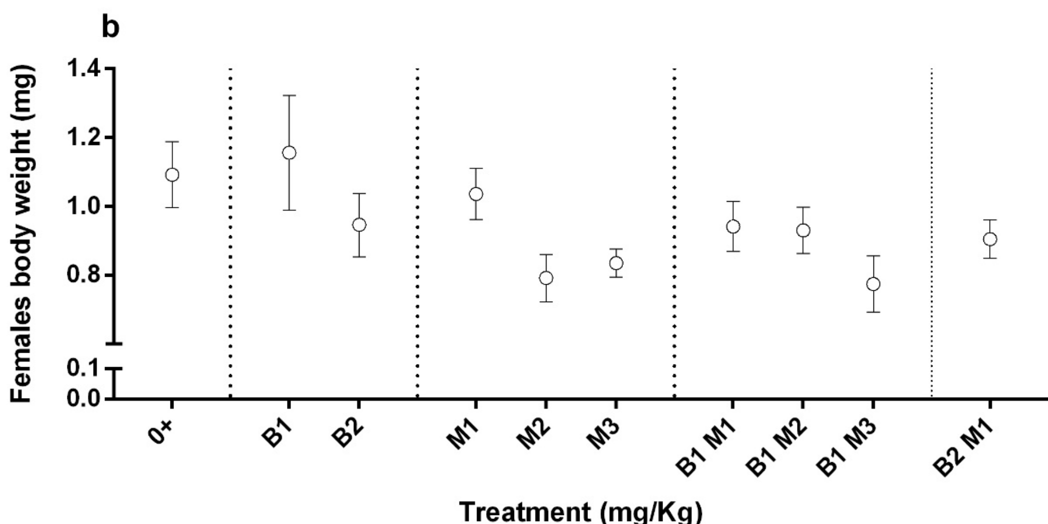


Figure 4 - Effects of BP3 (B), 4-MBC (M) and their binary mixture on *C. riparius* body weight of a) males and b) females (mean ± CI).

3.2 Effects of BP3, DEET and of their mixture on *C. riparius*

Exposure to the selected DEET concentrations did not elicited any effects in the emergence rate of *C. riparius* (figure 5, table 3). However, delayed emergence was observed for *C. riparius* female imagoes exposed to DEET (figure 6b), as well as a slight reduction of imagoes weight in males and females (figure 7a, b).

Relatively to effects of the combined exposure to BP3 and DEET, our results did not show any altered effect of BP3 on emergence rate caused by the presence of DEET (figure 5; table 3). However, GLM analysis revealed that, for both genders, the effects of BP3 on development time

were altered by DEET since the most parsimonious models for each of them included interaction (figure 6; table 3 and table 2SM). In fact, our results showed that DEET influences' BP3 effects on development time in opposite directions for females and males. Regarding males (figure 6a), the interaction regression coefficient was positive which indicates that higher-than-additive effects occur in development time when BP3 and DEET are combined (table 3; table 2SM). On the other hand, the negative interaction regression value found for females (figure 6b) implies that BP3 effect on female development time is decreased when combined with DEET, leading to lower-than-additive effects (table 3; table 2SM). These contrasting mixture effects were also supported by deviations to the IA model. For male *C. riparius*, observed development time was higher than predicted by the IA model. Regarding females, the IA-predicted development time was higher than our experimental observation, which indicates antagonistic effects. GLM analysis revealed that both BP3 and DEET reduced the weight of *C. riparius* imagoes, regardless of the gender, but no departure from additivity was identified for the mixture since the minimal adequate models for either males and females did not include interaction term (figure 7a and b; table 3 and table 2SM).

Table 3. Summary of the results for Generalized Linear Model for the effect of BP3 and DEET on *C. riparius* emergence rate, development time and imagoes weight. Only best minimal adequate models, i.e. model with lowest AIC, are presented.

Emergence rate	**β**	**Standard error**	**z value**	***p*-value**
BP3	-0.083	0.012	-7.025	2.15×10^{-12}

Development time (males)	**β**	**Standard error**	**z value**	***p*-value**
BP3	4.52×10^{-3}	2.32×10^{-3}	1.943	0.057
DEET	2.25×10^{-5}	3.26×10^{-5}	0.691	0.493
BP3: DEET	1.17×10^{-5}	4.31×10^{-6}	2.723	0.009

Development time (females)	**β**	**Standard error**	**z value**	***p*-value**
BP3	1.20×10^{-1}	3.38×10^{-2}	3.552	0.0008
DEET	1.66×10^{-3}	6.74×10^{-4}	2.465	0.0167
BP3: DEET	-1.05×10^{-4}	6.90×10^{-5}	-1.517	0.135

Imagoes weight (males)	**β**	**Standard error**	**z value**	***p*-value**
BP3	$- 5.05 \times 10^{-3}$	1.29×10^{-3}	-3.920	0.0003
DEET	$- 5.93 \times 10^{-5}$	1.49×10^{-5}	-3.979	0.0002

Imagoes weight (females)	**β**	**Standard error**	**z value**	***p*-value**
BP3	-7.603×10^{-3}	2.027×10^{-3}	-3.752	0.0004
DEET	$-1.216e \times 10^{-4}$	3.559×10^{-5}	-3.412	0.001

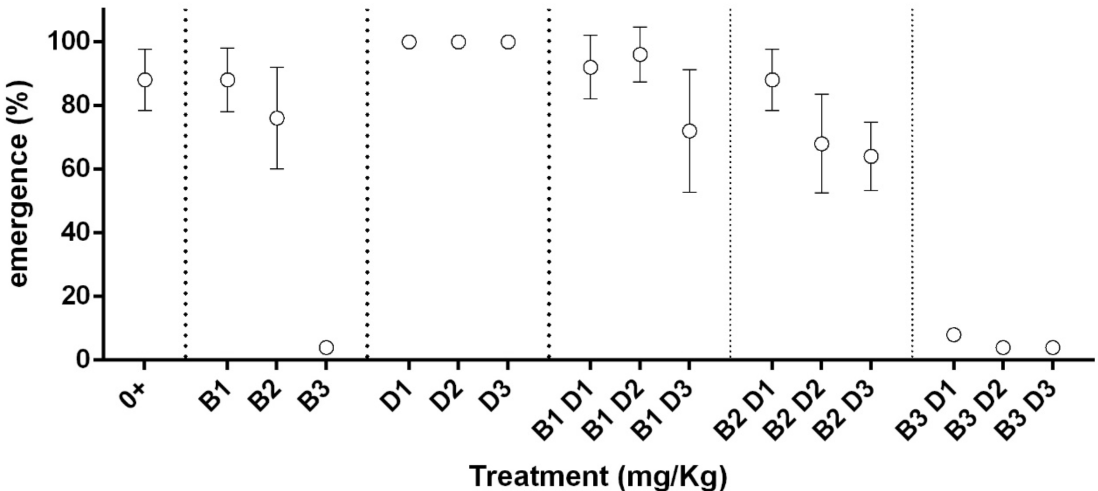
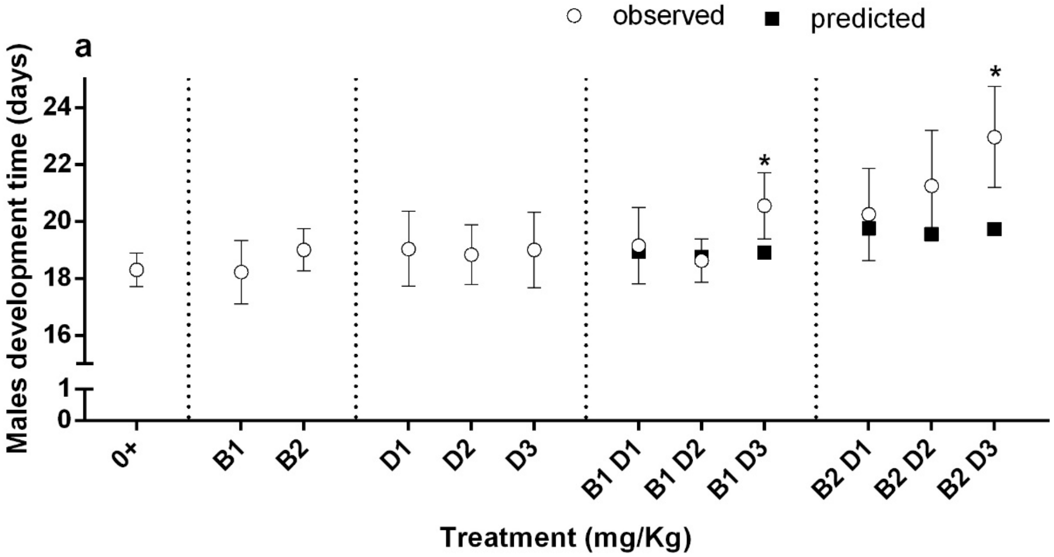


Figure 5 - Effects of BP3 (B), DEET (D) and their binary mixture on *C. riparius* emergence rate (mean ± CI).



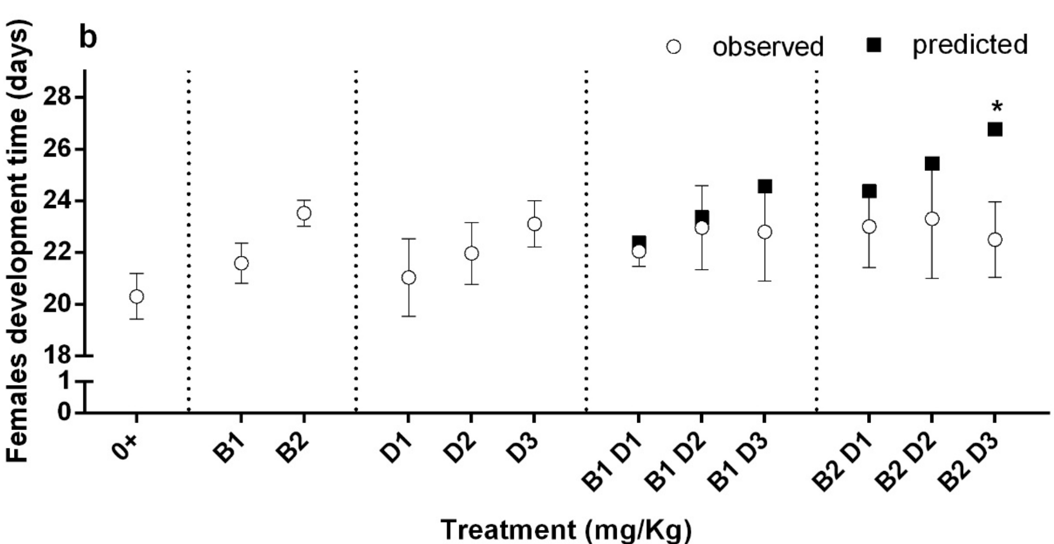
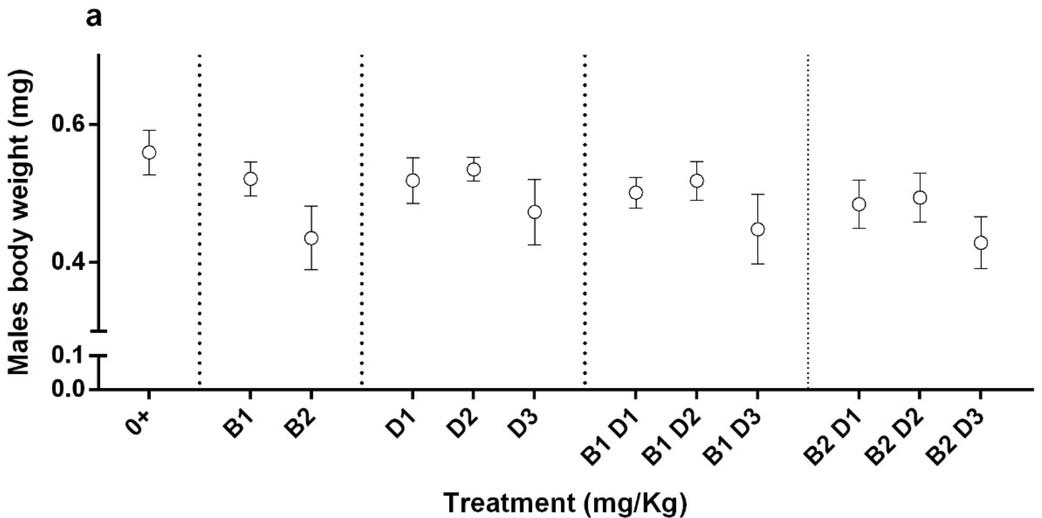


Figure 6 - Effects of BP3 (B), DEET (D) and their binary mixture on *C. riparius* development time of a) males and b) females. Empty symbols denote observed responses (mean ± CI) and filled symbols represent effects predicted by independent action reference model. * denotes values predicted by the independent action model that were significantly different from the observed values (i. e. were outside the confidence intervals (95%)).



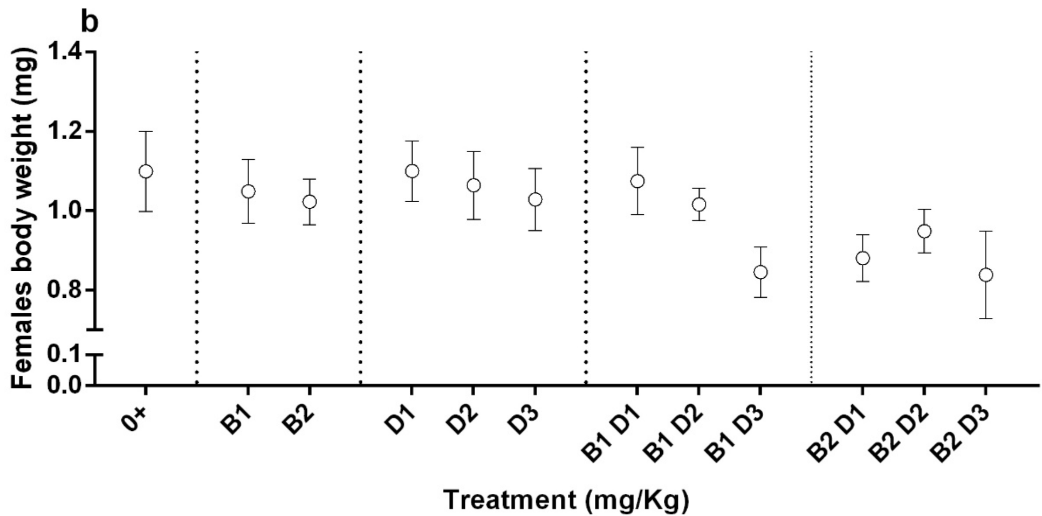


Figure 7 - Effects of BP3 (B), DEET (D) and their binary mixture on *C. riparius* body weight of a) males and b) females (mean ± CI).

4. Discussion

Organic UV-filters are emerging and persistent contaminants of freshwaters sediments where they co-occur with a variety of different compounds. This study showed evidences of greater than additive toxic effects of a binary mixture of two different organic UV-filters towards reproductive

traits on the midge *C. riparius.* Moreover, non-additive effects were also observed in response to the binary mixture containing BP3 and the insect repellent DEET.

Despite only few studies have been directly investigating the toxicity of chemical mixtures containing organic UV-filters, it has already been shown that toxicity of organic UV-filters is altered when in mixtures (Molins-Delgado et al., 2016; Ozáez et al., 2016b; Park et al., 2017). Results obtained by Ozáez et al., (2016b) suggested an antagonistic interaction after 96h of exposure to the binary mixture of 4-MBC and BP3 in terms of survival and in the ecdysone receptor gene expression in *C. riparius*. Likewise, exposure to binary and ternary mixtures of ethylhexyl methoxycinnamate, avobenzone and octocrylene showed reduced toxicity towards *Daphnia magna,* compared to single exposures (Park et al., 2017). Adding to this, our results showed that the toxicity of mixtures containing UV-filters can vary considerably concerning the different endpoints analysed. In fact, synergism was found for emergence rate of *C. riparius* larvae exposed to the mixture of BP3 and 4-MBC but the same was not observed for development time or imagoes body weight. Contrarily, non-additive effects were found for development time in *C. riparius* exposed to a mixture containing DEET and BP3, but no interaction was found for emergence rate or imagoes body weight. These differences illustrate the complexity of the physiological processes governing the combined effects of UV-filters in a holometabolous insect, whose metamorphosis is an endocrine controlled process. The effects of UV-filters on emergence are complex and can be due to multiple carry-over effects of larval exposure (i.e. effects on larvae development, feeding, energy requirements for detoxification). These differences in the joint toxicity in relation to different endpoints are common as showed in different studies (Ozáez et al., 2016b; Pavlaki et al., 2011; Silva et al., 2015) and can be associated with the susceptibility of the physiological processes affecting each endpoint (Cedergreen and Streibig, 2005).

The mode of action of UV-filters in invertebrates is not clear and thus explanation for such discrepancies in the toxicity of mixtures containing UV-filters are not straightforward. However, by acting as endocrine disruptors, UV-filters can affect reproductive traits, through effects in hormonal processes controlling pupal stage and the onset of metamorphosis. For instance, it has been shown that at the organismal level, BP3 and 4-MBC (single exposures) increase the development time of *C. riparius* females and reduce the imagoes body weigh of males (Campos et al., 2017b). Additionally, at the molecular level, 4-MBC induces the transcription of ecdysone receptor of *C. riparius* and increases the expression of the methoprene-tolerant gene (Met). Moreover, BP3 can induce activation of ecdysone receptor in *C. riparius*, by mimicking the hormone 20-hydroxyecdysone (Ozáez et al., 2013; Ozáez et al., 2014, Ozáez et al., 2016a). Thus,

the synergistic effects observed in our study for emergence rates might be related with endocrine disruption, namely with alterations in the levels of ecdysone and juvenile hormone. Both hormones have an important role in the endocrine pathways and the concentrations of these two hormones in insects mediate the development transitions, namelly regulate molting, metamorphosis and reproduction of insects (Johnson et al., 2014; Planelló et al., 2015; Ozáez et al., 2016a). Kunz and Fent (2006) also observed synergism to the exposure of UV-filters in a recombinant yeast with the human estrogen receptor alpha (hERα). The authors pointed out that this synergism might be related with changes in metabolic pathways, namely by the activation and co-activation of the hERα (Kunz and Fent, 2006).

The toxicity of UV-filters (single exposures) to *C. riparius* is also related with detoxification mechanisms. At similar concentrations, although neurotoxicity has not been observed, the exposure of *C. riparius* to BP3 and 4-MBC led to an increase in the energy consumption (increase in electron transport system) (Campos et al., 2017b). Moreover, in *C. riparius* the increase in the expression of Heat shock protein 70 (hsp70) gene and its activation were observed due to exposure to 4-MBC (Ozáez et al., 2016b) and to BP3, respectively (Ozáez et al., 2014). Additionally, Ozáez et al (2016b) observed that exposure to *C. riparius* to the binary mixture of BP3 and 4-MBC increased the expression of the *hsp70* gene comparatively to single exposure to both UV-filters. Therefore, the exposure of UV-filters in mixture might cause higher stress in the cell which can be another explanation to the observed synergism. The hsp70 are somehow associated with hormonal pathways as well, namely playing a role in the signalling of steroid hormone in cells (Echeverria and Picard, 2010), which might also contribute to the endocrine disruption.

At last, another possible explanation to the observed synergism can be related with the bioaccumulation of the UV-filters when in mixture. Possibly, UV-filters may bioaccumulate or cling to the *C. riparius* and somehow cause stronger effects. In fact, although in the presence of antagonism, Park et al. (2017) observed that UV-filters exhibit different behaviour when in mixture. The authors tested three different binary mixtures of UV-filters and observed that UV-filters could either clung to the body of *D. magna*, accumulate in the gut or accumulate in thoracic appendages. Different toxicity patterns resulting from different binary mixtures with UV-filters can therefore be related to the bioavailability, to different uptake and elimination kinetics and to different pathways inside the organisms' body.

It must be referred, however, that none of the above-mentioned explanations is mutually exclusive and a complex combination of factors must be considered to understand the effects of mixtures containing UV-filters for a certain organism, under certain conditions, at a certain time

point, a considering a certain endpoint. Ozáez et al. (2016b) suggests that the effects of UV-filters in mixtures are probably a result of the balance between their actions in different hormonal receptors. The same authors also suggested that the effect caused in terms of the endocrine system together with the effects on heat shock proteins gene expression could be indicative of different modes of action of BP3 and 4-MBC in *C. riparius* which can increase the toxicity of UV-filters when in mixture.

Our results also provide evidences that effects of organic UV-filters can be altered by the presence of other chemicals. The combination of BP3 with DEET induced non-additive and gender-dependent effects in terms of *C. riparius* development time, leading to greater-than-additive effects in males and lower-than-additive effects in females. These results are not in accordance with previous studies, where stronger effects on development were identified in females after exposure to these compounds, suggesting sex related developmental toxicity with males being more tolerant than females (Campos et al., 2017b; Campos et al., 2016b). Indeed, the present study corroborates the higher physiological susceptibility of females towards single exposures to BP3 and to DEET suggesting also that processes underlying the contrasting responses to mixtures can be of a different nature.

A possible explanation is that the combined action of these compounds has increased effect on the energy balance, triggering the adoption of different sex-related strategies as response. DEET, although considered as unlikely to cause effects in aquatic organisms at concentrations found in aquatic environment (Aronson et al., 2012; Campos et al., 2016a; Campos et al., 2016b; Costanzo et al., 2007), act in gustatory and olfactory receptor neurons of insects (Ditzen et al., 2008; Lee et al., 2010), probably impairing the ability of organisms to detect the presence of predators, food and even contaminants. Moreover, DEET caused neurophysiological impairments in mammals and insects, namely in *C. riparius*, by binding to the active site of cholinesterase (Campos et al., 2016b; Corbel et al., 2009), which may also alter feeding behaviour. Feeding inhibition was previously found to *S. vittatum*, rats and mice exposed to DEET (Campos et al., 2016a; Schoenig et al., 1999). Moreover, alterations in the detoxification mechanisms (e.g.: enzymatic/ non-enzymatic antioxidant defences) such as a decrease of the catalase and GST activities and total glutathione contents of *C. riparius* have also been observed under DEET exposure (Campos et al., 2016b). Because BP3 is known to increase the energy consumption on *C. riparius* (Campos et al., 2017) the combined effects on energy intake and metabolism could have contributed to the synergistic delayed development observed in males.

Female *C. riparius*, on the other hand showed less than additive effects in terms of development time when exposed to the mixture of both compounds which was not expected since a delay of emergence was observed as a response to single exposure to BP3 and DEET in this and other studies (Campos et al., 2016b, Campos et al., 2017b). However, and despite no significant differences for adults' body weight, female imagoes were also smaller in the combined exposures in comparison to single exposures and this premature metamorphosis is again a possible sign of endocrine disruption. The reason why this effect is observed only under the combined exposure to DEET and BP3 is unknown. However, this might be related to the already mentioned interactions of both compounds on energy intake and metabolism and or to differences in BP3 intake caused by DEET exposure that can alter accumulation and effects in female chironomids that require higher levels of energetic reserves for egg masses production and laying (Goedkoop et al., 2010). Nevertheless, it is important to note that *C. riparius* is a protandric species with males emerging slightly earlier than females (Armitage et al., 1995). Thus, synergistic effects of this mixture are plausible to occur on natural populations of *C. riparius* since any alteration of the natural patterns of emergence can lead to deleterious reproductive effects which are aggravated with the reduction in the reproductive output of smaller female imagoes (Honek, 1993; Ponlawat and Harrington, 2007; Sibley et al., 2001).

5. Conclusion

The toxicity of chemical mixtures containing organic UV-filters needs to be addressed given the ubiquity of these compounds in aquatic environments. Our results suggest that their effects are complex and that single exposure of organisms to organic UV-filters can, in some situations, underestimate the true risk in real scenarios. Because organic UV-filters have shown to alter endocrine activity it is important to address effects using relevant exposure scenarios and endpoints. In this sense, focusing on insects that have a complex life-cycle is detrimental to evaluate effects of these compounds and of their mixtures on natural populations.

References

Armitage P., Cranston P.S.. Pinder L.C.V., . 1995. The Chironomidae. The biology and ecology of non-biting midges, Chapman & Hall. London, 572 p.

Aronson, D., Weeks, J., Meylan, B., Guiney, P.D., Howard, P.H., 2012. Environmental release, environmental concentrations, and ecological risk of N,N-Diethyl-m-toluamide (DEET). Integr Environ Assess Manag 8, 135-166.

ASTM, 1980. Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates and amphibians. American Standards for Testing and Materials, Philadelphia, P.A.

Backhaus, T., Faust, M., 2012. Predictive Environmental Risk Assessment of Chemical Mixtures: A Conceptual Framework. Environmental Science & Technology 46, 2564-2573.

Brausch, J.M., Rand, G.M., 2011. A review of personal care products in the aquatic environment: Environmental concentrations and toxicity. Chemosphere 82, 1518-1532.

Campos, D., Gravato, C., Fedorova, G., Burkina, V., Soares, A.M.V.M., Pestana, J.L.T., 2017a. Ecotoxicity of two organic UV-filters to the freshwater caddisfly *Sericostoma vittatum*. Environmental Pollution 228, 370-377.

Campos, D., Gravato, C., Quintaneiro, C., Golovko, O., Žlábek, V., Soares, A.M.V.M., Pestana, J.L.T., 2017b. Toxicity of organic UV-filters to the aquatic midge *Chironomus riparius*. Ecotoxicology and Environmental Safety 143, 210-216.

Campos, D., Gravato, C., Quintaneiro, C., Koba, O., Randak, T., Soares, A.M., Pestana, J.L., 2016a. Are insect repellents toxic to freshwater insects? A case study using caddisflies exposed to DEET. Chemosphere 149, 177-182.

Campos, D., Gravato, C., Quintaneiro, C., Soares, A.M.V.M., Pestana, J.L.T., 2016b. Responses of the aquatic midge *Chironomus riparius* to DEET exposure. Aquatic Toxicology 172, 80-85.

Cedergreen, N., Streibig, J.C., 2005. Can the choice of endpoint lead to contradictory results of mixture-toxicity experiments? Environmental Toxicology and Chemistry 24, 1676-1683.

Corbel, V., Stankiewicz, M., Pennetier, C., Fournier, D., Stojan, J., Girard, E., Dimitrov, M., Molgo, J., Hougard, J.-M., Lapied, B., 2009. Evidence for inhibition of cholinesterases in insect and mammalian nervous systems by the insect repellent deet. BMC Biology 7, 47.

Coronado, M., De Haro, H., Deng, X., Rempel, M.A., Lavado, R., Schlenk, D., 2008. Estrogenic activity and reproductive effects of the UV-filter oxybenzone (2-hydroxy-4-methoxyphenyl-methanone) in fish. Aquatic Toxicology 90, 182-187.

Costanzo, S.D., Watkinson, A.J., Murby, E.J., Kolpin, D.W., Sandstrom, M.W., 2007. Is there a risk associated with the insect repellent DEET (N,N-diethyl-m-toluamide) commonly found in aquatic environments? Science of The Total Environment 384, 214-220.

Díaz-Cruz, M., Llorca, M., Barceló, D., Barceló, D., 2008. Organic UV filters and their photodegradates, metabolites and disinfection by-products in the aquatic environment. TrAC Trends in Analytical Chemistry 27, 873-887.

Ditzen, M., Pellegrino, M., Vosshall, L.B., 2008. Insect Odorant Receptors Are Molecular Targets of the Insect Repellent DEET. Science 319, 1838-1842.

Echeverria, P.C., Picard, D., 2010. Molecular chaperones, essential partners of steroid hormone receptors for activity and mobility. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research 1803, 641-649.

Fediuk, D.J., Wang, T., Chen, Y., Parkinson, F.E., Namaka, M.P., Simons, K.J., Burczynski, F.J., Gu, X., 2012. Metabolic Disposition of the Insect Repellent DEET and the Sunscreen Oxybenzone Following Intravenous and Skin Administration in Rats. International Journal of Toxicology 31, 467-476.

Gago-Ferrero, P., Díaz-Cruz, M.S., Barceló, D., 2012. An overview of UV-absorbing compounds (organic UV filters) in aquatic biota. Analytical and Bioanalytical Chemistry 404, 2597-2610.

Giokas, D.L., Sakkas, V.A., Albanis, T.A., Lampropoulou, D.A., 2005. Determination of UV-filter residues in bathing waters by liquid chromatography UV-diode array and gas chromatography–mass spectrometry after micelle mediated extraction-solvent back extraction. Journal of Chromatography A 1077, 19-27.

Goedkoop, W., Spann, N., Åkerblom, N., 2010. Sublethal and sex-specific cypermethrin effects in toxicity tests with the midge *Chironomus riparius* Meigen. Ecotoxicology 19, 1201-1208.

Golovko, O., Kumar, V., Fedorova, G., Randak, T., Grabic, R., 2014. Removal and seasonal variability of selected analgesics/anti-inflammatory, anti-hypertensive/cardiovascular pharmaceuticals and UV filters in wastewater treatment plant. Environmental Science and Pollution Research 21, 7578-7585.

Honek, Alois, 1993. Intraspecific Variation in Body Size and Fecundity in Insects: A General Relationship. Oikos 66, 483-492.

Johnson, E.C., Braco, J.T., Whitmill, M.A., 2014. Connecting nutrient sensing and the endocrine control of metabolic allocation in insects. Current Opinion in Insect Science 1, 66-72.

Kameda, Y., Kimura, K., Miyazaki, M., 2011. Occurrence and profiles of organic sun-blocking agents in surface waters and sediments in Japanese rivers and lakes. Environmental Pollution 159, 1570-1576.

Kasichayanula, S., House, J.D., Wang, T., Gu, X., 2007. Percutaneous characterization of the insect repellent DEET and the sunscreen oxybenzone from topical skin application. Toxicology and Applied Pharmacology 223, 187-194.

Kunz, P.Y., Fent, K., 2006. Estrogenic activity of UV filter mixtures. Toxicology and Applied Pharmacology 217, 86-99.

Langford, K.H., Reid, M.J., Fjeld, E., Øxnevad, S., Thomas, K.V., 2015. Environmental occurrence and risk of organic UV filters and stabilizers in multiple matrices in Norway. Environment International 80, 1-7.

Lee, Y., Kim, S.H., Montell, C., 2010. Avoiding DEET through Insect Gustatory Receptors. Neuron 67, 555-561.

Martin, H.L., Svendsen, C., Lister, L.J., Gomez-Eyles, J.L., Spurgeon, D.J., 2009. Measurement and modeling of the toxicity of binary mixtures in the nematode *Caenorhabditis elegans*—a test of independent action. Environmental Toxicology and Chemistry 28, 97-104.

Molins-Delgado, D., Gago-Ferrero, P., Díaz-Cruz, M.S., Barceló, D., 2016. Single and joint ecotoxicity data estimation of organic UV filters and nanomaterials toward selected aquatic organisms. Urban groundwater risk assessment. Environmental Research 145, 126-134

Morgado, R.G., Gomes, P.A.D., Ferreira, N.G.C., Cardoso, D.N., Santos, M.J.G., Soares, A.M.V.M., Loureiro, S., 2016. Toxicity interaction between chlorpyrifos, mancozeb and soil moisture to the terrestrial isopod *Porcellionides pruinosus*. Chemosphere 144, 1845-1853.

OECD, 2004a. Test no. 218: Sediment-water chironomid toxicity test using spiked sediment, in: Publishing, O. (Ed.), OECD Guidelines for the Testing of Chemicals.

OECD, 2004b. Test no. 219: sediment–water chironomid toxicity using spiked water, in: Publishing, O. (Ed.), OECD Guidelines for the testing of chemicals

Ozáez, I., Martínez-Guitarte, J.L., Morcillo, G., 2013. Effects of in vivo exposure to UV filters (4-MBC, OMC, BP-3, 4-HB, OC, OD-PABA) on endocrine signaling genes in the insect *Chironomus riparius*. Science of The Total Environment 456–457, 120-126.

Ozáez, I., Aquilino, M., Morcillo, G., Martínez-Guitarte, J.-L., 2016a. UV filters induce transcriptional changes of different hormonal receptors in *Chironomus riparius* embryos and larvae. Environmental Pollution 214, 239-247.

Ozáez, I., Martínez-Guitarte, J.L., Morcillo, G., 2014. The UV filter benzophenone 3 (BP-3) activates hormonal genes mimicking the action of ecdysone and alters embryo development in the insect *Chironomus rip*arius (Diptera). Environmental Pollution 192, 19-26.

Ozáez, I., Morcillo, G., Martínez-Guitarte, J.-L., 2016b. The effects of binary UV filter mixtures on the midge *Chironomus riparius*. Science of The Total Environment 556, 154-162.

Paredes, E., Perez, S., Rodil, R., Quintana, J.B., Beiras, R., 2014. Ecotoxicological evaluation of four UV filters using marine organisms from different trophic levels *Isochrysis galbana*, *Mytilus galloprovincialis*, *Paracentrotus lividus*, and *Siriella armata*. Chemosphere 104, 44-50.

Park, C.-B., Jang, J., Kim, S., Kim, Y.J., 2017. Single- and mixture toxicity of three organic UV-filters, ethylhexyl methoxycinnamate, octocrylene, and avobenzone on *Daphnia magna*. Ecotoxicology and Environmental Safety 137, 57-63.

Pavlaki, M.D., Pereira, R., Loureiro, S., Soares, A.M.V.M., 2011. Effects of binary mixtures on the life traits of *Daphnia magna*. Ecotoxicology and Environmental Safety 74, 99-110.

Planelló, R., Herrero, Ó., Gómez-Sande, P., Ozáez, I., Cobo, F., Servia, M.J., 2015. Ecdysone-Related Biomarkers of Toxicity in the Model Organism Chironomus riparius: Stage and Sex-Dependent Variations in Gene Expression Profiles. PLoS ONE 10, e0140239.

Ponlawat, A., Harrington, L.C., 2007. Age and body size influence male sperm capacity of the dengue vector *Aedes aegypti* (Diptera: Culicidae). J Med Entomol 44, 422-426.

Ramos, S., Homem, V., Alves, A., Santos, L., 2015. Advances in analytical methods and occurrence of organic UV-filters in the environment — A review. Science of The Total Environment 526, 278-311.

Ramos, S., Homem, V., Alves, A., Santos, L., 2016. A review of organic UV-filters in wastewater treatment plants. Environment International 86, 24-44.

Schmitt, C., Oetken, M., Dittberner, O., Wagner, M., Oehlmann, J., 2008. Endocrine modulation and toxic effects of two commonly used UV screens on the aquatic invertebrates *Potamopyrgus antipodarum* and *Lumbriculus variegatus.* Environmental Pollution 152, 322-329.

Schoenig, G.P., Osimitz, T.G., Gabriel, K.L., Hartnagel, R., Gill, M.W., Goldenthal, E.I., 1999. Evaluation of the chronic toxicity and oncogenicity of N,N-diethyl-m-toluamide (DEET). Toxicol Sci 47, 99-109.

Sibley, P.K., Ankley, G.T., Benoit, D.A., 2001. Factors affecting reproduction and the importance of adult size on reproductive output of the midge *Chironomus tentans*. Environ Toxicol Chem 20, 1296-1303.

Silva A.R.R., Cardoso D.N., Cruz A., Lourenco J., Mendo S., Soares A.M.V.M., Loureiro S., 2015. Ecotoxicity and genotoxicity of a binary combination of triclosan and carbendazim to *Daphnia magna*. Ecotoxicology and Environmental Safety. 115, 279-290.

Tsui, M.M.P., Leung, H.W., Wai, T.-C., Yamashita, N., Taniyasu, S., Liu, W., Lam, P.K.S., Murphy, M.B., 2014. Occurrence, distribution and ecological risk assessment of multiple classes of UV filters in surface waters from different countries. Water Research 67, 55-65.

Wang, J., Pan, L., Wu, S., Lu, L., Xu, Y., Zhu, Y., Guo, M., Zhuang, S., 2016. Recent Advances on Endocrine Disrupting Effects of UV Filters, International journal of environmental research and public health.

Iwasaki, Y., Brinkman, S.F., 2015. Application of a generalized linear mixed model to analyze mixture toxicity: Survival of brown trout affected by copper and zinc. Environmental Toxicology and Chemistry 34, 816-820.

Suplementary Material

Table 1SM. Candidate models to describe different endpoints of C*hironomus riparius* after exposure to the binary mixture of BP3 vs 4-MBC. AIC is the Akaike information criterion.

	Model	AIC value
Emergence (%)	BP3 + 4 MBC + BP3*4-MBC	**184.87**
	BP3 + 4 MBC	185.97
	BP3	205.43
Male development time	BP3 + 4 MBC + BP3*4-MBC	162.46
	BP3 + 4 MBC	160.46
	4-MBC	**158.60**
Female development time	BP3 + 4 MBC + BP3*4-MBC	-94.36
	BP3 + 4 MBC	**-95.69**
	4-MBC	-94.04
Male body weight	BP3 + 4 MBC + BP3*4-MBC	-43.75
	BP3 + 4 MBC	-45.21
	4-MBC	**-47.21**
Female body weight	BP3 + 4 MBC + BP3*4-MBC	-55.21
	BP3 + 4 MBC	**-56.86**
	4-MBC	-55.42

Table 2SM. Candidate models to describe different endpoints of C*hironomus riparius* after exposure to the binary mixture of BP3 vs DEET. AIC is the Akaike information criterion.

		AIC: BP3 vs DEET
Emergence (%)	BP3 + DEET + BP3*DEET	222.03
	BP3 + DEET	220.21
	BP3	**218.84**
Male development time	BP3 + DEET + BP3*DEET	**-141.78**
	BP3 + DEET	-136.31
Female development time	BP3 + DEET + BP3*DEET	**225.68**
	BP3 + DEET	226.09
	BP3	228.04
Male body weight	BP3 + DEET + BP3*DEET	-179.55
	BP3 + DEET	**-180.77**
Female body weight	BP3 + DEET + BP3*DEET	-87.35
	BP3 + DEET	**-89.35**
	BP3	-80.18

Chapter 5:
Two-generational effects of Benzophenone-3 on the aquatic midge *Chironomus riparius*

Two-generational effects of Benzophenone-3 on the aquatic midge *Chironomus riparius*

Abstract

Organic UV-filters are emergent contaminants continuously released into the aquatic ecosystems. These compounds are persistent showing potential for bioaccumulation. Partial life-cycle tests might underestimate the toxicity of UV-filters especially since they have shown to be endocrine disruptors. In the present study, a benthic aquatic insect *Chironomus riparius* was exposed to a gradient of Benzophenone-3 (BP3) concentrations over two generations to assess effects over a full life cycle from the first-instar larvae in the parental (P) generation (emergence, fecundity and fertility) until emergence in the subsequent generation (filial – F1). Our results showed that concentrations of up to 8 mg BP3/Kg, elicited no effects in terms of emergence rate and development time of *C. riparius* in the P generation. Our results also showed that *C. riparius* fecundity was not affected by BP3 exposure but a strong dose-response was observed for fertility with none of the egg ropes hatching at 8 mg BP3/Kg. Concerning effects in the F1 generation, emergence and development time were delayed by continuous exposure to BP3. Moreover, reduced emergence and changes in development time were observed in the F1 generation maintained under control/clean conditions but whose parents were previously exposed to BP3. Results found in this multigenerational test clearly show reproductive effects of BP3 on *C. riparius* that would not be detected using standard tests. Full life cycle and multigenerational assays are critical to properly evaluate the population level effects of endocrine disrupting compounds such as organic UV-filters.

Key words: multigenerational effects; toxicity; UV-filters; aquatic organisms; Personal care products

1. Introduction

The most part of standard ecotoxicological tests focus on evaluation of effects of contaminants at organismal level of only a single generation. However, in the environment, natural populations are exposed to contaminants over several generations and the responses of organism can be different over the time. Indeed, the toxicity of the contaminants can be altered (i.e: increase or decrease over the generations) or even appear in later generations (Barata et al., 2017, Bona et al. 2016, Hochmuth et al. 2015, Kafel et al., 2012; Waissi et al., 2017) due to latent or carry over effects (Anway et al., 2006; Bhandari et al., 2015; Burton and Metcalfe, 2014). Therefore, multigenerational tests are essential to better assess the effects of contaminants in natural populations. This is especially critical concerning persistent and endocrine disruptors substances known to impair the development and the reproduction of organisms causing epigenetic and transgenerational effects (Anway et al 2005; Oppold et al., 2015).

Detection of Organic ultraviolet filters (UV-filters) in the aquatic environment has been increasing in the last decades. These compounds, are present in personal care products to protect skin and also in plastic products and materials to avoid damages caused by ultraviolet radiation (Brausch and Rand, 2011; Díaz-Cruz et al., 2008; Sambandan and Ratner, 2011). Previously, exposure to UV-filters has been shown to affect the feeding activity, cell viability and growth, as well as endpoints related with reproduction in diverse aquatic organisms (Campos et al., 2017a; Campos et al., 2017b; Gao et al., 2013; Kaiser et al., 2012; Kunz and Fent, 2006; Schmitt et al., 2008; Sieratowicz et al., 2011). Moreover, UV-filters also showed to be endocrine disruptors (Coronado et al., 2008; Ozáez et al., 2013; Wang et al., 2016). UV-filters are considered persistent, have low water solubility, high values of octanol - water partition coefficients (log K_{ow} > 3) and organic carbon - water partition coefficient (log K_{oc}) with consequent potential to accumulation in biota and sediments (Díaz-Cruz et al., 2008; Brausch and Rand, 2011). Taking into account the endocrine activity of UV-filters, their physic-chemicals properties and their continuous release in the aquatic environments, it is expected that aquatic organisms are exposed to UV-filters throughout their entire life, with possible consequences in the development and reproduction of the organisms throughout generations which calls for multigenerational ecotoxicity investigation.

In the present study, the aquatic non-biting midge *Chironomus riparius* was chosen to assess the effects of UV-filters over two consecutive generations. *C. riparius* is a benthic aquatic organism used as a model species in freshwater ecotoxicology (OECD, 2004a, b). *C. riparius* traits make them a good organism to be used in multigenerational assays since it is a multivoltine species, displaying sexual reproduction and a short life cycle in laboratory conditions.

Multigenerational studies have been conducted using *C. riparius* as model species to assess the effects of the endocrine disruptors (Tassou and Schulz, 2009; Tassou and Schulz, 2011; Vogt et al., 2007a; Vogt et al., 2007b; Watts et al., 2001) and a guideline fully covering the first generation (i.e.: fertility and fecundity) and part of the second generation has already been proposed for *C. riparius* (OECD, 2010). Moreover, *C. riparius* spend most part of their life cycle buried in the sediment, making them ideal to assess effects of lipophilic persistent contaminants such as organic UV-filters.

The aim of this study was to evaluate the impact of long-term exposure of Benzophenone-3 (BP3) in *C. riparius*. In order to do that, *C. riparius* was exposed over two consecutive generations to a gradient of BP3 concentrations using spiked sediments and endpoints such as emergence rate, development time, fecundity and fertility were assessed for the parental (P) generation as well as the emergence and development time for filial (F1) generation. BP3 was selected as a model organic UV-filter due to its presence in several sunscreens to protect against UVA and UVB radiation and because it is allowed by legislation in different regions such as European Union, United States and Japan (Ahmed et al., 2008; Wahie et al., 2007) being one of the most widely detected UV-filers around the world in aquatic ecosystems. Furthermore developmental, reproductive and endocrine disruption caused by exposure to BP3 have been shown in several vertebrate and invertebrate species including *C. riparius* (Campos et al., 2017b; Kim et al., 2014; Ozáez et al., 2014; Wang et al., 2016).

2. Materials & methods

2.1 Model organism and culture conditions

C. riparius egg ropes were collected from laboratory cultures established at University of Aveiro, Portugal. *C. riparius* cultures were maintained in an acrylic box, with the larvae in plastic containers with 1-2 cm inorganic fine sand (burnt at 500 ºC during 4h) and American Society for Testing and Materials (ASTM) hard water medium (ASTM, 1980) in the proportions of 1:4. The medium was aerated, changed every week and the organisms were fed three times a week with macerated Tetramin® (TetraWerk, Melle, Germany). The cultures were maintained at 20 ± 2ºC with a photoperiod of 16:8 h light/dark.

2.2 Experimental design

BP3 (2-hydroxy-4-methoxybenzophenone; CAS No. 131-57-7; purity ≥ 98%) was provided from Sigma-Aldrich (Portugal). BP3 has a molecular weight of 228.24 g/mol and a log K_{ow}= 3.79

(Gago-Ferrero et al., 2012). Due to low water solubility (0.10 g/L – 25ºC) (Gago-Ferrero et al., 2012), a stock solution of BP3 and subsequent gradient of concentrations were performed in ethanol (96%).

The experimental design was performed according to the guideline OECD 233 (OECD, 2010) with a slight modification: organisms were exposed via sediment to three concentrations of BP3 (2, 4 and 8 mg/Kg). The artificial sediment was composed by 75% of inorganic fine sediment (<1mm), 20% of kaolin, 5% of cellulose and 0.1% of calcium carbonate; 50 g of sediment spiked with 10 mL of respective BP3 solution were added to each test vessel according to Campos et al. (2017b). The same volume of ethanol (10 mL -96%) was also added to solvent control treatment. After contamination, the glass vessels were left to evaporate during 72h in a fume hood. Afterwards and to obtain a homogenous sediment paste, 10 mL of ASTM were added in all treatments and the sediment thoroughly mixed. Immediately after, 150 mL of ASTM were carefully added to the vessel to avoid the resuspension of the sediment. Vessels were then left to equilibrate during 48h.

The experimental design is depicted in figure 1. For the entire test (P and F1 generation) larvae with less than 24h were added to fifteen replicates of each treatment. During the test (28 days) larvae were fed with 0.25mg/org/day of a suspension of macerated fish food (Tetramin®) and all experiments were conducted at 20ºC ± 1ºC with a photoperiod of 16:8h light-dark. Concerning the parental generation, emergence was checked twice per day to determine the total number of emerged imagoes and their development (i.e. time in days until emergence). Within each treatment, imagoes were transferred to a breeding cage (53.5 cm x 34.5cm x 29 cm) where they could mate and oviposit. The females laid the egg ropes into crystallizing dishes with 200 g of artificial sediment previously spiked with respective BP3 solution and 450 mL of ASTM. The presence of egg ropes was checked every day, and egg ropes were collected and kept in small crystallizing dishes (Ø 4 cm) with 10 mL of the respective experimental solution (water only). The structure, the fecundity and the fertility were assessed in each egg rope. The total number of eggs of each egg ropes was determined by the ring count method (applicable only for egg ropes that have a normal C-shape form) (Benoit et al., 1997). Briefly, the method consists in multiplying the number of rings by the mean number of eggs of at least five rings selected along of the egg ropes length. When the egg ropes do not present a normal C-shape the total number of eggs was counted or larvae were counted after hatching. Fecundity is defined as the total number of egg ropes per total number of females added to each breeding cage. Hatchability was checked during the 6 days after egg ropes were laid. Egg ropes were considered fertile when at least 1/3 of eggs

hatched. Fertility is defined as the total number of fertile egg ropes per total number of females added to each breeding cage (OECD, 2010).

Egg ropes from each treatment were used to start the next generation test (F1) in their respective treatment and, simultaneously, in clean conditions (figure 1). Due to inexistence of fertile egg ropes it was not possible to start the F1 generation in the highest tested concentration, 8 mg BP3/Kg. To start the F1 generation a total of 6 egg ropes were used per treatment, except at 4mg BP3/Kg, where only 4 egg ropes were used (due to low hatching). The emergence rate and development time was checked every day in the F1 generation.

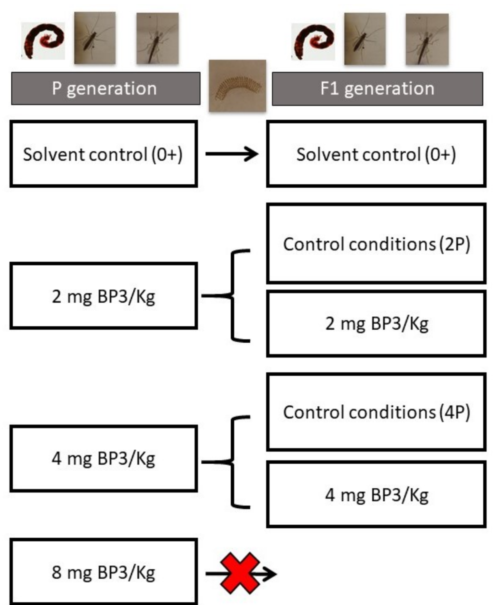


Figure 1 - Experimental design used for *C. riparius* multigenerational experiment (1st (P) and 2nd (F1) generations).

2.3 Statistical analyses

Effects of BP3 in all *C. riparius* endpoints were evaluated using analysis of variances (ANOVA) with multiple comparisons against the control treatment examined by Dunnett's *post-hoc* test. To check normality the Shapiro-Wilk were applied on the residuals and homoscedascy were assessed by Levene's test. Emergence (P and F1 generation exposed to control conditions), development time of males (F1 exposed to control conditions) and females (P and F1 generation) and the total number of eggs per egg ropes were analysed with Kruskal-Wallis test followed by Dunn's *post-hoc*

test. For all statistical tests, the significance level was set at $p < 0.05$. All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, La Jolla California USA).

3. Results

3.1 Effects of BP3 on *C. riparius* P generation

Emergence rate in the solvent control (P and F1 generation) was above 90% (figure 2). Our results showed no effects of BP3 in terms of emergence rate when comparing with the solvent control treatment (H= 8.507; Df=3; p = 0.037; figure 2a). Exposure to BP3 did not alter *C. riparius* development time of males ($F_{(3;52)}$= 2.050; p=0.118; figure 3a) and females (H= 0.391; Df=2; p=0.942; figure 3b) in the P generation. The fecundity (number of laid egg ropes per female) is presented in figure 4a. The total number of egg ropes was similar between all experimental treatments and no significant effects of BP3 exposure were observed for the number of eggs per egg ropes (H= 4.889; Df= 3; p=0.180; figure 4b). Concerning fertility, a strong dose dependent decrease in the number of fertile egg ropes was observed. In fact, at 4 mg BP3/Kg, 78% of egg ropes were infertile and at 8 mg/Kg BP3 none of the egg ropes laid by *C. riparius* females hatched (figure 4a).

3.2 Effects of BP3 on *C. riparius* F1 generation

Concerning the effects in F1 generation exposed to the same treatments as the P generation, BP3 exposure caused a significant and dose dependent reduction in emergence rates ($F_{(2;42)}$= 9.164; p< 0.001; figure 2b) with reductions of 14.09 and 26.77% compared to solvent control treatment, upon exposure to 2 and 4 mg BP3/Kg respectively. However, significant differences were only observed at 4 mg BP3/Kg comparatively with solvent control treatment. Regarding F1 generation maintained under control conditions whose parental generation was previously exposed to 4 mg BP3/Kg, a significant decrease in emergence rate of 14.44% was observed in comparison with the solvent control treatment (H= 9.389; Df=2; p<0.009; figure 2b).

Development time of *C. riparius* males in the F1 generation was significantly affected by BP3 exposure ($F_{(2;37)}$= 15.44; p<0.001; figure 3b). The *C. riparius* males of F1 generation exposed to the same treatments as the parents, emerged significantly earlier at 2 mg BP3/Kg and delayed their development at 4 mg BP3/Kg when compared with the solvent control treatment. Also, development time of females exposed to the same treatments in the F1 generation as their parents was significantly affected by BP3 exposure (H= 10.04; Df=2; p=0.007; figure 3d), but the effects were only observed at 2 mg BP3/Kg comparatively with solvent control treatment.

Moreover, both males and female imagoes whose F1 generation was under control conditions but whose parents were previously exposed to 2 and 4 mg BP3/Kg emerged significantly earlier comparatively with the solvent control treatment (H= 17.60; Df=2; $p<0.001$; figure 3b; H= 20.53; Df=2; $p<0.001$; figure 3d).

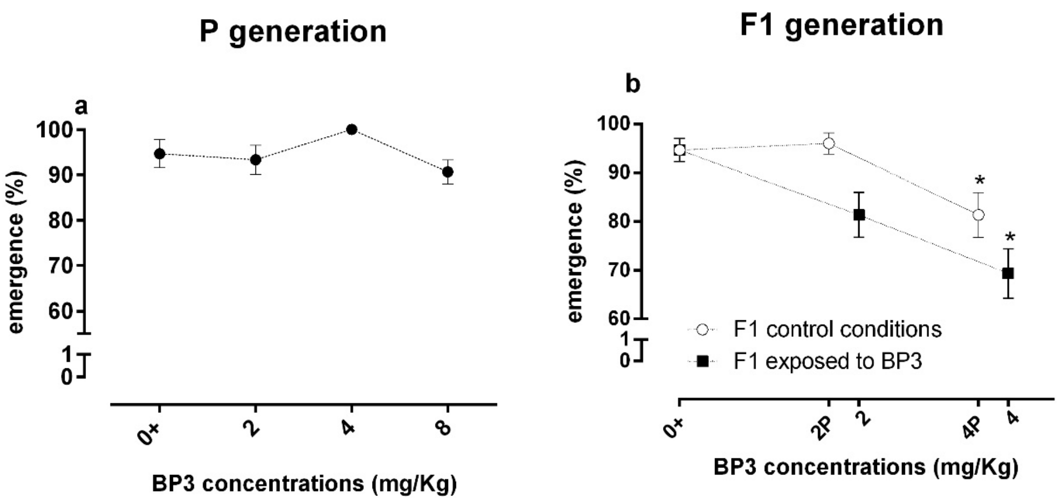


Figure 2 - Effects of BP3 on *C. riparius* emergence (%; mean ± SEM) during two consecutive generations. a) P generation and b) F1 generation. Open circles represent F1 generation larvae exposed under control conditions whose P generation was exposed to BP3. The black squares represent F1 generation larvae exposed to the same treatments as the P generation. Asterisks denote a significant difference compared with solvent control treatment (0+)

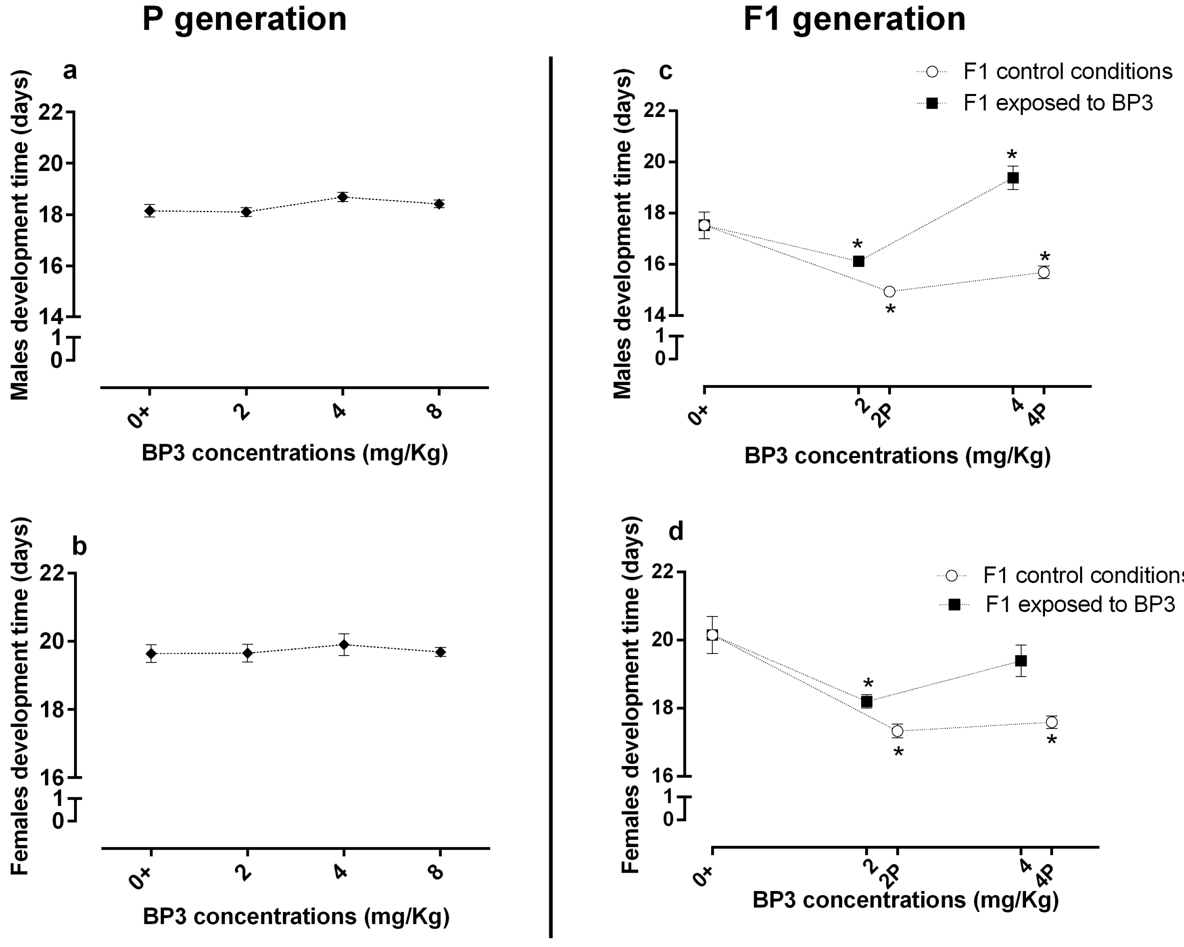


Figure 3 - Development time of males (a,c) and females (b,d) of P (a,b) and F1 (c,d) generations (days; mean ± SE) of *C. riparius.* Open circles represent F1 generation larvae exposed under control conditions whose P generation was exposed to BP3. The black squares represent F1 generation larvae exposed to the same conditions as the P generation. Asterisks denote a significant difference compared with solvent control treatment (0+).

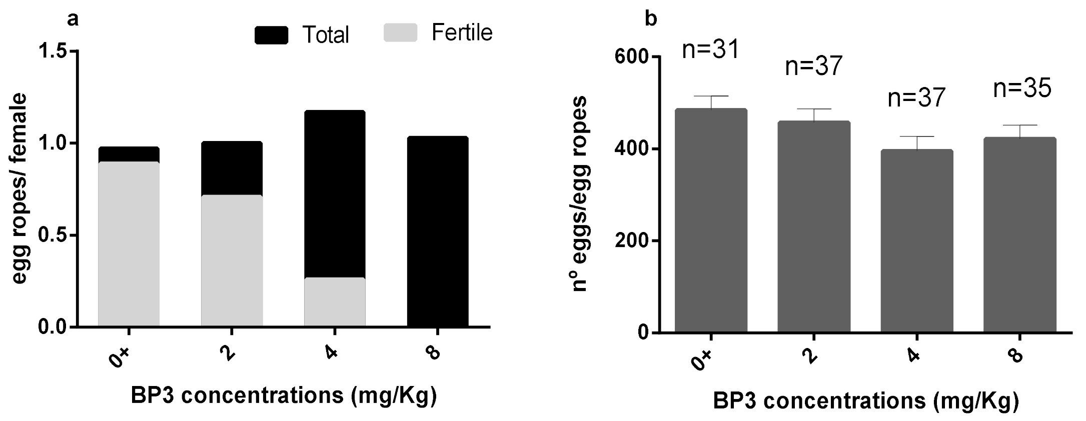


Figure 4 - Effects of BP3 on a) total and fertile egg ropes per female of *C. riparius* and b) number of eggs per egg ropes (mean ± SE) produced by *C. riparius* female.

4. Discussion

In the present study, we investigated the toxicity of BP3 in *C. riparius* during two consecutive generations. Obtained results showed that the toxicity of BP3 increased throughout exposed generations and in P generation, although no effects were reported for *C. riparius* emergence rates and development time, their fertility was strongly compromised. Moreover, the data shows that emergence rates and developmental time of F1 generation were altered even when they are not exposed to BP3 (but whose parents were previously exposed), which clearly imply carry-over effects of BP3. These results indicate clear reproductive and developmental toxicity of environmentally relevant concentrations of BP3 on *C. riparius* and call for the importance of full life-cycle/multigenerational testing. Although time consuming and somewhat laborious these multigenerational assays are critical to better evaluate the long-term ecological consequences of persistent compounds that are endocrine disruptors and have high bioaccumulation/ bioconcentration potential such as organic UV-filters (Díaz-Cruz et al., 2008).

Our results show stronger effects of BP3 on fertility than on emergence rate, which might be considered a survival trait, in the P generation suggesting that energetic costs related with defence mechanisms and detoxification (already observed for *C. riparius* and other aquatic insect larvae exposed to BP3 (Campos et al., 2017a, Campos et al., 2017b)) can lead to detrimental effects in terms of embryonic development and hatching likely due to less energy allocated into reproduction (Servia et al., 2006; Sokolova et al., 2012). It is however interesting to note that effects on fertility occurred without significant changes in terms of developmental time (i.e. time to emergence), fecundity (number of egg ropes produced by female) and number of eggs per egg ropes. Effects on reproduction (i.e. decrease in hatching) due to BP3 exposure were previously reported in the fish Japanese medaka (*Oryzias latipes*) (Coronado et al., 2008) and zebrafish (*Danio rerio*) (Balázs et al., 2016). The decrease in fertility of *C. riparius* without previous effects in the development time of males and females were also reported to the endocrine disruptor tebufenozide at 26.2 µg/L (Tassou and Schulz, 2013). Furthermore, reductions in fertility of *C. riparius* have been reported due to exposure to other contaminants like organometals, metals (Vogt et al., 2007a; Vogt et al., 2007b) and pharmaceuticals (Heye et al., 2016). Nevertheless, and since the main link between individual and population is reproduction (Barata et al., 2002, Tassou and Schulz, 2013), impairments of *C. riparius* fertility caused by exposure to BP3 show population level effects of this organic UV-filter and underline the importance of using this sensitive endpoint to complement standard *C. riparius* partial life cycle tests.

Besides the effects at physiological level it is known that UV-filters are endocrine disruptors (Kim et al., 2014; Ozáez et al., 2013; Schlumpf et al.,2008; Wang et al., 2016) and as such insect endocrine systems may be targeted by exposure to BP3. Hormones like juvenile hormone (JH) and Ecdysone, play an important role in the development and reproduction of the insects controlling moulting, pupation, and metamorphosis (Riddiford, 2012; Riddiford et al., 2000; Yamanaka et al., 2013). Alterations in the levels of these hormones may affect the development and the reproduction of *C. riparius* (Planelló et al., 2015) and probably compromise the energetic reserves that parents transfer to offspring as well (Johnson et al., 2014). It was shown before that BP3 causes hormonal effects, namely activating the expression of the ecdysone responsive genes and altering the transcript levels of the insulin-like receptor gene in *C. riparius* larvae; and also altering the embryo development of *C. riparius* (Ozáez et al., 2016; Ozáez et al., 2013).

Moreover, our results show that *C. riparius* F1 generation was more affected than P generation by exposure to BP3. In fact, reduced emergence rates and significant differences in the development time were observed in F1 generation for both *C. riparius* males and females exposed to BP3, while no effects were observed in the P generation. These results provide evidences of parental or latent effects of BP3 on *C. riparius*. In fact, previous studies have reported that *C. riparius* F1 generation, exposed to endocrine disruptors such as pyriproxyfen, teflubenzuron and bisphenol A was more affected than the P generation (Tassou and Schulz, 2009; Tassou and Schulz, 2011; Watts et al., 2001). This migth be related with the reduced energy allocation of exposed midges to their progeny which could have altered their sensitivity to the tested compound. Also, and despite no data of imagoes weight is available in the present study, for the parental generation previous work has shown that exposure to higher concentrations of BP3 can also lead to reduction in imagoes weight with potential effects on progeny (Campos et al., 2017b).

Concerning effects on development time, earlier emergence was observed in F1 midges exposed to lower concentrations of BP3, but no effects or delayed emergence of *C. riparius* females and males, respectively, were also observed in the highest BP3 concentration tested. These results are somewhat inconsistent with standard partial life-cycle tests showing that BP3 exposure causes reductions in larval growth and development rates in *C. riparius* (Campos et al., 2017b). In fact, endocrine disruptors are known to show no clear patterns according to the dose (Santillo et al., 1998), and sometimes the effects are greatest at lower concentrations (Patlak, 1996). Nevertheless, increases as well as decreases in development time have been observed before in insects exposed to endocrine disruptors (Quesada-Claderón et al., 2016; Tassou and Schulz 2011; Watt et al., 2001). Either way, changes in the synchrony of male and female

developmental rates can affect mating in *C. riparius* swarms and thus may have consequences in terms of their reproduction and population dynamics.

Interestingly we also observed reduced emergence and altered development time in F1 generation reared under control conditions but whose parental generation had been exposed to BP3, showing that effects of BP3 parental exposure persisted even after *C. riparius* were transferred to uncontaminated medium. This suggests the possibility of transgenerational epigenetic effects of BP3. The observation of epigenetic effects due to exposures to endocrine disruptors with consequences in the subsequent generations were previously observed in *Daphnia magna* (Vandegehuchte et al. 2010a, 2010b), in the mosquito *Aedes albopictus* (Oppold et al., 2015) and also in vertebrates (Anway et al., 2006). However, to confirm this hypothesis of epigenetic alterations and transgenerational effects induced by organic UV-filters, and considering the life cycle of *C. riparius*, at least 3 consecutive generations should be tested to avoid exposure of germ lines of the unexposed generation (Skinner et al., 2011 Vandegehuchte et al., 20010a).

This study suggests transgenerational or parental effects of BP3 in *C. riparius,* showed by the higher sensitivity of the F1 generation in comparison with the parental generation. However and even using only contaminated sediment, it is important to note that these effects might also be a consequence of exposure of F1 eggs inside the gelatinous matrix to BP3 present in the overlying water. Because in the case of the P generation, organisms were only exposed to contaminated sediments as larvae; this might explain not only the stronger effects on F1 generation but also the reduced hatchability of egg ropes in BP3 treatments. In fact, Ozáez et al. (2014), observed that exposure to BP3 delayed the hatching of *C. riparius* egg ropes which lengthened the time to embryos development. Additionally, Ozáez et al. (2016), observed that embryos seem to be more affected than larvae (4th instar) after exposure to BP3 (via water), once the expression of genes related with hormonal pathways (i.e.: Hormonal receptor 38, Methoprene-tolerant) was altered mainly in embryos. If this is the case, then effects observed in F1 generation not exposed to BP3 arise by carry-over effect within one generation (exposure of eggs with latent effects observed at emergence of larvae) also suggesting ecological effects that might be underestimated using standard partial life-cycles assays only.

5. Conclusion

This study clearly indicates that exposure to low, environmentally relevant concentrations of BP3, can compromise reproduction and population dynamics of an aquatic insect species. Since BP3 and other organic UV-filters are persistent in sediments and known endocrine disruptors their

environmental risk assessment needs to rely on multigenerational tests. By focusing on insects with a short life cycle like chironomids, the inclusion of complete life cycles and relevant life stages (eggs, larvae, pupae, imagoes) and measurement of effects on endpoints (fertility, development, and emergence patterns) which are controlled by hormones, they can complement other endocrine and epigenetic responses and thus, be used to better evaluate the long-term effects of organic UV-filters in the aquatic environment.

References

Ahmed, F.K., 2008. Worldwide regulation of UV-filters: current status and future trends. In: Lim, H.W., Draelos, Z.D. (Eds.), Clinical Guide to Sunscreens and Photoprotection. Informa Healthcare, New York, 65–81.

Anway, M.D., Cupp, A.S., Uzumcu, M., Skinner, M.K., 2005. Epigenetic Transgenerational Actions of Endocrine Disruptors and Male Fertility. Science 308, 1466-1469.

Anway, M.D., Memon, M.A., Uzumcu, M., Skinner, M.K., 2006. Transgenerational Effect of the Endocrine Disruptor Vinclozolin on Male Spermatogenesis. Journal of Andrology 27, 868-879.

ASTM, 1980. Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates and amphibians. American Standards for Testing and Materials, Philadelphia, P.A.

Balázs, A., Krifaton, C., Orosz, I., Szoboszlay, S., Kovács, R., Csenki, Z., Urbányi, B., Kriszt, B., 2016. Hormonal activity, cytotoxicity and developmental toxicity of UV filters. Ecotoxicology and Environmental Safety 131, 45-53.

Barata, C., Baird, D.J., Soares, A.M.V.M., 2002. Food supply on density-dependent effects on demographic responses of the cladoceran *Moinodaphnia macleaya* to heavy metal exposure. Ecological Applications, 12, 552-56.

Barata, C., Campos, B., Rivetti, C., LeBlanc, G.A., Eytcheson, S., McKnight, S., Tobor-Kaplon, M., de Vries Buitenweg, S., Choi, S., Choi, J., Sarapultseva, E.I., Coutellec, M.-A., Coke, M., Pandard, P., Chaumot, A., Quéau, H., Delorme, N., Geffard, O., Martínez-Jerónimo, F., Watanabe, H., Tatarazako, N., Lopes, I., Pestana, J.L.T., Soares, A.M.V.M., Pereira, C.M., De Schamphelaere, K., 2017. Validation of a two-generational reproduction test in *Daphnia magna*: An interlaboratory exercise. Science of The Total Environment 579, 1073-1083

Benoit, D.A., Sibley, P.K., Juenemann, J.L., Ankley, G.T., 1997. *Chironomus tentans* life-cycle test: Design and evaluation for use in assessing toxicity of contaminated sediments. Environmental Toxicology and Chemistry 16, 1165-1176.

Bhandari, R.K., vom Saal, F.S., Tillitt, D.E., 2015. Transgenerational effects from early developmental exposures to bisphenol A or 17α-ethinylestradiol in medaka, *Oryzias latipes*. Scientific Reports 5, 9303.

Bona, M. Dalla, Lizzi, F., Borgato, A., De Liguoro, M., 2016. Increasing toxicity of enro- floxacin over four generations of *Daphnia magna*, Ecotoxicology Environmental Safety 132, 397–402.

Brausch, J.M., Rand, G.M., 2011. A review of personal care products in the aquatic environment: Environmental concentrations and toxicity. Chemosphere 82, 1518-1532.

Burton, T., Metcalfe, N.B., 2014. Can environmental conditions experienced in early life influence future generations? Proceedings of the Royal Society B: Biological Sciences 281, 20140311.

Campos, D., Gravato, C., Fedorova, G., Burkina, V., Soares, A.M.V.M., Pestana, J.L.T., 2017a. Ecotoxicity of two organic UV-filters to the freshwater caddisfly *Sericostoma vittatum*. Environmental Pollution 228, 370-377.

Campos, D., Gravato, C., Quintaneiro, C., Golovko, O., Žlábek, V., Soares, A.M.V.M., Pestana, J.L.T., 2017b. Toxicity of organic UV-filters to the aquatic midge *Chironomus riparius*. Ecotoxicology and Environmental Safety 143, 210-216

Coronado, M., De Haro, H., Deng, X., Rempel, M.A., Lavado, R., Schlenk, D., 2008. Estrogenic activity and reproductive effects of the UV-filter oxybenzone (2-hydroxy-4-methoxyphenyl-methanone) in fish. Aquatic Toxicology 90, 182-187.

Díaz-Cruz, M., Llorca, M., Barceló, D., Barceló, D., 2008. Organic UV filters and their photodegradates, metabolites and disinfection by-products in the aquatic environment. TrAC Trends in Analytical Chemistry 27, 873-887.

Gago-Ferrero, P., Díaz-Cruz, M.S., Barceló, D., 2012. An overview of UV-absorbing compounds (organic UV filters) in aquatic biota. Analytical and Bioanalytical Chemistry 404, 2597-2610.

Gao, L., Yuan, T., Zhou, C., Cheng, P., Bai, Q., Ao, J., Wang, W., Zhang, H., 2013. Effects of four commonly used UV filters on the growth, cell viability and oxidative stress responses of the *Tetrahymena thermophila*. Chemosphere 93, 2507-2513.

Heye, K., Becker, D., Lütke Eversloh, C., Durmaz, V., Ternes, T.A., Oetken, M., Oehlmann, J., 2016. Effects of carbamazepine and two of its metabolites on the non-biting midge *Chironomus riparius* in a sediment full life cycle toxicity test. Water Research 98, 19-27.

Hochmuth, J.D., De Meester, L., Pereira, C.M.S., Janssen, C.R., De Schamphelaere, K. A. C., 2015. Rapid adaptation of a *Daphnia magna* population to metal stress is associated with heterozygote excess, Environmental Science & Technology's, 49, 9298-9307.

Johnson, E.C., Braco, J.T., Whitmill, M.A., 2014. Connecting nutrient sensing and the endocrine control of metabolic allocation in insects. Current Opinion in Insect Science 1, 66-72.

Kafel, A., Zawisza-Raszka, A., Szulińska, E., 2012. Effects of multigenerational cadmium exposure of insects (*Spodoptera exigua* larvae) on anti-oxidant response in haemolymph and developmental parameters. Environmental Pollution 162, 8-16.

Kaiser, D., Sieratowicz, A., Zielke, H., Oetken, M., Hollert, H., Oehlmann, J., 2012. Ecotoxicological effect characterisation of widely used organic UV filters. Environmental Pollution 163, 84-90.

Kim, S., Jung, D., Kho, Y., Choi, K., 2014. Effects of benzophenone-3 exposure on endocrine disruption and reproduction of Japanese medaka (*Oryzias latipes*)—A two generation exposure study. Aquatic Toxicology 155, 244-252.

Kunz, P.Y., Fent, K., 2006. Multiple hormonal activities of UV filters and comparison of in vivo and in vitro estrogenic activity of ethyl-4-aminobenzoate in fish. Aquatic Toxicology 79, 305-324.

Patlak, M., 1996. A testing deadline for endocrine disrupters. Environmental Science & Technology 30, 540A-544A.

OECD, 2004a. Test no. 218: Sediment-water chironomid toxicity test using spiked sediment, in: Publishing, O. (Ed.), OECD Guidelines for the Testing of Chemicals.

OECD, 2004b. Test no. 219: sediment–water chironomid toxicity using spiked water, in: Publishing, O. (Ed.), OECD Guidelines for the testing of chemicals

OECD, 2010. OECD guideline for testing of chemicals: Sediment-Water Chironomid Life-Cycle Toxicity Test Using Spiked Water or Spiked Sediment. OECD Publishing.

Oppold, A., Kreß, A., Vanden Bussche, J., Diogo, J.B., Kuch, U., Oehlmann, J., Vandegehuchte, M. B., Müller, R., 2015. Epigenetic alterations and decreasing insecticide sensitivity of the Asian tiger mosquito *Aedes albopictus* Ecotoxicology Environmental Safety, 122, 45-53

Ozáez, I., Aquilino, M., Morcillo, G., Martínez-Guitarte, J.-L., 2016. UV filters induce transcriptional changes of different hormonal receptors in *Chironomus riparius* embryos and larvae. Environmental Pollution 214, 239-247.

Ozáez, I., Martínez-Guitarte, J.L., Morcillo, G., 2013. Effects of in vivo exposure to UV filters (4-MBC, OMC, BP-3, 4-HB, OC, OD-PABA) on endocrine signaling genes in the insect *Chironomus riparius*. Science of The Total Environment 456–457, 120-126.

Ozáez, I., Martínez-Guitarte, J.L., Morcillo, G., 2014. The UV filter benzophenone 3 (BP-3) activates hormonal genes mimicking the action of ecdysone and alters embryo development in the insect *Chironomus riparius* (Diptera). Environmental Pollution 192, 19-26.

Planelló, R., Herrero, Ó., Gómez-Sande, P., Ozáez, I., Cobo, F., Servia, M.J., 2015. Ecdysone-Related Biomarkers of Toxicity in the Model Organism *Chironomus riparius*: Stage and Sex-Dependent Variations in Gene Expression Profiles. PLoS ONE 10, e0140239.

Quesada-Calderón, S, Bacigalupe, L. D., Toro-Vélez, A. F., Madera-Parra, C. A., Peña-Varón, M. R. Cárdenas-Henao, H., 2017. The multigenerational effects of water contamination and endocrine disrupting chemicals on the fitness of *Drosophila melanogaster.* Ecology and Evolution 7:6519–6526

Riddiford, L.M., 2012. How does juvenile hormone control insect metamorphosis and reproduction? General and Comparative Endocrinology 179, 477-484.

Riddiford, L.M., Cherbas, P., Truman, J.W., 2000. Ecdysone receptors and their biological actions. Vitam Horm 60, 1-73.

Sambandan, D.R., Ratner, D., 2011. Sunscreens: An overview and update. Journal of the American Academy of Dermatology 64, 748-758.

Santillo, D., Stringer, R.L., Johnston, P.A., Tickner, J., 1998. The precautionary principle: Protecting against failures of scientific method and risk assessment. Marine Pollution Bulletin 36, 939-950.

Schlumpf, M., Durrer, S., Faass, O., Ehnes, C., Fuetsch, M., Gaille, C., Henseler, M., Hofkamp, L., Maerkel, K., Reolon, S., Timms, B., Tresguerres, J.A.F., Lichtensteiger, W., 2008. Developmental toxicity of UV filters and environmental exposure: a review. International Journal of Andrology 31, 144-151.

Schmitt, C., Oetken, M., Dittberner, O., Wagner, M., Oehlmann, J., 2008. Endocrine modulation and toxic effects of two commonly used UV screens on the aquatic invertebrates Potamopyrgus antipodarum and *Lumbriculus variegatus*. Environmental Pollution 152, 322-329.

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

Sieratowicz, A., Kaiser, D., Behr, M., Oetken, M., Oehlmann, J., 2011. Acute and chronic toxicity of four frequently used UV filter substances for *Desmodesmus subspicatus* and *Daphnia magna*. J Environ Sci Health A Tox Hazard Subst Environ Eng 46, 1311-1319.

Skinner, M.K., Manikkam, M., Guerrero-Bosagna, C., 2011. Epigenetic transgenerational actions of Endocrine Disruptors Reproductive toxicology (Elmsford, N.Y.) 31, 337-343.

Sokolova, I.M., Frederich, M., Bagwe, R., Lannig, G., Sukhotin, A.A., 2012. Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. Mar Environ Res 79, 1-15.

Tassou, K.T., Schulz, R., 2009. Effects of the insect growth regulator pyriproxyfen in a two-generation test with *Chironomus riparius*. Ecotoxicology and Environmental Safety 72, 1058-1062.

Tassou, K.T., Schulz, R., 2011. Two-generation effects of the chitin synthesis inhibitor, teflubenzuron, on the aquatic midge *Chironomus riparius*. Ecotoxicology and Environmental Safety 74, 1203-1209.

Tassou, K.T., Schulz, R., 2013. Low field-relevant tebufenozide concentrations affect reproduction in *Chironomus riparius* (Diptera: Chironomidae) in a long-term toxicity test. Environmental Science and Pollution Research 20, 3735-3742.

Vandegehuchte, M.B., Lemière, F., Vanhaecke, L., Vanden Berghe, W., Janssen, C.R., 2010a. Direct and transgenerational impact on *Daphnia magna* of chemicals with a known effect on DNA methylation. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 151, 278-285.

Vandegehuchte, M.B., De Coninck, D., Vandenbrouck, T., De Coen, W.M., Janssen, C.R., 2010b. Gene transcription profiles, global DNA methylation and potential transgenerational

epigenetic effects related to Zn exposure history in *Daphnia magna*. Environ Pollut 158, 3323-3329.

Vogt, C., Belz, D., Galluba, S., Nowak, C., Oetken, M., Oehlmann, J., 2007a. Effects of cadmium and tributyltin on development and reproduction of the non-biting midge *Chironomus riparius* (Diptera)—baseline experiments for future multi-generation studies. Journal of Environmental Science and Health, Part A 42, 1-9.

Vogt, C., Nowak, C., Diogo, J.B., Oetken, M., Schwenk, K., Oehlmann, J., 2007b. Multi-generation studies with *Chironomus riparius* – Effects of low tributyltin concentrations on life history parameters and genetic diversity. Chemosphere 67, 2192-2200.

Wahie, S., Lloyd, J.J., Farr, P.M., 2007. Sunscreen ingredients and labelling: a survey of products available in the UK. Clinical and Experimental Dermatology 32, 359-364.

Waissi, G. C., Vaananen, K., Nybom, I., Pakarinen, K., Akkanen, J., Leppanen, M. T., Kukkonen, J. V. K., 2017. The chronic effects of fullereneC60-associated sediments in the midge *Chironomus riparius* - Responses in the first and the second generation. Environmental Pollution. 229, 423-430.

Wang, J., Pan, L., Wu, S., Lu, L., Xu, Y., Zhu, Y., Guo, M., Zhuang, S., 2016. Recent Advances on Endocrine Disrupting Effects of UV Filters, International journal of environmental research and public health.

Watts, M.M., Pascoe, D., Carroll, K., 2001. Chronic exposure to 17α-ethinylestradiol and bisphenol A-effects on development and reproduction in the freshwater invertebrate *Chironomus riparius* (Diptera: Chironomidae). Aquatic Toxicology 55, 113-124.

Yamanaka, N., Rewitz, K.F., O'Connor, M.B., 2013. Ecdysone Control of Developmental Transitions: Lessons from *Drosophila* Research. Annu Rev Entomol 58, 497-516.

Chapter 6:

Effects of the organic UV-filter 4-MBC on freshwater benthic invertebrate communities and ecosystem function

Effects of the organic UV-filter 4-MBC on freshwater benthic invertebrate communities and ecosystem function

Abstract

In the last decades, the use of organic Ultraviolet-filters (UV-filters) is increasing worldwide and these compounds are now emerging contaminants of many freshwater ecosystems. The aim of the present study was to assess the ecological effects of 3-(4-methylbenzylidene) camphor (4-MBC) on freshwater invertebrate communities. For that artificial streams were used and a natural invertebrate benthic community was exposed to sediments contaminated with two concentrations of 4-MBC. Effects were evaluated in terms of macroinvertebrate abundance and community's structure, as well as leaf decomposition and primary production. Results showed that macroinvertebrates community parameters as well as leaf decomposition rates were not affected by exposure to 4-MBC. On the other hand, primary production was strongly reduced in the presence of this compound. This study highlights the importance of higher tier ecotoxicity experiments for the assessment of effects of low concentrations of organic UV-filters on community structure and ecosystem functioning.

Keywords: freshwaters, structure and functioning of the ecosystem, UV-filters effects; primary production; mesocosms

1. Introduction

The increase use of personal care products (PCPs) has led to contamination of natural aquatic ecosystems, mainly due to inefficient removal during wastewater treatment plants, but also due to outdoor recreational activities (Braush and Rand, 2011). In the last decade, organic ultraviolet-filters (UV-filters), synthetic compounds mainly used to protect the skin and several materials against solar UV radiation (Chisvert and Salvador, 2007), have been frequently detected in aquatic environments. Organic UV-filters have low water solubility and are expected to accumulate in sediments and biota, being considered persistent in the environment (Díaz-Cruz et al., 2008; Gago-Ferrero et al., 2012) with concentrations in sediments reaching 2.4 mg/Kg (Gago-Ferrero et al., 2011). Thus, freshwater benthic macroinvertebrate communities, especially those living in areas with high human pressure, are prone to exposure to these contaminants.

Standard laboratory toxicity tests already demonstrated that exposure to organic UV-filters can alter feeding activity, development and reproduction of some benthic macroinvertebrates such as the trichoptera *Sericostoma vittatum* (Campos et al., 2017a), the midge *Chironomus riparius* (Campos et al., 2017b) and the oligochaete *Lumbriculus variegatus* (Schmitt et al., 2008). However, those traditional single species tests convey uncertainties since they cannot be used directly to predict the effects of contaminants in the structure and function of ecosystems (Stewart et al., 2013). Thus, information on effects of UV-filters using realistic exposure scenarios are necessary for a more accurate ecological risk assessment. Therefore, a higher-tier approach is critical to include the diversity of species and habitat conditions found in natural systems (Relyea and Hoverman, 2006; Stewart et al., 2013) while integrating functional and structural parameters for a better understanding of the effects of these compounds on ecosystem processes (Gessner and Tlili, 2016).

For many years, the structure of macroinvertebrates communities has been used in ecosystem monitoring. Alterations in macroinvertebrates communities can be a consequence of stress responses (Blijswijk et al., 2004; Vidal et al., 2014), but functional parameters should be considered for a more accurate evaluation of ecosystem health and of effects on key ecological processes (Young et al., 2008; Dalu et al., 2017). So, in this study, functional effects of 4-MBC were assessed using leaf decomposition (by measuring leaf loss weight) and primary production (by measuring the chlorophyll *a* contents of periphyton communities). Leaf decomposition and primary production are important functional endpoints since they represent the main energy sources in freshwater food webs and those processes have been used as sensitive and reliable indicators of ecosystems health (Abelho et al., 2016; Evans et al., 2014; Peter et al., 2013; Piggott

et al., 2015; Xu et al., 1999). Density and trait mediated effects of stressors on detritivores and grazers can lead to alterations in the leaf decomposition or primary production (Dossena et al., 2012; Fleeger et al., 2003; Gessner et al., 2016; Hasenbein et al., 2017; Peters et al., 2013; Guasch et al., 2016; Graça, 2001) compromising thus, the functioning of the ecosystem. Moreover, direct effects of contaminants on producers can also lead to indirect effects in macroinvertebrates communities.

The purpose of this work was to contribute with sound ecotoxicity data concerning effects of a selected UV-filter on a natural freshwater community of benthic macroinvertebrates. For that, indoor artificial streams were used to assess possible effects of 4-MBC on community structure and functional parameters. The UV-filter 3-(4-methylbenzylidene) camphor (4-MBC) was chosen due to its frequent detection in European aquatic environments (Ramos et al., 2015). 4-MBC is expected to accumulate in sediments and biota due to his low biodegradation rate (Gago-Ferrero et al., 2012; Ramos et al., 2015), being considered as a high priority compound (Environment Agency, 2008).

2. Materials and methods

2.1 Chemical compound and sediment spiking

The 3-(4-methylbenzylidene) camphor (4-MBC; CAS No. 36861-47-9, purity _98%) was obtained from Sigma-Aldrich (Portugal). The compound has a molecular weight of 254.37 g/mol, water solubility of 0.017 g/L and a log K_{ow} of 4.95 (Gago-Ferrero et al., 2012). The stock solutions of 4-MBC were prepared in ethanol (96%). The sediment composed of 99 % of sand (< 2 mm, previously burn at 500 ºC during 4h) and 1 % of grounded alder leaves (*Alnus glutionosa*), was spiked with 4-MBC stock solutions to obtain two nominal concentrations of 2 and 20 mg 4-MBC/Kg. Spiked sediments were left in the fume hood during 72h for ethanol evaporation.

2.2 Leaf collection and macroinvertebrate community characterization

Alder leaves were collected from the riparian vegetation at São Pedro de Alva (40º28’N, 8º 19’W) during autumn and then air dried and stored in the darkness. Macroinvertebrate community was sampled in the Mau river (Sever do Vouga, Portugal), located in an unpolluted area (Vidal et al., 2014). A previous surber macroinvertebrate sampling was performed in the

same sampling spot to determine the adequate composition and density of the community to be introduced in each artificial stream.

2.3 Experimental design

The experiment was conducted in an indoor mesocosm system (15 ± 1 ºC and photoperiod: 16h light: 8h dark). A total of nine artificial streams were used (2 m long, 0.200 m width and 0.225 m depth). Three replicates (3 artificial streams) were used for each treatment (solvent control (0+), 2 and 20 mg 4-MBC/Kg). To simulate natural conditions, the average flow in each artificial stream was maintained at a constant rate of approx. 4 L/min. Artificial pond water (APW) (Naylor et al., 1989) enriched with phosphate and nitrate was used to simulate actual mineral concentrations on the Mau river (Vidal et al., 2014).

Each artificial stream contained approximately 280 L of APW, 7 Kg of sediment, 3 leaf packs (10 mm mesh size) and 5 unglazed ceramic tiles (20 cm^2) (Figure 1). Each leaf pack contained approx. 1 g of *Alnus glutinosa* leaves which was conditioned during 15 days in Mau river water before being placed in artificial streams. Ceramic tiles were placed in the Mau River for 2 weeks before the experiment to allow biofilm colonization. At day 0 of the exposure period, macroinvertebrates were collected by kick-sampling in gentle riffle habitats in the Mau river. Organisms were transported to the laboratory using river water, sorted by taxa and then allocated evenly to each artificial stream. In total, 159 organisms representing a total of 15 taxa were inoculated in each artificial stream, being the majority, in terms of abundance, collectors (Hydropsychidae; Ephemera sp.; Chironomidae; Oligochaeta - 65.41%) followed by grazers (Baetidae, Leptophebiidae, Ephemerellidae, Leutridae - 18.24%), shredders (Sericostoma sp, Lepidostoma sp. - 12.58%) and lastly predators (Boyeria sp, Onychogomphus sp, Calopteryx sp., Athericidae, Sialis sp - 3.77%). Water physico-chemical parameters (pH, temperature, conductivity and dissolved oxygen) were measured every three days. After 7 days of exposure, leaf packs were carefully removed from the respective treatments and cleaned with soft paintbrushes to remove possible attached organisms and sediment particles. Leaves were then dried (50ºC, 4 days) and weighed. Ceramic plates of the respective artificial streams were scrubbed with a soft brush and rinsed in water. The samples were then filtered with GF/C filters (1.2 µm) and stored at -20ºC in the darkness until analyses. Chlorophyll *a* was extracted with 90% acetone and measured spectrophotometrically (Jeffrey and Humphrey, 1975). At the end of the test all the macroinvertebrates were picked with the help of soft tip tweezers and by sieving the sediment. Any organisms found in leaf packs were combined with these benthic samples from the respective

stream replicates. Macroinvertebrates were preserved in 70% ethanol and finally identified with the help of a stereomicroscope (MS5, Leica Microsystems, Houston, USA).

Figure 1 – Detailed view of a) artificial stream; b) *A. glutinosa* leaf packs; c) ceramic plates with periphyton and d) artificial stream system (overview).

2.4 Statistical analysis

Effects of 4-MBC exposure on chlorophyll *a*, total macroinvertebrates abundance (i.e. total number of invertebrates recorded), species richness (i.e. number of different macroinvertebrate families recorded), % of "EPT family abundance" (i.e. total number of invertebrates belonging to Ephemeroptera, Plecoptera and Trichoptera), grazers density and shredders density were evaluated using analysis of variances (ANOVA) with multiple comparisons examined by Dunnett's *post-hoc* test. To check normality the Shapiro-Wilk were applied on residuals and homoscedascy were assessed by Levene's test. Chlorophyll *a* data were log transformed and Kruskal-Wallis test was used for analysis of the leaf mass loss. The influence of UV-filters on the structure of the benthic macroinvertebrate communities was assessed by using permutational multivariate analysis of variance, PERMANOVA (999 permutations; Anderson, 2001). All data was analysed using Prism 6.0. (GraphPad Software, La Jolla California USA) with significance level set at $p < 0.05$, except PERMANOVAs that were performed on R software (version 3.2.0, R Foundation for Statistical Computing, Vienna, Austria).

3. Results and discussion

In this study, mesocosms systems were used to investigated effects of organic 4-MBC, an organic UV-filter, on freshwater invertebrate communities and ecosystem function. Results revealed no effects on invertebrate structural endpoints and no changes were observed in leaf litter processing comparing to control streams. Exposure to 4-MBC elicited nevertheless a strong reduction in periphyton primary production. These results add ecotoxicological data concerning potential ecological effects of UV-filters within freshwaters. In our study the exposure to 4-MBC did not affect the species richness (p= 0.600; $F_{(2,6)}$ = 0.5787; figure 2a), the % EPT group abundance (p= 0.6542; $F_{(2,6)}$ = 0.4559; figure 2b) neither total macroinvertebrates abundance (p= 0.6809; $F_{(2,6)}$ = 0.4100; figure 2c). Moreover, no significant effects were found in terms of shredders density (p= 0.620; $F_{(2,6)}$ = 0.5181; data not shown) neither for macroinvertebrates community structure (PERMANOVA; p = 0.302 $F_{(2,6)}$= 0.2852). These results partially agree with the somewhat low acute toxicity of organic UV-filters and 4-MBC reported before for benthic invertebrates (Schmitt et al., 2008). This is also in agreement with our preliminary studies where LC_{50} values higher than 100 mg 4-MBC/Kg for two benthic insects were observed.

The leaf litter processing was used as a functional endpoint and is generally an important indicator of the ecological status of freshwaters specially in the case of benthic communities dominated by detritivores such as this one (Gessner and Chauvet, 2002; McKie et al., 2008; Pestana et al., 2009; Woodward et al., 2012). In the present study, no significant effects of 4-MBC in terms of leaf litter processing were observed in comparison with control artificial streams (p= 0.829; H=0.622; Df= 2; figure 3a). This is a clear indication that 4-MBC did not elicit density mediated effects (no effect on the density of shredders) nor trait mediated effects (e.g. alteration of feeding behaviour). Indeed, although effects in macroinvertebrates structure communities were not expected since sub-lethal concentrations of 4-MBC were chosen, a decrease in leaf litter processing was to be expected given the toxic anorexia reported before for shredders species. In fact, and contrary to the present study, Campos et al., (2017a) observed a 50% reduction on *S. vittatum* feeding rates exposed to lower concentrations of 4-MBC within same periods of exposure. However, it should be noted that the study of Campos et al. (2017a) was conducted under 20ºC, while this mesocosms experiment was conducted at 15ºC. Thus, this disagreement can be partially explained by a decreased toxicity of 4-MBC due to lower metabolic rate of organisms at 15ºC in comparison with data from laboratory single species tests (Díaz Villanueva et al., 2011; Mas-Martí et al., 2015). This temperature dependent toxicity has been observed before for a wide range of contaminants and invertebrate species (Camp and Buchwalter 2016; Gomiero

and Viarengo, 2014; Nieto et al., 2016; Satpute et al., 2007; Weston et al 2009). Moreover, shredders and other benthic invertebrates may have been mostly attached to leaves within leaf packs therefore reducing they direct contact with the contaminated sediment and consequently direct exposure to the compound.

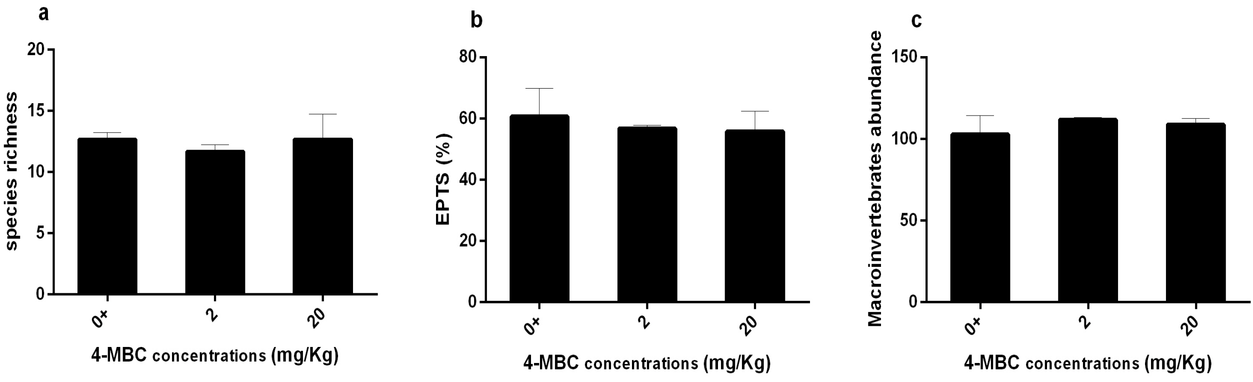


Figure 2 – Effects of 4-MBC exposure on a) species richness; b) Ephemeroptera, Plecoptera and Trichoptera (EPTs) (%-abundance) and c) macroinvertebrates abundance. All values are presented as mean ± SE.

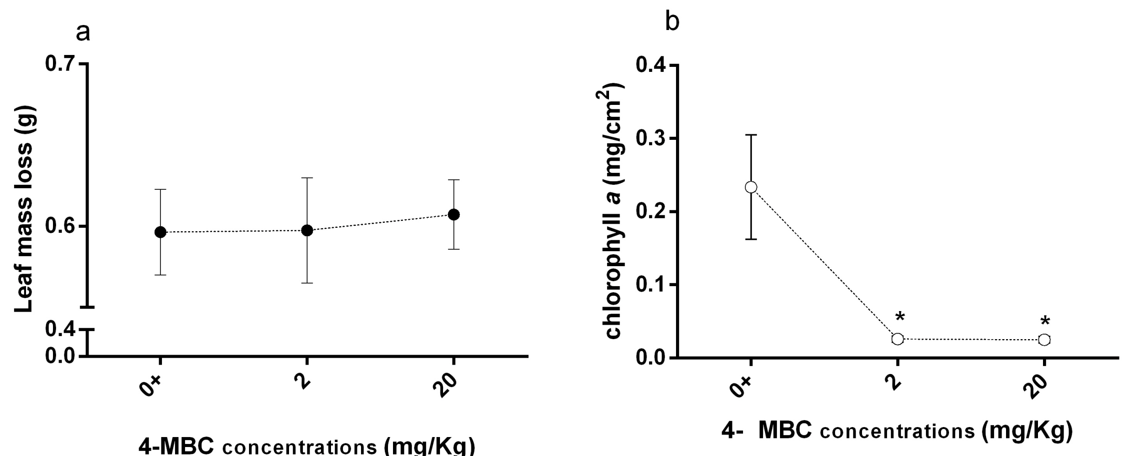


Figure 3 - Effects of 4-MBC exposure on a) leaf mass loss (g) and b) chlorophyll *a* concentration (mg/cm^2). All values are presented as mean ± SE. * denotes significant differences comparing with control treatments (0+); $p<0.05$.

In addition to allochthonous matter input, primary production is other source of organic matter in streams but few studies have been addressing the adverse effects of the contaminants in this fundamental process within freshwater ecosystems (Peters et al., 2013). In the present study, a strong effect in the primary production was observed with a higher decrease in the

chlorophyll *a* (p= 0.001; $F_{(2,6)}$ = 28.07; figure 3b) in contaminated artificial streams when compared with the control treatment. Generally, and when considering contaminants other than herbicides, changes on periphyton biomass and productivity are attributed to indirect effects of contaminants, when grazers density is directly affected by the compounds (Abelho et al., 2016; Rogers et al., 2016). However, in this study, no effects were observed in grazers density (p= 0.925; $F_{(2,6)}$ = 0.079; data not shown) and since we observed a reduction in chlorophyll *a* within periphyton samples , there is a clear suggestion that 4-MBC is directly impairing primary production and not indirectly through density effects or feeding inhibition from grazers. These results are in contrast to previous studies that revealed low toxicity of 4-MBC to freshwater algae (Rodil et al., 2009; Sieratowicz et al., 2011). The drastic effect observed in primary production might have been due to the adsorption and accumulation of the 4-MBC by the periphyton (Guasch et al., 2010; Writer et al 2011) even if only contaminated sediment was used. It has been however reported high sensitivity of some marine algae to 4MBC (EC50 = 171.45 µg/L) (Paredes et al., 2014) and significant inhibition of primary production can have profound effects in invertebrate communities and nutrient dynamics in streams (Gessner et al 2016; Hasenbein et al., 2017, Perschbacher and Ludwig 2004, Zou 2016). Effects on periphyton production caused by exposure to 4-MBC call for additional research on effects of UV-filters on freshwater ecosystems. This research would have to consider longer exposure periods to correctly assess effects on primary production and evaluate the long-term consequences of bottom up indirect effects. Also, the inclusion of other algae species in the toxicity assessments is desirable. In fact, periphyton samples are often dominated by diatoms in small to medium sized streams (Ghosh and Gaur et al., 1998) and diatom species composition and abundance are known indicators of ecological status of freshwater streams and lakes (Almeida and Feio, 2012; Delgado et al., 2017; Larras et al., 2017; Lavoie et al., 2014). Based on results presented here is probable that diatom species can be even more sensitive than phytoplankton species used in ecotoxicity studies and be putative good indicators of effects of organic UV-filters in freshwaters

To conclude, our results highlight the importance of using an approach that includes structural and functional parameters in the ecotoxicological studies to better understand the direct and indirect effects of organic-UV-filters in freshwater ecosystems. Moreover, the disconnection between laboratory ecotoxicity results pointing to impairment of feeding, growth and development of freshwater detritivore invertebrates (Campos et al., 2017a, b) exposed to organic UV-filters and the lack of effects on macroinvertebrate structural endpoints and leaf litter processing needs further research in terms of the mediating effects that temperature might elicit

on toxicity of organic UV-filters. Also, it is essential to monitor the seasonal presence and levels of these compounds in hotspots within freshwaters such as fluvial beaches and lakes. Ecological effects should also be studied *in situ* aiming to understand the long-term consequences of their presence in freshwater sediments and in important ecosystems functional parameters such as primary productivity.

References

Abelho, M., Martins, T.F., Shinn, C., Moreira-Santos, M., Ribeiro, R., 2016. Effects of the fungicide pyrimethanil on biofilm and organic matter processing in outdoor lentic mesocosms. Ecotoxicology 25, 121-131.

Almeida, S.F.P., Feio, M.J., 2012. DIATMOD: Diatom predictive model for quality assessment of Portuguese running waters. Hydrobiologia 695, 185-197.

Blijswijk, W. V., Coimbra, C. N., Graça, M. A. S., 2004. The use of biological methods based on macroinvertebrates to an Iberian stream (cental Portugal) receiving paper mil efluente. Limnetica 233-4, 307-314.

Camp, A.A., Buchwalter, D.B., 2016. Can't take the heat: Temperature-enhanced toxicity in the mayfly *Isonychia bicolor* exposed to the neonicotinoid insecticide imidacloprid. Aquatic Toxicology 178, 49-57.

Campos, D., Gravato, C., Fedorova, G., Burkina, V., Soares, A.M.V.M., Pestana, J.L.T., 2017a. Ecotoxicity of two organic UV-filters to the freshwater caddisfly *Sericostoma vittatum*. Environmental Pollution 228, 370-377.

Campos, D., Gravato, C., Quintaneiro, C., Golovko, O., Žlábek, V., Soares, A.M.V.M., Pestana, J.L.T., 2017b. Toxicity of organic UV-filters to the aquatic midge *Chironomus riparius*. Ecotoxicology and Environmental Safety 143, 210-216.

Chisvert, A., Salvador, A., 2007. 3.1 - UV Filters in Sunscreens and other Cosmetics. Regulatory Aspects and Analytical Methods, Analysis of Cosmetic Products. Elsevier, Amsterdam, pp. 83-120.

Dalu, T., Wasserman, R.J., Tonkin, J.D., Alexander, M.E., Dalu, M.T.B., Motitsoe, S.N., Manungo, K.I., Bepe, O., Dube, T., 2017. Assessing drivers of benthic macroinvertebrate community structure in African highland streams: An exploration using multivariate analysis. Science of The Total Environment 601-602, 1340-1348.

Delgado, C., Almeida, S.F.P., Elias, C.L., Ferreira, V., Canhoto, C., 2017. Response of biofilm growth to experimental warming in a temperate stream. Ecohydrology 10, e1868-n/a.

Díaz-Cruz, M., Llorca, M., Barceló, D., Barceló, D., 2008. Organic UV filters and their photodegradates, metabolites and disinfection by-products in the aquatic environment. TrAC Trends in Analytical Chemistry 27, 873-887.

Díaz Villanueva, V., Albariño, R., Canhoto, C., 2011. Detritivores feeding on poor quality food are more sensitive to increased temperatures. Hydrobiologia 678, 155-165.

Dossena, M., Yvon-Durocher, G., Grey, J., Montoya, J.M., Perkins, D.M., Trimmer, M., Woodward, G., 2012. Warming alters community size structure and ecosystem functioning. Proceedings of the Royal Society B: Biological Sciences 279, 3011-3019.

Environment Agency, 2008. UV-filters in cosmetics – prioritisation for environmental assessment. Environment Agency, Bristol, BS32 4UD

Evans, C.D., Bonn, A., Holden, J., Reed, M.S., Evans, M.G., Worrall, F., Couwenberg, J., Parnell, M., 2014. Relationships between anthropogenic pressures and ecosystem functions in UK blanket bogs: Linking process understanding to ecosystem service valuation. Ecosystem Services 9, 5-19.

Fleeger, J.W., Carman, K.R., Nisbet, R.M., 2003. Indirect effects of contaminants in aquatic ecosystems. Science of The Total Environment 317, 207-233.

Gago-Ferrero, P., Díaz-Cruz, M.S., Barceló, D., 2011. Fast pressurized liquid extraction with in-cell purification and analysis by liquid chromatography tandem mass spectrometry for the determination of UV filters and their degradation products in sediments. Analytical and Bioanalytical Chemistry 400, 2195-2204.

Gago-Ferrero, P., Díaz-Cruz, M.S., Barceló, D., 2012. An overview of UV-absorbing compounds (organic UV filters) in aquatic biota. Analytical and Bioanalytical Chemistry 404, 2597-2610.

Gessner, M.O., Chauvet, E., 2002. A case for using litter breakdown to assess functional stream integrity. Ecological Applications 12, 498-510.

Gessner, M.O., Tlili, A., 2016. Fostering integration of freshwater ecology with ecotoxicology. Freshwater Biology 61, 1991-2001.

Ghosh, M., Gaur, J.P., 1998. Current velocity and the establishment of stream algal periphyton communities. Aquatic Botany 60, 1-10.

Gomiero, A., Viarengo, A., 2014. Effects of elevated temperature on the toxicity of copper and oxytetracycline in the marine model, Euplotes crassus: A climate change perspective. Environmental Pollution 194, 262-271.

Graça, M.A.S., 2001. The Role of Invertebrates on Leaf Litter Decomposition in Streams – a Review. International Review of Hydrobiology 86, 383-393.

Guasch, H., Serra, A., Corcoll, N., Bonet, B., Leira, M., 2010. Metal Ecotoxicology in Fluvial Biofilms: Potential Influence of Water Scarcity, in: Sabater, S., Barceló, D. (Eds.), Water Scarcity in the Mediterranean: Perspectives Under Global Change. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 41-53.

Hasenbein, S., Lawler, S.P., Connon, R.E., 2017. An assessment of direct and indirect effects of two herbicides on aquatic communities. Environmental Toxicology and Chemistry 36, 2234-2244.

Jeffrey, S.W., Humphrey, G.F., 1975. New spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher plants, algae and natural phytoplankton. Biochemie und Physiologie der Pflanzen 167, 191-194.

Larras, F., Coulaud, R., Gautreau, E., Billoir, E., Rosebery, J., Usseglio-Polatera, P., 2017. Assessing anthropogenic pressures on streams: A random forest approach based on benthic diatom communities. Science of The Total Environment 586, 1101-1112.

Lavoie, I., Campeau, S., Zugic-Drakulic, N., Winter, J.G., Fortin, C., 2014. Using diatoms to monitor stream biological integrity in Eastern Canada: An overview of 10years of index development and ongoing challenges. Science of The Total Environment 475, 187-200.

Mas-Martí, E., Romaní, A.M., Muñoz, I., 2015. Consequences of Warming and Resource Quality on the Stoichiometry and Nutrient Cycling of a Stream Shredder. PLoS ONE 10, e0118520.

McKie, B.G., Woodward, G., Hladyz, S., Nistorescu, M., Preda, E., Popescu, C., Giller, P.S., Malmqvist, B., 2008. Ecosystem functioning in stream assemblages from different regions: contrasting responses to variation in detritivore richness, evenness and density. Journal of Animal Ecology 77, 495-504.

Naylor, C., Maltby, L., Calow, P., 1989. Scope for growth in Gammarus pulex, a freshwater benthic detritivore. Hydrobiologia 188, 517-523.

Nieto, E., Hampel, M., González-Ortegón, E., Drake, P., Blasco, J., 2016. Influence of temperature on toxicity of single pharmaceuticals and mixtures, in the crustacean A. desmarestii. Journal of Hazardous Materials 313, 159-169.

Paredes, E., Perez, S., Rodil, R., Quintana, J.B., Beiras, R., 2014. Ecotoxicological evaluation of four UV filters using marine organisms from different trophic levels *Isochrysis galbana*, *Mytilus galloprovincialis*, *Paracentrotus lividus*, and *Siriella armata*. Chemosphere 104, 44-50.

Perschbacher PW, Ludwig GM. 2004. Effects of diuron and other aerially applied cotton herbicides and defoliants on the plankton communities of aquaculture ponds. Aquaculture 233:197–203.

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

Peters, K., Bundschuh, M., Schäfer, R.B., 2013. Review on the effects of toxicants on freshwater ecosystem functions. Environmental Pollution 180, 324-329.

Ramos, S., Homem, V., Alves, A., Santos, L., 2015. Advances in analytical methods and occurrence of organic UV-filters in the environment — A review. Science of The Total Environment 526, 278-311.

Relyea, R., Hoverman, J., 2006. Assessing the ecology in ecotoxicology: a review and synthesis in freshwater systems. Ecology Letters 9, 1157-1171.

Rodil, R., Moeder, M., Altenburger, R., Schmitt-Jansen, M., 2009. Photostability and phytotoxicity of selected sunscreen agents and their degradation mixtures in water. Analytical and Bioanalytical Chemistry 395, 1513.

Rodil, R., Moeder, M., Altenburger, R., Schmitt-Jansen, M., 2009. Photostability and phytotoxicity of selected sunscreen agents and their degradation mixtures in water. Analytical and Bioanalytical Chemistry 395, 1513-1524.

Rogers, H.A., Schmidt, T.S., Dabney, B.L., Hladik, M.L., Mahler, B.J., Van Metre, P.C., 2016. Bifenthrin Causes Trophic Cascade and Altered Insect Emergence in Mesocosms: Implications for Small Streams. Environmental Science & Technology 50, 11974-11983.

Satpute, N.S., Deshmukh, S.D., Rao, N.G.V., Tikar, S.N., Moharil, M.P., Nimbalkar, S.A., 2007. Temperature-Dependent Variation in Toxicity of Insecticides Against *Earias vitella* (Lepidoptera: Noctuidae). Journal of Economic Entomology 100, 357-360.

Schmitt, C., Oetken, M., Dittberner, O., Wagner, M., Oehlmann, J., 2008. Endocrine modulation and toxic effects of two commonly used UV screens on the aquatic invertebrates *Potamopyrgus antipodarum* and *Lumbriculus variegatus*. Environmental Pollution 152, 322-329.

Sieratowicz, A., Kaiser, D., Behr, M., Oetken, M., Oehlmann, J., 2011. Acute and chronic toxicity of four frequently used UV filter substances for *Desmodesmus subspicatus* and *Daphnia magna*. J Environ Sci Health A Tox Hazard Subst Environ Eng 46, 1311-1319.

Stewart, R. I. A., M. Dossena, D. A. Bohan, E. Jeppesen, R. L. Kordas, M. E. Ledger, M. Meerhoff, B. Moss, C. Mulder, J. B. Shurin, B. Suttle, R. Thompson, M. Trimmer & G. Woodward, 2013. Mesocosm experiments as a tool for ecological climate change research. Advances in Ecological Research 48, 71–181.

Vidal, T., Santos, J.I., Marques, C.R., Pereira, J.L., Claro, M.T., Pereira, R., Castro, B.B., Soares, A., Gonçalves, F., 2014. Resilience of the macroinvertebrate community of a small mountain river (Mau River, Portugal) subject to multiple stresses. Marine and Freshwater Research 65, 633-644.

Weston, D.P., You, J., Harwood, A.D., Lydy, M.J., 2009. Whole sediment toxicity identification evaluation tools for pyrethroid insecticides: III. Temperature manipulation. Environmental Toxicology and Chemistry 28, 173-180.

Woodward, G., Gessner, M.O., Giller, P.S., Gulis, V., Hladyz, S., Lecerf, A., Malmqvist, B., McKie, B.G., Tiegs, S.D., Cariss, H., Dobson, M., Elosegi, A., Ferreira, V., Graça, M.A.S., Fleituch, T., Lacoursière, J.O., Nistorescu, M., Pozo, J., Risnoveanu, G., Schindler, M., Vadineanu, A., Vought, L.B.-M., Chauvet, E., 2012. Continental-Scale Effects of Nutrient Pollution on Stream Ecosystem Functioning. Science 336, 1438-1440.

Writer, J.H., Ryan, J.N., Barber, L.B., 2011. Role of biofilms in sorptive removal of steroidal hormones and 4-nonylphenol compounds from streams. Env. Sci. Technol. 45, 7275–7283

Young, R.G., Matthaei, C.D., Townsend, C.R., 2008. Organic matter breakdown and ecosystem metabolism: functional indicators for assessing river ecosystem health. Journal of the North American Benthological Society 27, 605-625.

Zou, K., Thébault, E., Lacroix, G., Barot, S., 2016. Interactions between the green and brown food web determine ecosystem functioning. Functional Ecology 30, 1454-1465.

Chapter 7:
General Discussion

General Discussion

The continuous use of organic UV-filters increases every day and consequently, it is expected that their concentrations in the environment increase over the time as well. These compounds are now considered emerging contaminants at a global scale with both marine and freshwater environments and biota as final recipients of such compounds. Organic UV-filters thus present a new threat to the ecological status of such ecosystems that urge consideration. Additionally, the persistence of organic UV-filters in sediments within complex chemical mixtures together with their potential for bioaccumulation and recent evidences of estrogenic activity call for an in-depth evaluation of their ecological effects.

Despite the above mentioned, studies assessing the effects of UV-filters are mainly based in standard single species tests. There is a lack of understanding of what concerns possible effects of these compounds under different levels of biological organization and realistic exposure scenarios. As such, this work was directed at some of these research gaps and focused on generating sound ecotoxicological data that can be used for regulation and for a better evaluation of ecological effects of organic UV-filters in freshwaters: i) evaluation of organismal and sub-organismal level effects in benthic aquatic organisms; ii) assessment of effects of binary mixtures containing UV-filters; iii) investigation of long-term, multigenerational effects; and iv) evaluation of effects towards natural benthic macroinvertebrates communities and on ecosystem functioning. Several conclusions can be summarized from all these results as well as future avenues for research.

Firstly, the individual effects of UV-filters on the performance of two benthic insect species *C. riparius* and *S. vittatum* were evaluated. Results clearly showed that **environmental relevant concentrations of UV-filters can indeed cause deleterious effects in these freshwater insects**. The two invertebrate species appear to be sensitive to UV-filters exposure and as such are suitable organisms to evaluate the effects of these contaminants on sediments. These results demonstrate the importance of monitoring the ecological effects of organic UV-filters using model and non-model invertebrate species.

Considering the above mentioned, and based on data for chironomids, the risk quotient (RQ) was determined to characterize the risk that UV-filters might present to sediment dwelling organisms. The RQ is calculated as a MEC/PNEC ratio, being the MEC the Measured Effect Concentration and the PNEC the Predicted No-Effect Concentration. If RQ > 1 means that the UV-filters present a risk (EPA, 2017). In this study, to perform the risk characterization the data used

to calculate the PNEC was obtained in the present work and the MEC was obtained from the literature. As showed in the table 1, **BP3 and 4-MBC present high risk to sediment dwelling organisms** highlighting the importance and the need of studies with benthic species in the environmental risk assessments of contaminants with high lipophilicity.

Table 1 - Risk assessment of UV-filters in sediments. NOEC: No observed effect concentration; PNEC: Predicted no-effect concentration; MEC: Measured environmental concentration; RQ: Risk quotient.

	NOEC (mg/Kg)	Assessment factor	PNEC	MEC (mg/Kg)	RQ	Environmental risk
BP3	1.55 [a]	100 [b]	0.015	0.051 [c]	3.290	Yes
4-MBC	0.80 [a]	100 [b]	0.008	0.049 [c]	6.125	Yes

a- NOEC obtained in chapter 2 (endpoint: development time of *C. riparius*).
b- Assessment factor for derivation of $PNEC_{sediment}$ considering NOEC of one long-term test (ECHA, 2008).
c- To our knowledge, maximum concentration of BP3 and 4-MBC reported in sediments (Mizukawa et al., 2017).

These results clearly indicate that investigation should continue towards the effects of these compounds in order to identify the most sensitive ecological receptor to UV-filters in the aquatic environment. For that and given the low solubility of organic UV-filters, efforts should be directed at evaluating chronic, sub-lethal responses in an array of species and trophic guilds paving the way for other approaches including the estimation of Species Sensitivity Distribution curves. Also and to complement laboratory studies, environmental chemical and biological monitoring and "*in situ*" assays with benthic invertebrate species can be instrumental to evaluate the true ecological effects of organic UV-filters that are expected to be present in sediments of aquatic systems under anthropogenic pressure (e.g downstream of effluents and fluvial beaches). As far as we known, there is only one study that assesses and reports the presence of UV-filters in the Portuguese coast (Picot Groz et al., 2014), however, no information was reported for Portuguese freshwaters.

The scarcity of studies assessing the effects of UV-filters at sub-organismal level together with their unknown specific mode of action in invertebrates, led us to assess the effects of these contaminants using a biomarker approach. The results suggest that the *C. riparius* and *S. vittatum* presented different patterns of responses to UV-filters at sub-organismal level indicating different strategies for detoxification of different compounds in these two insect species. However, and **despite evidences of metabolic costs related with detoxification, UV-filters did not cause oxidative stress neither neurotoxicity at the tested concentrations in both organisms.** It is

plausible that other biochemical endpoints could show higher sensitivity and specificity towards exposure to organic UV-filters based on their endocrine activity. Even if not easily translated to population level effects, endocrine screening biochemical assays (focusing on hormones, vitellogenin, etc.) should be pursued and applied in invertebrates in order to complement reproductive endpoints and mechanistically address toxicity of these compounds and differences in sensitivity of different larval stages or different species.

As stated before UV-filters, as most contaminants, are present in natural environments in complex mixtures which may lead to additive, synergistic or antagonistic effects. Given the lack of research considering effects of complex mixtures containing organic UV-filters, effects of binary mixtures of BP3 - 4-MBC and BP3 - DEET (an insect repellent) on *C. riparius* were evaluated. **Synergistic interactions in both mixtures (i.e.: UV-filters mixtures and mixtures of UV filters and insect repellents) were found for development and emergence rates of exposed organisms.** Despite variation in response to these mixtures were observed, considering the different life-history traits analysed, **our results indicate thus that individual chemical testing can underestimate toxicity of organic UV-filters to freshwater organisms.** Given their physical-chemical properties (e.g. high log K_{ow}), and given that many personal care products contain a mixture of different organic UV-filters, these compounds have a great potential to simultaneously contaminate and be present on freshwaters. This is also the case with UV filters and insect repellents used concomitantly by the human population. As such, and given the toxicity of UV-filters, our results suggest that the actual risk for freshwater invertebrates can even be greater than expected from standard tests with single chemical exposures**.** Thus, more studies assessing the effects of different combinations of UV-filters and also UV-filters with others personal care products are important since the probability of its occurrence together in the environment is high. Moreover, UV-filters are also used in several materials such as plastics. Thus, studies assessing the combined effects of UV-filters with microplastics (also present in cosmetics (Fendall et al., 2009)) might be interesting and extremely relevant, taking also into account that microplastics are a recent global concern. In fact, given that microplastics can be vectors of hydrophobic compounds, altering their bioavailability to biota (Bakir et al., 2014, Cole et al., 2013; Oliveira et al., 2013, Vethaak and Leslie, 2016) and that organic UV-filters are used also to coat plastic materials studying the combined effects of these two stressors can be a fruitful area of research.

Given the persistence of organic UV-filters in sediments it is important to address their long-term effects to biota. Here a two-generational approach was used to address effects of BP3 on *C. riparius*. Our results also showed a strong dose-response in fertility of exposed organisms.

Furthermore, **continuous exposure to BP3 concentrations used, delayed emergence and development time in the F1 generation in contrast with no effects observed for the parental generation. These results clearly show reproductive effects of BP3 on *C. riparius* that would not be detected using standard tests**. As such, it is advisable to include reproductive endpoints in the evaluation of chronic effects of chemicals with Chironomus including fecundity and fertility as measured endpoints in the standard 28-days partial life cycle assays. However, the higher sensitivity observed in the F1 generation illustrate the need for multigenerational assays that are critical to properly evaluate the population level effects of endocrine disrupting compounds such as organic UV-filters. Moreover, our results also illustrate that effects of organic UV-filters might not always be dose-dependent which is a common feature between several endocrine disruptors with lower concentrations causing more severe effects than higher ones (Coronado et al., 2008; Kim et al., 2014 Patlak, 1996; Santillo et al., 1998). Furthermore, **carry-over effects of BP3 with reduced emergence and changes in development time were observed in *C. riparius* F1 generation maintained under control/clean conditions but whose parents were previously exposed to BP3. These results thus suggest that BP3 might cause transgenerational effects in *C. riparius* population.** Although a standardized protocol for multigenerational exposures with *C. riparius* is available some refinement on the protocol including the exposure of at least two more generations to chemicals would certainly improve the sensitivity of the test and allow to distinguish true epigenetic and transgenerational effects (Skinner et al., 2011) and also ascertain how sensitivity is altered throughout generations.

Despite the importance of the above mentioned data from laboratory assays, the evaluation of ecological effects of UV-filters in freshwaters cannot be complete without the consideration of more ecological relevant scenarios of exposure, namely the inclusion of multispecies assemblages and possible trait and density mediated indirect effects. It is also detrimental to assess if and how effects observed in individual organisms and laboratory exposures are translated into altered ecosystems function. Mesocosms are considered high tier approaches in ecotoxicology and they were used to evaluate the effects of 4-MBC on freshwater benthic invertebrate communities and ecosystem functions such as primary production and leaf decomposition. **Exposure to sediments contaminated with 4-MBC did not cause alterations on the structure of the macroinvertebrates community nor altered rates of leaf decomposition.** These results were somehow surprising since they were not related with effects observed in single species toxicity tests using *S. vittatum* and *C. riparius,* two detritivore species. We previously stated that *S. vittatum* and *C. riparius* were sensitive organisms to assess the effects of UV-filters in the aquatic ecosystems (according to

single standard tests) and the results obtained in mesocosms experiment can lead to different conclusions. However, this mismatch between effects observed in laboratory assays with insect species and lack of effects at the invertebrate community level may also arise from different experimental conditions namely a lower temperature used in mesocosms set-up that could have altered the toxicity of 4-MBC (Abdel-Tawwab and Wafeek, 2017; DeLorenzo et al., 2009). Thus, the mediating effects of temperature on organic-UV-filters should be investigated in the future. Moreover, the fact that only artificial sediment (but not food, i.e. leaf litter) was contaminated in the streams used, may also have allowed invertebrates to avoid the contaminant and remain in the leaf packs where they were feeding. Consequently, assays using contaminated food will be interesting to test this hypothesis. Although the results obtained in mesocosms trials showed no clear effects, after 7 days of 4-MBC exposure, on the benthic macroinvertebrates, **there was a drastic decrease in primary production caused by 4-MBC exposure**. This unexpected effect clearly calls for more research on the sensitivity of primary producers, namely diatoms, to organic UV-filters, as well as on the consequent bottom-up indirect effects caused by reductions in primary production. Although laborious, community ecotoxicology approaches using mesocosms systems are ideal to test for such effects. Future work should also consider the use of more natural sediments to better address the bioavailability of organic UV-filters to natural benthic communities. Finally, multispecies assays should also be used to address bioaccumulation and transport of organic UV-filters along food webs.

In conclusion, organic UV-filters are considered global emergent contaminants of aquatic ecosystems whose ecological effects urge to address. All findings reported in this thesis demonstrate the toxicity and potential deleterious effects of these compounds. At the same time, all results illustrate the validity and importance of an integrated ecotoxicological approach using aquatic invertebrates and different levels of biological organization and can surely contribute to a better environmental risk assessment of organic UV-filters in freshwaters.

References

Abdel-Tawwab, M., Wafeek, M., 2017. Fluctuations in water temperature affected waterborne cadmium toxicity: Hematology, anaerobic glucose pathway, and oxidative stress status of Nile tilapia, *Oreochromis niloticus* (L.). Aquaculture 477, 106-111.

Bakir, A., Rowland, S. J. and Thompson, R. C. (2014) "Enhanced desorption of persistent organic pollutants from microplastics under simulated physiological conditions," Environmental Pollution: 185, pp. 16–23.

Cole, M., Lindeque, P., Fileman, E., Halsband, C., Goodhead, R., Moger, J., Galloway, T.S., 2013. Microplastic ingestion by zooplankton. Environ. Sci. Technol. 47, 6646-6655.

Coronado, M., De Haro, H., Deng, X., Rempel, M.A., Lavado, R., Schlenk, D., 2008. Estrogenic activity and reproductive effects of the UV-filter oxybenzone (2-hydroxy-4-methoxyphenyl-methanone) in fish. Aquatic Toxicology 90, 182-187.

DeLorenzo, M.E., Wallace, S.C., Danese, L.E., Baird, T.D., 2009. Temperature and salinity effects on the toxicity of common pesticides to the grass shrimp, *Palaemonetes pugio*. Journal of Environmental Science and Health, Part B 44, 455-460

ECHA: European Chemicals Agency, 2008. Characterization of dose [concentration]-response for environment. Guidance on Information Requirements and Chemical Safety Assessment.

EPA, U.S., 2017.Ecological Risk assessment. U.S. EPA: https://www.epa.gov/risk/conducting-ecological-risk-assessment, acedido em 2017.

Fendall, L. S.; Sewell, M. A., 2009. Contributing to marine pollution by washing your face: Microplastics in facial cleansers. Mar. Pollut. Bull. 58, 1225−1228.

Kim, S., Jung, D., Kho, Y., Choi, K., 2014. Effects of benzophenone-3 exposure on endocrine disruption and reproduction of Japanese medaka (*Oryzias latipes*)—A two generation exposure study. Aquatic Toxicology 155, 244-252.

Mizukawa, A., Molins-Delgado, D., Azevedo, J.C.R., Fernandes C. V. S., Díaz-Cruz S., Barceló, D., 2017. Sediments as a sink for UV filters and benzotriazoles: the case study of Upper Iguaçu watershed, Curitiba (Brazil). Environ Sci Pollut Res 24, 18284–18294.

Oliveira, M., Ribeiro, A., Hylland, K., Guilhermino, L., 2013.Single and combined effects of microplastics and pyrene on juveniles (0+ group) of the common goby *Pomatoschistus microps* (Teleostei, Gobiidae). Ecological Indicators 34:641–647.

Patlak, M., 1996. A testing deadline for endocrine disrupters. Environmental Science & Technology 30, 540A-544A.

Picot Groz, M., Martinez Bueno, M.J., Rosain, D., Fenet, H., Casellas, C., Pereira, C., Maria, V., Bebianno, M.J., Gomez, E., 2014. Detection of emerging contaminants (UV filters, UV stabilizers and musks) in marine mussels from Portuguese coast by QuEChERS extraction and GC–MS/MS. Science of The Total Environment 493, 162-169.

Santillo, D., Stringer, R.L., Johnston, P.A., Tickner, J., 1998. The precautionary principle: Protecting against failures of scientific method and risk assessment. Marine Pollution Bulletin 36, 939-950.

Skinner, M.K., Manikkam, M., Guerrero-Bosagna, C., 2011. Epigenetic transgenerational actions of Endocrine Disruptors Reproductive toxicology (Elmsford, N.Y.) 31, 337-343.

Vethaak, A. Dick, and Heather A. Leslie. 2016. “Plastic Debris Is a Human Health Issue.” Environmental Science and Technology 50 (13): 6825–26.

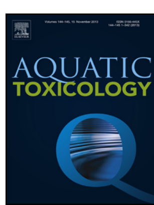
Annex I

Aquatic Toxicology 172 (2016) 80–85

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox



Responses of the aquatic midge *Chironomus riparius* to DEET exposure

Diana Campos, Carlos Gravato, Carla Quintaneiro, Amadeu M.V.M. Soares, João L.T. Pestana*

Departamento de Biologia & CESAM, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

ARTICLE INFO

Article history:
Received 25 September 2015
Received in revised form
18 December 2015
Accepted 29 December 2015
Available online 4 January 2016

Keywords:
Personal care products
Freshwater insects
Emergent pollutants
Biochemical responses
N,N-diethyl-3-methylbenzamide

ABSTRACT

N,N-diethyl-3-methylbenzamide (DEET) is the active ingredient of many commercial insect repellents. Despite being detected worldwide in effluents, surface water and groundwater, there is still limited information on DEET's toxicity toward non-target aquatic invertebrates. Thus, our main objective was to assess the effects of DEET in the life cycle of *Chironomus riparius* and assess its biochemical effects. Laboratory assays showed that DEET reduced developmental rates (reduced larval growth, delayed emergence) of *C. riparius* larvae and also caused a decrease in the size of adult midges. Concerning the biochemical responses, a short exposure to DEET caused no effects in lipid peroxidation, despite the significant inhibition of catalase and glutathione-*S*-transferase activities and of total glutathione contents. Moreover, inhibition of acetylcholinesterase activity was also observed showing neurotoxic effects. Environmental risk assessment of insect repellents is needed. Our results showed moderate toxicity of DEET toward *C. riparius*, however, due to their mode of action, indirect ecological effects of DEET and of other insect repellents cannot be excluded and should be evaluated.

© 2015 Elsevier B.V. All rights reserved.

1. Introduction

Personal care products (PCPs), including insect repellents, are prone to reach the environment through wastewater treatment plants (Stuart et al., 2012), since this group of emerging contaminants can be excreted via urine if it is first absorbed in the body after skin wash (Brausch and Rand, 2011; Pedrouzo et al., 2011). Insect repellents are used for protection against insect bites, whereby they prevent the spread of diseases and play an important role in the prevention and control of outbreaks (Liu et al., 2013).

Initially, *N,N*-diethyl-3-methylbenzamide (DEET), the synthetic active ingredient present in most commercial insect repellents (Costanzo et al., 2007), was considered to be a non-bioaccumulative and non-persistent compound (USEPA, 1998) and environmental risk assessment was not considered necessary (Costanzo et al., 2007). However, DEET has been routinely detected in aquatic environments worldwide and in different matrices (Costanzo et al., 2007). For example, in Australia a maximum concentration of 0.14 μg/L, 0.49 μg/L and 1.5 μg/L was found in effluent, surface water and in influent respectively (Costanzo et al., 2007) and in the USA, DEET was also found in effluents (2.1 μg/L) and surface waters (0.64 μg/L) (Glassmeyer et al., 2005). In Europe, DEET is routinely found in river water at concentrations reaching 0.16 μg/L (Calza et al., 2011) and in seawater at concentrations of 0.013 μg/L (Weigel et al., 2004). In South Korea, Yoon et al. (2010) also detected DEET in surface water and in effluents (0.09 and 0.19 μg/L, respectively).

DEET's repellent properties have been studied mostly in mosquito species which are harmful to human health, as, for example, *Culex quinquefasciatus*, *Aedes aegypti* and *Anopheles albimanus* (Leal, 2014). The mode of action of DEET still causes controversy amongst scientists. Initially, it was though that DEET interfered with the detection of lactic acid on host by insects (Dogan et al., 1999) but this hypothesis has already been discarded. DEET has been shown to cause avoidance behavior by activation of olfactory neurons (Syed and Leal, 2008) in *C. quinquefasciatus* (Syed and Leal, 2008) and *A. aegypti* (Stanczyk et al., 2010) and in *Drosophila melanogaster* the data obtained by Pellegrino et al. (2011) suggested that DEET acts by disturbing the insect odor sense. On the other hand, Lee et al. (2010) showed that DEET inhibited feeding behavior in *D. melanogaster* by direct activation of its gustatory receptor neurons. Recent studies showed that DEET can act through direct contact or by distance (Leal, 2014), affecting the gustatory and olfactory receptor neurons of insects (Ditzen et al., 2008; Lee et al., 2010). Together these studies highlight that DEET has a neurotoxic effect on insects that can result in behavioral (feeding, avoidance) impairment with potentially important physiological consequences.

Due to its presence in many freshwater systems, understanding the ecological effects of DEET is a pertinent issue. *Chironomus riparius* Meigen (Diptera: Chironomidae) is a benthic species widely

* Corresponding author.
E-mail addresses: jpestana@ua.pt, jltpestana9@gmail.com (J.L.T. Pestana).

http://dx.doi.org/10.1016/j.aquatox.2015.12.020
0166-445X/© 2015 Elsevier B.V. All rights reserved.

used in ecotoxicological tests to evaluate the toxicity of different environmental stressors, their mixtures on different organismal and biochemical endpoints (Campos et al., 2014; Pestana et al., 2009; Rodrigues et al., 2015a,b). Although chironomids are non-biting midges, they have a very important role in the ecosystem because they serve as food for many other macroinvertebrates and vertebrates, and also in the detritus processing of organic matter (Pery et al., 2002). It is plausible that exposure to DEET can elicit deleterious effects on non-target insects through neurotoxicity and/or physiological impairment. Laboratory tests using chironomids and a set of organismal and biochemical endpoints are thus ideal to investigate the effects and mechanisms of action of insect repellents in non-target aquatic insects.

The aim of this study was thus to investigate individual and biochemical effects of DEET on the freshwater insect, *C. riparius*. Larval growth, percentage of emergence, time to emergence and adult body size were the individual-level responses selected to assess the chronic effects of DEET. Acetylcholinesterase activity, oxidative stress biomarkers and antioxidant defences were selected as endpoints to assess the biochemical effects of DEET in these aquatic larvae.

2. Materials and methods

2.1. Test organism

C. riparius used in all experiments were obtained from a laboratory culture established for more than 15 years in the University of Aveiro. Organisms in these cultures are maintained at constant room temperature (20 °C ± 1 °C) in plastic containers with previously burnt (500° C for 4 h) inorganic fine sediment (<1 mm), American Society for Testing Materials (ASTM) hard water, and a 16:8 h light-dark photoperiod. Larvae are fed *ad libitum* every two days with a suspension of macerated Tetramin® (Germany) and the medium is changed weekly.

2.2. Experimental design

2.2.1. Chronic 28-days full life cycle test

The chronic toxicity experiments were performed according to OECD 219 guideline (OECD, 2004) and first instar (less than 48 h post hatching) larvae were used. Larvae were exposed to a gradient of five concentrations (8, 12, 18, 27 and 40.5 mg/L) of DEET (CAS number: 134-62-3; molecular weight: 191.27; Sigma–Aldrich, Germany) (chosen on the basis of preliminary tests) plus a control treatment (ASTM hard water only) and in each treatment twelve replicates with five larvae each were used. The glass vials contained 150 mL of medium and 1 cm layer of sediment. During the test, organisms were fed every two days with a suspension of macerated Tetramin® (0.5 mg per organism per day) and the test conditions were the same as described for culturing. After ten days, six replicates of each treatment were sacrificed, larvae were counted and placed in 70% ethanol for measurement of body length. The total length of each larvae was measured with a stereo dissecting microscope fitted with a calibrated eyepiece micrometer. The initial size of larvae at the beginning of the test (pool of 20 larvae) was subtracted from final larvae length to estimate *C. riparius* larval growth over this 10-day period. The six remaining replicates were used to assess the cumulative percentage of emergence, the male/female ratio and the mean time to emergence of organisms in all treatments by day 28. For that, adult midges (imagos) were collected daily from emergent traps with the aid of an aspirator and preserved in 70% ethanol. Imagos were then dried at 50 °C for 24 h and weighed in a microbalance (Mettler UMT2).

2.2.2. Biochemical responses

Fourth instar *C. riparius* larvae (12 days) were exposed for 48 h to a gradient of three DEET concentrations (8, 18 and 40.5 mg/L) plus a control treatment (ASTM hard water only) under the same conditions as in culturing. Seven replicates with fifteen larvae were used. Each crystalizing dish contained 200 mL of medium and 1 cm layer of sediment. No food was provided during exposure. After 48 h, larvae were quickly dried on filter paper, immediately weighed, frozen in liquid nitrogen and stored at −80 °C until use.

In order to determine lipid peroxidation (LPO), protein and enzyme activities each frozen pool of larvae was homogenized by sonication in 1600 μL of Milli-Q water. For LPO an aliquot of 150 μL was removed and 4 μL of 4% BHT (2,6-Di-*tert*-butyl4-methylphenol) in methanol were added. To the remaining volume was added a K-phosphate buffer (pH 7.4, 0.2 M) following centrifugation at 10,000 × *g* for 20 min at 4 °C and the post-mitochondrial supernatant (PMS) was separated in aliquots for catalase (CAT), glutathione-*S*-transferase (GST), and acetylcholinesterase (AChE) activity, total glutathione (TG) and protein concentration quantification.

2.2.2.1. Lipid peroxidation. LPO was measured by thiobarbituric acid reactive substances (TBARS) assay (Bird and Draper, 1984; Ohkawa et al., 1979). The reaction included a mixture of 150 μL of homogenate separated for LPO, 4 μL 4% BHT, 500 μL of 12% (w/v) trichloroacetic acid sodium salt, 50 μL of 0.73% (w/v) 2-thiobarbituric acid and 400 μL of 60 mM Tris–HCl with 0.1 mM diethylenetriaminepentaacetic acid. The mixture was incubated for 60 min at 100 °C, centrifuged for 5 min at 11,500 rpm, kept away from the light and absorbance was read at 535 nm. The results were expressed as nmol TBARS per g of weight using a $\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

2.2.2.2. Catalase activity. CAT activity was determined by measuring decomposition of the substrate hydrogen peroxide (H_2O_2) (Clairborne, 1985). The reaction included a mixture of 135 μL of K-phosphate (pH 7.0; 0.05 M), 150 μL of 30% H_2O_2 and 15 μL of PMS. Absorbance was read at 240 nm during 2 min. The results were expressed as μmol per min per mg of protein using a $\varepsilon = 40\ M^{-1}\ cm^{-1}$.

2.2.2.3. Glutathione-S-transferase activity. GST activity was measured based on the method describe by Habig et al. (1974). Two hundred microliters of reaction solution containing 4950 μL of phosphate buffer (0.1 M; pH 6.5), 150 μL 10 mM 1-chloro-2,4-dinitrobenzene and 900 μL of 10 mM reduced L-glutathione were mixed with 100 μL of PMS. The absorbance was read at 340 nm, for 5 min. The enzymatic activity was expressed in nmol per min per mg of protein using a $\varepsilon = 9.6 \times 10^3\ M^{-1}\ cm^{-1}$.

2.2.2.4. Acetylcholinesterase activity. The enzymatic activity of AChE was measured following Ellman's method (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996). Fifty microliters of PMS were mixed with 250 μL of reaction solution. The reaction solution was made with 30 mL of K-phosphate buffer (0.1 M; pH 7.2), 200 μL of 0.075 M acetylthiocholine iodide solution and 1 mL of 10 mM 5,5′-dithiobis(2-nitrobenbenzoic acid). The absorbance was read at 414 nm at 10 and 15 min. The enzymatic activity was expressed in nmol per min per mg of protein using a $\varepsilon = 13.6 \times 10^3\ M^{-1}\ cm^{-1}$.

2.2.2.5. Total glutathione content. Total glutathione levels were measured by addition of 250 μL of reaction solution and 50 μL of PMS following the method described by Baker et al. (1990). The reaction solution was made with 18 mL of Na-K phosphate buffer (0.2 M; pH 8.0), 3 mL of β-nicotinamide adenine dinucleotide

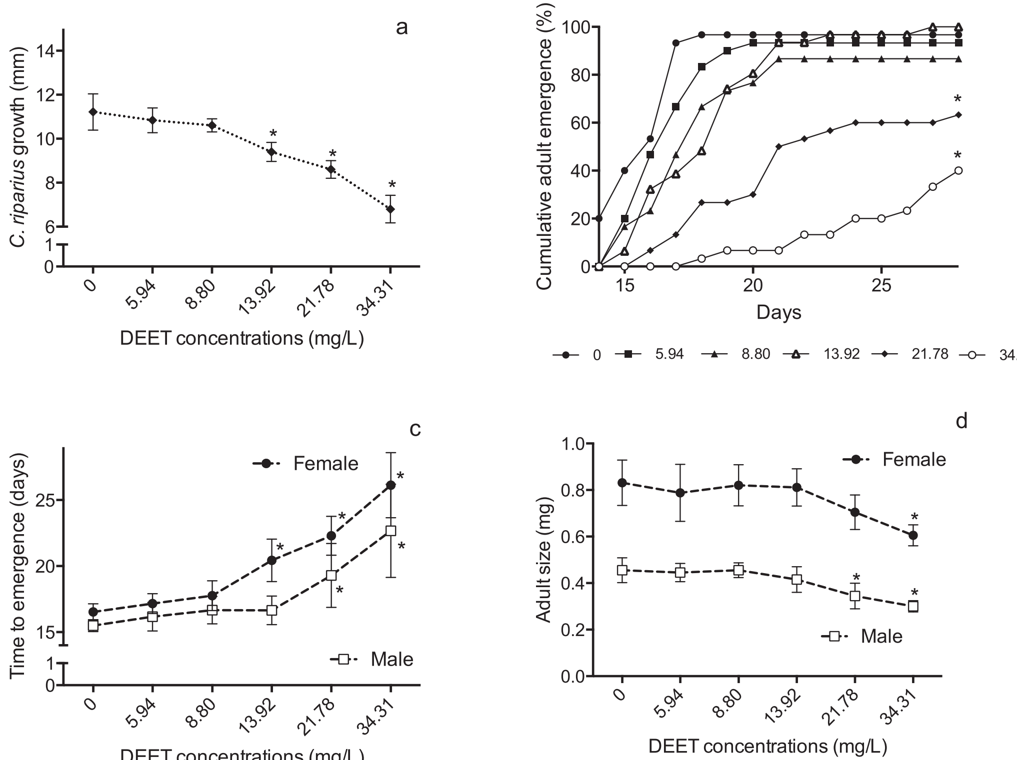


Fig. 1. Effects of DEET exposure on (a) *C. riparius* larval growth over 10 days (mm; mean ± SD), (b) *C. riparius* cumulative emergence (%), (c) time to emergence (days; mean; ± SD) and (d) adult body weight (mg; mean ± SD) * denotes a significant difference compared to control (0) treatment at $p < 0.05$ (Dunnett's test). * in cumulative emergence corresponds to differences in total percentage of emergence.

2′-phosphate reduced tetrasodium salt, 6 mL of 10 mM 5,5′-dithiobis(2-nitrobenzoic acid) and 1.5 mL of glutathione reductase (25 µL from stock with 1 U/mL). The absorbance was read at 412 nm for 3 min following the recycling reaction of reduced glutathione in the presence of an excess of glutathione reductase. Total glutathione levels, expressed as µmol per mg of protein, were then calculated using a standard curve with L-GSH as a standard.

2.2.2.6. Protein quantification. The protein concentration was quantified with Bradford method (Bradford, 1976) adapted from BioRad's Bradford micro-assay, using γ-globulin as a standard. For reaction 10 µL of PMS and 250 µL of BioRad solution were mixed and 15 min later the absorbance was read at 590 nm.

2.3. Chemical analysis

Determinations of DEET in water samples were performed by liquid chromatography-tandem mass spectrometry (LCMS/MS) using a water and methanol as a mobile phase and a C18 analytical column. 6460 system with electrospray ionisation in positive polarity was used for the determination of DEET concentrations in water samples. The lower detection limit in water was 10 ng/L. The real concentrations of DEET in water of the life cycle test were measured ten days after contamination and for each treatment three composite replicates were analyzed. For the biomarker exposure, DEET measurements were performed at the end of the exposure period (48 h).

2.4. Statistical analysis

Significant effects of DEET exposure on *C. riparius* biochemical and life-history responses were evaluated using analysis of variance (ANOVA) with multiple comparisons examined by Dunnett's *post hoc* test. For all statistical tests the significance level was set at $p < 0.05$. All variables were assessed for normality using residual probability plots while Levene's and Bartlett's tests verified the homoscedascity of data ($p > 0.05$). The emergence time of males, LPO and AChE data were Log-transformed to correct for unequal variances. All statistical analyses were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA).

3. Results

3.1. DEET concentrations in water

The values of nominal and measured concentrations for the chronic (10 days) and biochemical exposure (48 h) are presented in Table 1. Measured DEET concentrations in water were up to 27% lower than nominal concentrations after 10 days of exposure. The half time of DEET in surface water is estimated to be up to 15 days (Weeks et al., 2012). Besides photodegradation and microbial degradation of DEET in our experimental system we cannot exclude the adsorption of DEET to the sediment and/or added food. Measured concentrations are presented in all results and figures.

3.2. Life cycle endpoints

Larval growth was affected in *C. riparius* exposed to DEET with a LOEC of 13.92 mg/L (Fig. 1a; $F_{(5,35)} = 55.48$; $p < 0.001$). In the three highest concentrations of DEET, *C. riparius* larval growth was significantly reduced by 16, 23 and 39%, respectively.

At the end of the test (28 days), and in the two highest concentrations, some larvae, which did not reach the pupal or adult stage,

Table 1
DEET concentrations measured in overlying water (mg/L) after ten days in chronic exposure and after 48 h in biomarkers exposure (mean ± SD).

	Nominal concentrations (mg/L)	Real concentrations (mg/L)
Chronic exposure (10 days)	8	5.94 (±0.04)
	12	8.80 (±0.46)
	18	13.92 (±0.44)
	27	21.78 (±0.61)
	40.5	34.31 (0.55)
Biomarkers exposure (48 h)	8	6.90
	18	16.20
	40.5	38.94

were still recovered from the sediment. The percentage of emergence was 96.67% in the control treatment. The total percentage of emergence was significantly affected by DEET (Fig. 1b; $F_{5,35} = 11.19$; $p < 0.001$) with significant reductions of 33.34 and 56.67% in concentrations of 21.78 and 34.31 mg/L, respectively, when compared with the control treatment. Time to emergence of female and male *C. riparius* adults decreased significantly in response to DEET exposure (Fig. 1c) with a LOEC of 13.92 mg/L for females ($F_{5,29} = 31.60$; $p < 0.001$) and 21.78 mg/L for males ($F_{5,32} = 10.89$; $p < 0.001$). Also, *C. riparius* imagos were smaller than the control ones. Size of female imagos was significantly lower at 34.31 mg/L compared to control imagos (Fig. 1d; $F_{5,28} = 4.001$; $p < 0.01$), while *C. riparius* males imagos' size decreased significantly in response to DEET, when compared to control treatment (Fig. 1d; $F_{5,33} = 9.958$; $p < 0.001$, LOEC of 21.78 mg/L).

3.3. Biochemical responses

Levels of LPO were not significantly altered in *C. riparius* larvae exposed to DEET for 48 h (Fig. 2a; $F_{3,24} = 20.33$; $p > 0.05$). However, *C. riparius* larvae exposed to DEET showed a significant decrease of catalase activity in all tested concentrations compared to the control treatment (Fig. 2b; $F_{3,26} = 6.592$; $p < 0.01$). Moreover, GST activity in larvae of *C. riparius* was significantly reduced with a LOEC of 38.94 mg/L (Fig. 2c; $F_{3,25} = 4.105$; $p < 0.05$) compared to control. Furthermore, AChE activity was significantly inhibited by DEET in larvae of *C. riparius* in a concentration-dependent manner, with a LOEC of 6.90 μg/L (Fig. 2e; $F_{3,24} = 20.33$; $p < 0.001$). Levels of TG were also significantly decreased by exposure to DEET (Fig. 2d ($F_{3,27} = 3.918$; $p < 0.05$)) compared to control.

4. Discussion

Insect repellents such as DEET, are currently found in many freshwater systems in increasing concentrations and their ecological effects need to be evaluated. Our study shows that DEET is only moderately toxic to chironomids. Exposure to high concentrations of DEET cause significant changes in growth and emergence patterns of *C. riparius*. Neurotoxicity, inhibition of antioxidant defences and phase II conjugation enzymes (CAT and GST) and reduction of glutathione levels were also observed in *C. riparius* larvae under short-term exposures to sub-lethal concentrations of DEET.

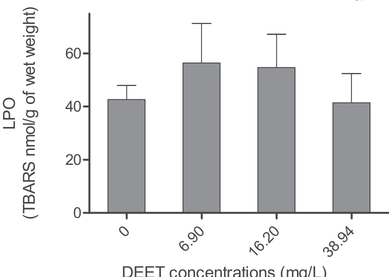
Studies concerning the ecotoxicity of DEET to aquatic organisms are scarce and mainly focused on acute toxicity studies (Costanzo et al., 2007). Deleterious effects of DEET concentrations usually found in natural environment, in the μg/L order, are not expected, based on DEET's ecotoxicological data for crustaceans such as *Daphnia magna* with an 48 h-LC_{50} of 160 mg/L (Seo et al., 2005), and fish, i.e., *Gambusia affinis* and *Oncorhynchus mykiss* with an 48 h-LC_{50} of 235 mg/L and 96 h-LC_{50} of 71.3 mg/L (Michael and Grant, 1974; USEPA, 1998), respectively. Acute tests conducted with 2nd instar *C. riparius* larvae showed that 96 h-LC_{50} was higher than 70 mg/L (unpublished data).

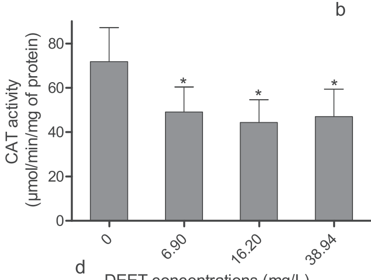
Life history parameters are key endpoints used to assess detrimental effects of contamination to aquatic insects and the effects of DEET exposure on *C. riparius* growth and delayed emergence are indicative of population level effects. DEET concentrations tested in our study, and eliciting such effects, are, however, much higher than the ones found in the aquatic environment. Costanzo et al. (2007) and Aronson et al. (2012) had already stated that it was unlikely for DEET to cause effects in aquatic ecosystems at environmentally relevant concentrations. In literature, reports of sub-lethal effects of DEET and of other insect repellents in aquatic organisms are scarce and are focused on potential of repellency and pharmacological mode of action (Bohbot and Dickens, 2012; Xu et al., 2014). Nevertheless, DEET exposure has been shown to cause chronic toxicity toward *D. magna* growth and reproduction with NOEC's of 3.7 mg/L (length) and 14 mg/L (reproduction assessments) respectively (Aronson et al., 2012).

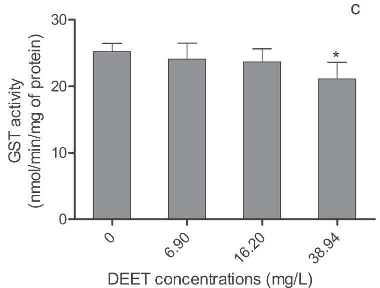
Our results show that, besides altering the emergence pattern (reduction in developmental rates) of *C. riparius*, DEET exposure also caused a decrease in adult's body size. Body size of imagos have been scarcely used as an endpoint to evaluate sub-lethal effects of contaminants, but reproductive output is dependent of the body size of aquatic midges since there is evidence that larger females are more fecund and that male body size is also positively correlated with the total number of gametes, as well as with reproductive success (Ponlawat and Harrington, 2007; Lilley et al., 2012). Thus, the reduction in size of male and female *C. riparius* imagos caused by exposure to DEET is an indication of even stronger reproductive effects showing that this can be a sensitive and relevant parameter for a better evaluation of population level effects of contaminants (Rodrigues et al., 2015b).

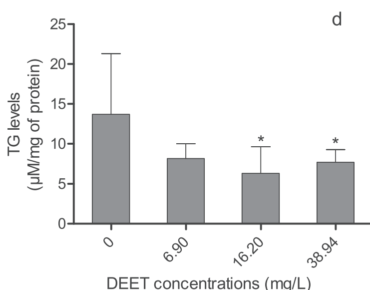
Because DEET interferes with the olfactory receptors of mosquitos which are present in both adults and aquatic larvae (Syed and Leal, 2008; Xia et al., 2008; Crespo, 2011), the effects in *C. riparius* life history traits including body size of adults are probably due to a reduction in food consumption by interference of DEET with their olfactory system. Study with *Anopheles gambiae* larvae show that larval odor receptor is sensitive to DEET and DEET also caused potential repulsive behavior in these larvae (Xia et al., 2008). Furthermore, it has been shown that DEET causes feeding inhibition and reduction in body weight of rats and mice (Schoenig et al., 1999).

Concerning biochemical responses our results show that short-term exposure to DEET did not cause oxidative stress in *C. riparius* larvae contrary to observations of oxidative damage caused by DEET in fish (Slaninova et al., 2014) and rats (Abu-Qare and Abou-Donia, 2000; Abu-Qare et al., 2001). *C. riparius* larvae exposed to DEET showed no alteration of LPO levels, i.e. no oxidative stress, and a concomitant decrease in activity of the biotransformation (GST) and antioxidant (CAT) enzymes. We hypothesize that glutathione (TG) consumption was the main pathway involved in detoxification processes in order to cope with DEET itself or reactive by products formed during its detoxification, thus avoiding oxidative stress (Rodrigues et al., 2015b). However, it is also possible that









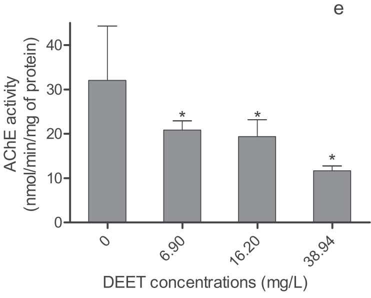


Fig. 2. Effects of short-term (48 h) DEET exposure on (a) lipid peroxidation (TBARS nmol/g wet weight; mean ± SD); (b) catalase activity (µmol/min/mg protein; mean ± SD); (c) glutathione-*S*-transferase activity (nmol/min/mg protein; mean ± SD); (d) total glutathione content (µmol/mg protein; mean ± SD); (e) acetylcholinesterase activity (nmol/min/mg protein; mean ± SD). All endpoints measured in *C. riparius* 4th instar larvae. * denotes significant differences compared to the control treatment at $p < 0.05$ (Dunnett's test).

an increase in ROS, caused by enzymatic inhibition (CAT and GST activities) can lead to an increased consumption of glutathione. Although the literature on these indirect biochemical effects of DEET is scarce, Hellestad et al. (2011) showed increased GST activities caused by DEET exposure in cell cultures of *Aedes albopictus* while Slaninova et al. (2014) showed no effects in terms of LPO levels, and CAT and GST activities in common carp exposed to 1 mg/L DEET.

Regarding possible neurotoxic effects of DEET, we found an inhibition of AChE in *C. riparius* larvae. AChE inhibition caused by DEET exposure in neuronal preparations of mammals and insects was already shown by Corbel et al. (2009) and in fact symptoms associated to DEET intoxication in invertebrates, mammals and humans reflect an apparent action on the central nervous system (Corbel et al., 2009) and it has been shown that DEET binds to the active site of cholinesterases (Corbel et al., 2009). This inhibition of AChE activity can also explain the behavioral effects observed at the organism level in the chronic experiments (reduced food intake), which may in turn lead to population level effects. Crane et al. (2002) showed that inhibition of AChE activity in *C. riparius* exposed to an organophosphate insecticide was related to a decreased larval weight and adult fecundity. In fact, together with CAT activity, AChE was the most sensitive parameter, showing that biochemical effects can be evident at lower concentrations than the ones used in our study. Inhibition of AChE activity can also explain why DEET can increase the toxicity of neurotoxic insecticides such as carbamates, a class of insecticides acting on this enzyme (Corbel et al., 2009).

Although DEET has been shown to be moderately toxic to *C. riparius*, our study unravels some of the biochemical changes and chronic effects induced by DEET in insects. Moreover, long-term and multigenerational effects of exposure to insect repellents like DEET should also be assessed. Because DEET and other insect repellents are also simultaneously used with other compounds, such as UV-filters in sunscreens and insecticides, the results reported here are useful to understand possible synergistic effects occurring on natural aquatic environments.

Moreover, it should be noted that insect repellents could also have potential deleterious effects that were not possible to detect or measure in these laboratory-controlled conditions. By interfering with their gustatory and olfactory organs it is likely that insects, which sense their surrounding environment using chemical cues, may lose or reduce the ability to detect food and predators (Klaschka, 2008; Pestana et al., 2009) or find suitable sites for oviposition when exposed to low concentrations of insect repellents (Prajapati et al., 2005; Yi et al., 2014). This could also lead to important effects at the population (mortality, reduced growth) and community levels. Thus, ecotoxicological experiments designed to include such realistic scenarios (different types of food, presence of predators) and the measurement of ecologically meaningful endpoints (e.g., oviposition, avoidance, feeding preferences, etc.,) are critical to correctly evaluate the ecological risk of insect repellents in aquatic ecosystems.

Acknowledgments

This work was supported by the Portuguese Foundation for Science and Technology (FCT) through CESAM: UID/AMB/50017/2013. FCT also financed a doctoral grant to Diana Campos (SFRH/BD/87370/2012), a post-doctoral research grant to João L.T. Pestana (SFRH/BPD/45342/2008) and Carla Quintaneiro (SFRH/BPD/89951/2012). We also thank FCT and POPH/FSE (Programa Operacional Potencial Humano/Fundo Social Europeu) for the contract of C. Gravato (IF/1401/2014).

References

Abu-Qare, A., Abou-Donia, M., 2000. Increased 8-hydroxy-2-deoxyguanosine, a biomarker of oxidative DNA damage in rat urine following a single dermal dose of DEET (*N*,*N*-diethyl-*m*-toluamide), and permethrin, alone and in combination. Toxicol. Lett. 117, 151–160.

Abu-Qare, A.W., Suliman, H.B., Abou-Donia, M.B., 2001. Induction of urinary excretion of 3-nitrotyrosine, a marker of oxidative stress, following administration of pyridostigmine bromide, DEET (*N*,*N*-diethyl-*m*-toluamide) and permethrin, alone and in combination in rat. Toxicol. Lett. 121, 127–134.
Aronson, D., Weeks, J., Meylan, B., Guiney, P.D., Howard, P.H., 2012. Environmental release, environmental concentrations, and ecological risk of *N*,*N*-diethyl-*m*-toluamide (DEET). Integr. Environ. Assess. Manage. 8, 135–166.
Baker, M.A., Cerniglia, G.J., Zaman, A., 1990. Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. Anal. Biochem. 190, 360–365.
Bird, R.P., Draper, H.H., 1984. Comparative studies on different methods of malonaldehyde determination. Methods Enzymol. 105, 299–305.
Bohbot, J.D., Dickens, J.C., 2012. Odorant receptor modulation: ternary paradigm for mode of action of insect repellents. Neuropharmacology 62, 2086–2095.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Brausch, J.M., Rand, G.M., 2011. A review of personal care products in the aquatic environment: environmental concentrations and toxicity. Chemosphere 82, 1518–1532.
Calza, P., Medana, C., Raso, E., Giancotti, V., Minero, C., 2011. *N*,*N*-diethyl-*m*-toluamide transformation in river water. Sci. Total Environ. 409, 3894–3901.
Campos, D., Alves, A., Lemos, M.L., Correia, A., Soares, A.V.M., Pestana, J.T., 2014. Effects of cadmium and resource quality on freshwater detritus processing chains: a microcosm approach with two insect species. Ecotoxicology 23, 830–839.
Clairborne, A., 1985. Catalase activity. In: Greenwald, R.A.E. (Ed.), Handbook of Methods for Oxygen Radical Research. CRC Press, Boca Raton, pp. 283–284.
Corbel, V., Stankiewicz, M., Pennetier, C., Fournier, D., Stojan, J., Girard, E., Dimitrov, M., Molgo, J., Hougard, J.-M., Lapied, B., 2009. Evidence for inhibition of cholinesterases in insect and mammalian nervous systems by the insect repellent deet. BMC Biol. 7, 47.
Costanzo, S.D., Watkinson, A.J., Murby, E.J., Kolpin, D.W., Sandstrom, M.W., 2007. Is there a risk associated with the insect repellent DEET (*N*,*N*-diethyl-*m*-toluamide) commonly found in aquatic environments? Sci. Total Environ. 384, 214–220.
Crane, M., Sildanchandra, W., Kheir, R., Callaghan, A., 2002. Relationship between biomarker activity and developmental endpoints in *Chironomus riparius* Meigen exposed to an organophosphate insecticide. Ecotoxicol. Environ. Saf. 53, 361–369.
Crespo, J.G., 2011. A review of chemosensation and related behavior in aquatic insects. J. Insect Sci. 11, 62.
Ditzen, M., Pellegrino, M., Vosshall, L.B., 2008. Insect odorant receptors are molecular targets of the insect repellent DEET. Science 319, 1838–1842.
Dogan, E.B., Ayres, J.W., Rossignol, P.A., 1999. Behavioural mode of action of deet: inhibition of lactic acid attraction. Med. Vet. Entomol. 13, 97–100.
Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.
Glassmeyer, S.T., Furlong, E.T., Kolpin, D.W., Cahill, J.D., Zaugg, S.D., Werner, S.L., Meyer, M.T., Kryak, D.D., 2005. Transport of chemical and microbial compounds from known wastewater discharges: potential for use as indicators of human fecal contamination. Environ. Sci. Technol. 39, 5157–5169.
Guilhermino, L., Lopes, M.C., Carvalho, A.P., Soares, A.M.V.M., 1996. Acetylcholinesterase activity in juveniles of *Daphnia magna* Straus. Bull. Environ. Contam. Toxicol. 57, 979–985.
Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione-*S*-transferases: the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.
Hellestad, V.J., Witthuhn, B.A., Fallon, A.M., 2011. The insect repellent DEET (*N*,*N*-diethyl-3-methylbenzamide) increases the synthesis of glutathione-*S*-transferase in cultured mosquito cells. Cell Biol. Toxicol. 27, 149–157.
Klaschka, U., 2008. The infochemical effect—a new chapter in ecotoxicology. Environ. Sci. Pollut. Res. 15, 452–462.
Leal, W.S., 2014. The enigmatic reception of DEET—the gold standard of insect repellents. Curr. Opin. Insect Sci. 6, 93–98.
Lee, Y., Kim, S.H., Montell, C., 2010. Avoiding DEET through insect gustatory receptors. Neuron 67, 555–561.
Lilley, T.M., Ruokolainen, L., Pikkarainen, A., Laine, V.N., Kilpimaa, J., Rantala, M.J., Nikinmaa, M., 2012. Impact of tributyltin on immune response and life history traits of *Chironomus riparius*: single and multigeneration effects and recovery from pollution. Environ. Sci. Technol. 46, 7382–7389.
Liu, F., Chen, L., Appel, A.G., Liu, N., 2013. Olfactory responses of the antennal trichoid sensilla to chemical repellents in the mosquito, *Culex quinquefasciatus*. J. Insect Physiol. 59, 1169–1177.
Michael, A.G., Grant, G.S., 1974. Toxicity of the repellent DEET (*N*,*N*-diethyl-meta-toluamide) to *Gambusia affinis* (Baird and Girard). Mosq. News 34, 32–35.
OECD, 2004. OECD Guidelines for the Testing of Chemicals. In: Section 2: Effects on Biotic Systems Test No. 219: sediment-water chironomid toxicity using spiked water. Organization for Economic Cooperation and Development, Paris, France.
Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351–358.
Pedrouzo, M., Borrull, F., Marcé, R.M., Pocurull, E., 2011. Analytical methods for personal-care products in environmental waters. TrAC Trends Anal. Chem. 30, 749–760.
Pellegrino, M., Steinbach, N., Stensmyr, M.C., Hansson, B.S., Vosshall, L.B., 2011. A natural polymorphism alters odour and DEET sensitivity in an insect odorant receptor. Nature 478, 511–514.
Pery, A.R., Mons, R., Flammarion, P., Lagadic, L., Garric, J., 2002. A modeling approach to link food availability, growth, emergence, and reproduction for the midge *Chironomus riparius*. Environ. Toxicol. Chem. 21, 2507–2513.
Pestana, J.L.T., Loureiro, S., Baird, D.J., Soares, A.M.V.M., 2009. Fear and loathing in the benthos: responses of aquatic insect larvae to the pesticide imidacloprid in the presence of chemical signals of predation risk. Aquat. Toxicol. 93, 138–149.
Ponlawat, A., Harrington, L.C., 2007. Age and body size influence male sperm capacity of the dengue vector *Aedes aegypti* (Diptera: Culicidae). J. Med. Entomol. 44, 422–426.
Prajapati, V., Tripathi, A.K., Aggarwal, K.K., Khanuja, S.P.S., 2005. Insecticidal, repellent and oviposition-deterrent activity of selected essential oils against *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus*. Bioresour. Technol. 96, 1749–1757.
Rodrigues, A.C.M., Gravato, C., Quintaneiro, C., Barata, C., Soares, A.M.V.M., Pestana, J.L.T., 2015a. Sub-lethal toxicity of environmentally relevant concentrations of esfenvalerate to *Chironomus riparius*. Environ. Pollut. 207, 273–279.
Rodrigues, A.C.M., Gravato, C., Quintaneiro, C., Golovko, O., Žlábek, V., Barata, C., Soares, A.M.V.M., Pestana, J.L.T., 2015b. Life history and biochemical effects of chlorantraniliprole on *Chironomus riparius*. Sci. Total Environ. 508, 506–513.
Schoenig, G.P., Osimitz, T.G., Gabriel, K.L., Hartnagel, R., Gill, M.W., Goldenthal, E.I., 1999. Evaluation of the chronic toxicity and oncogenicity of *N*,*N*-diethyl-*m*-toluamide (DEET). Toxicol. Sci. 47, 99–109.
Seo, J., Lee, Y.G., Kim, S.D., Cha, C.J., Ahn, J.H., Hur, H.G., 2005. Biodegradation of the insecticide *N*,*N*-diethyl-*m*-toluamide by fungi: identification and toxicity of metabolites. Arch. Environ. Contam. Toxicol. 48, 323–328.
Slaninova, A., Modra, H., Hostovsky, M., Sisperova, E., Blahova, J., Matejova, I., Vicenova, M., Faldyna, M., Zelnickova, L., Tichy, F., Svobodova, Z., 2014. Effects of subchronic exposure to *N*,*N*-diethyl-*m*-toluamide on selected biomarkers in Common Carp (*Cyprinus carpio* L.). Biomed. Res. Int. 2014, 8.
Stanczyk, N.M., Brookfield, J.F.Y., Ignell, R., Logan, J.G., Field, L.M., 2010. Behavioral insensitivity to DEET in *Aedes aegypti* is a genetically determined trait residing in changes in sensillum function. Proc. Natl. Acad. Sci. U. S. A. 107, 8575–8580.
Stuart, M., Lapworth, D., Crane, E., Hart, A., 2012. Review of risk from potential emerging contaminants in UK groundwater. Sci. Total Environ. 416, 1–21.
Syed, Z., Leal, W.S., 2008. Mosquitoes smell and avoid the insect repellent DEET. Proc. Natl. Acad. Sci. U. S. A. 105, 13598–13603.
USEPA (U.S. Environmental Protection Agency), 1998. Reregistration eligibility decision (RED). DEET. Office of Prevention, Pesticides, and Toxic Substances. EPA738-R-98-010. Washington, DC.
Weeks, J.A., Guiney, P.D., Nikiforov, A.I., 2012. Assessment of the environmental fate and ecotoxicity of *N*,*N*-diethyl-*m*-toluamide (DEET). Integr. Environ. Assess. Manage. 8, 120–134.
Weigel, S., Berger, U., Jensen, E., Kallenborn, R., Thoresen, H., Hühnerfuss, H., 2004. Determination of selected pharmaceuticals and caffeine in sewage and seawater from Tromsø/Norway with emphasis on ibuprofen and its metabolites. Chemosphere 56, 583–592.
Xia, Y., Wang, G., Buscariollo, D., Pitts, R.J., Wenger, H., Zwiebel, L.J., 2008. The molecular and cellular basis of olfactory-driven behavior in *Anopheles gambiae* larvae. Proc. Natl. Acad. Sci. U. S. A. 105, 6433–6438.
Xu, P., Choo, Y.-M., De La Rosa, A., Leal, W.S., 2014. Mosquito odorant receptor for DEET and methyl jasmonate. Proc. Natl. Acad. Sci. U. S. A. 111, 16592–16597.
Yi, X., Zhao, H., Wang, P., Hu, M., Zhong, G., 2014. BdorOrco is important for oviposition-deterring behavior induced by both the volatile and non-volatile repellents in *Bactrocera dorsalis* (Diptera: Tephritidae). J. Insect Physiol. 65, 51–56.
Yoon, Y., Ryu, J., Oh, J., Choi, B.-G., Snyder, S.A., 2010. Occurrence of endocrine disrupting compounds, pharmaceuticals, and personal care products in the Han River (Seoul, South Korea). Sci. Total Environ. 408, 636–643.

Annex II

Chemosphere 149 (2016) 177–182

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Are insect repellents toxic to freshwater insects? A case study using caddisflies exposed to DEET

Diana Campos [a], Carlos Gravato [a], Carla Quintaneiro [a], Olga Koba [b], Tomas Randak [b], Amadeu M.V.M. Soares [a], João L.T. Pestana [a,*]

[a] Departamento de Biologia & CESAM, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
[b] University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zatisi 728/II, 389 25 Vodnany, Czech Republic

HIGHLIGHTS

- Ecotoxicological data on the effects of insect repellents in aquatic systems is needed.
- Effects of DEET were assessed in the caddisfly *Sericostoma vittatum*.
- Deleterious effects of DEET were only observed at concentrations above environmental levels.
- DEET exposure decreased feeding rate and carbohydrates contents in *S. vittatum*.

ARTICLE INFO

Article history:
Received 27 November 2015
Received in revised form
5 January 2016
Accepted 23 January 2016
Available online 6 February 2016

Handling Editor: Jim Lazorchak

Keywords:
Freshwater insects
Sericostoma vittatum
Feeding behaviour
Biomarkers
N,N-Diethyl-3-methylbenzamide
Energy reserves

ABSTRACT

Stream ecosystems face ever-increasing pressures by the presence of emergent contaminants, such as, personal care products. *N, N*-diethyl-3-methylbenzamide (DEET) is a synthetic insect repellent that is being found in surface waters environments in concentrations up to 33.4 μg/L. Information concerning DEET's toxicity in the aquatic environment is still limited and focused only on its acute effects on model species. Our main objective was to assess the effects of DEET exposure to a caddisfly non-target species using sub-lethal endpoints. For that, we chose *Sericostoma vittatum*, an important shredder in Portuguese freshwaters that has been already used in different ecotoxicological assays. Besides acute tests, *S. vittatum* were exposed during 6 days to a gradient of DEET concentrations (8, 18 and 40.5 mg/L) to assess effects on feeding behaviour and biochemical responses, such as, lipid peroxidation levels (LPO), catalase and acetylcholinesterase (AChE) activities, and also assess effects on energy reserves and consumption. Acute tests revealed a 48 h-LC_{50} of 80.12 mg/L and DEET exposure caused feeding inhibition with a LOEC of 36.80 mg/L. Concerning the biochemical responses, DEET caused no effects in LPO nor on catalase activity. A non-significant decrease in AChE activity was observed. Regarding energetic reserves, exposure to DEET caused a significant reduction in *S. vittatum* carbohydrates levels. These results add important information for the risk assessment of insect repellents in the aquatic environment and suggest that reported environmental concentrations of DEET are not toxic to non-target freshwater insects.

© 2016 Elsevier Ltd. All rights reserved.

1. Introduction

Insect repellents are a class of personal care products that are applied to skin, clothes or other surfaces to prevent arthropod biting and consequently control dissemination of diseases (Costanzo et al., 2007). These compounds can be based on synthetic or natural substances and information about their chronic effects in aquatic environment is still lacking (Pedrouzo et al., 2011). The most widely used substance in commercial insect repellents is *N*, *N*-diethyl-3-methylbenzamide (DEET), an active ingredient that was first synthetized in 1946 by the U. S. Army (Costanzo et al., 2007). DEET has been detected in different matrices of aquatic environments, such as wastewater treatment plants influents and effluents (Costanzo et al., 2007; Glassmeyer et al., 2005), surface water (Calza

* Corresponding author.
E-mail address: jpestana@ua.pt (J.L.T. Pestana).

http://dx.doi.org/10.1016/j.chemosphere.2016.01.098
0045-6535/© 2016 Elsevier Ltd. All rights reserved.

et al., 2011; Costanzo et al., 2007; Yoon et al., 2010), seawater (Weigel et al., 2004) and even drinking water (Stackelberg et al., 2004). DEET has been detected in different regions of the world, such as, Europe (Calza et al., 2011), USA (Glassmeyer et al., 2005), Australia (Costanzo et al., 2007) or South Korea (Yoon et al., 2010) in concentrations ranging from 0.001 to 33.4 μg/L in surface waters worldwide (for more detail see Aronson et al. (2012)).

DEET's mode of action has been the subject of investigations using different insect species, namely *Drosophila melanogaster* (Pellegrino et al., 2011), *Culex quinquefasciatus*, *Aedes aegypti* and *Anopheles albimanus* (Leal, 2014). Recent studies have shown that DEET modify insect's behaviour by activation or modulation of olfactory receptors (Ditzen et al., 2008; Pellegrino et al., 2011) and can directly activate gustatory receptors neurons mimicking feeding deterrents (Lee et al., 2010). DEET has also been shown to inhibit the activity of acetylcholinesterase (AChE) in neuronal preparations of mammals and insects (Corbel et al., 2009). Collectively these studies suggest that although not designed to have biocidal properties, exposure to insect repellents such as DEET can affect non-target insects through behaviour impairment (feeding, predator and prey attack-escape performance) and neurotoxicity. Thus, it is important to evaluate their ecological effects in the aquatic environment.

However, only a few studies were conducted using aquatic organisms exposed to DEET and the majority of those investigations are related with its acute toxicity (Aronson et al., 2012). DEET appears to be slightly toxic, but taking into account the frequency of detection in surface waters and its persistence more studies are required to assess the chronic toxicity of DEET for an accurate risk assessment (Brausch and Rand, 2011; Costanzo et al., 2007). Moreover, it is also important that this assessment is conducted with different non-target species.

Caddisflies are used as model species for the assessment of effects of different contaminants in lotic ecosystems (Campos et al., 2014; Damásio et al., 2011; Pestana et al., 2009). The caddisfly *Sericostoma vittatum* Rambur (Trichoptera: Sericostomatidae) is an endemic species present in streams of the Iberian Peninsula during all year with an annual life cycle. They are benthic organisms with an important role in the fragmentation of allochthonous organic matter in streams being efficient shredders (Feio and Graça, 2000).

Due to constant detection of DEET in freshwaters and also due to their mode of action is expectable that DEET exposure can cause effects in non-target aquatic insects through feeding inhibition and/or neurotoxicity. Although the concentrations tested in our study (in order of mg/L) are above environmental relevant concentration (in order to μg/L), understanding ecological effects of DEET in aquatic ecosystems, its biochemical effects and tolerance of non-target organisms is a pertinent issue. So the aim of this study was to evaluate the ecotoxicological responses of *S. vittatum*, a freshwater caddisfly, to DEET exposure at different levels of biological organization. The endpoints chosen included feeding rate as organismal endpoint and oxidative stress (lipid peroxidation; LPO), antioxidant enzymes (catalase; CAT), and neurophysiological activity (AChE) as biochemical endpoints. We also wanted to evaluate the energy available (E_a) (measuring levels of carbohydrates, lipids and proteins contents) and energy consumption (E_c) (measuring electron transport system- ETS - activity).

2. Methods

2.1. Animals

S. vittatum were collected from Ribeira de São João, Lousã, Portugal (40°06′N, 8°14′W) using an hand net. Organisms were acclimated to laboratory conditions (20 ± 1 °C, light–dark cycle of 16:8 h) for one week in plastic containers with inorganic fine sediment (<1 mm) previously burnt (500 °C for 4 h), and filled with American Society for Testing Materials ASTM (1980) hard water. Following the protocol described in Pestana et al. (2009), organisms were fed *ad libitum* with unconditioned alder leaves (*Alnus glutinosa*), which provide adequate nutrition for maintenance and reproduction of this species under laboratory conditions.

2.2. S. vittatum acute experiments

S. vittatum were exposed to a range of DEET concentrations (39.05, 50.77, 66.00, 85.80, 111.54 and 145 mg/L) during 48 h plus control treatment (ASTM hard water only). The experimental setup consisted in five replicates with five organisms each, for each treatment. The organisms were exposed in glass vials with 150 mL of respective medium at 20 ± 1 °C and 16:8 h light: dark photoperiod. No food or sediment was added during the exposure period. In the end of 48 h all organisms in control treatment were alive.

2.3. S. vittatum feeding experiments

Based on preliminary experiments *S. vittatum*, were exposed to a gradient of three concentrations (8, 18 and 40.5 mg/L) of DEET (CAS number: 134-62-3; molecular weight: 191.27; Sigma–Aldrich, Germany) plus a control treatment (ASTM hard water only). Feeding trials were based on previous laboratory toxicity assays conducted with *S. vittatum* (Campos et al., 2014; Pestana et al., 2009). Briefly, we used ten replicates with one animal per replicate. In each replicate *S. vittatum* were allocated to glass vials containing 1 cm layer of inorganic fine sediment (<1 mm), 150 mL of respective solution and 6 conditioned alder leaf discs as food. Alder leaves used in these assays were collected from riparian vegetation of Alfusqueiro river near Destriz (40°38′N, 8°16′W). The leaves were air dried and stored in the darkness. Before use in feeding trials, the leaves were soaked in distilled water and leaf discs (Ø 10 mm) were prepared with a cork borer. Alder leaf discs were then autoclaved and conditioned during one week in 1500 mL of local river water, at 20 ± 1 °C, 16:8 h light: dark photoperiod and with aeration. After conditioning, alder leaf discs were dried at 50 °C during 96 h and weighed.

Alder leaf discs used in each replicate are soaked in the respective DEET solutions during 96 h before use. The test were conducted at 20 ± 1 °C with a photoperiod of 16 h light: 8 h dark. After 6 days of exposure, *S. vittatum* were collected, removed from their case, quickly dried on filter paper, immediately weighted, frozen in liquid nitrogen and stored at −80 °C. In the end of the test no mortality was observed in the control treatment. In this control treatment one of the caddisfly was in the pupal stage and thus this replicate was removed from the feeding calculations.

Alder leaf discs were also collected and dried at 50 °C during 96 h. Feeding rate was calculated as the difference between the initial and final leaf disc dry mass (mg) and divided by the wet mass of organism (mg) and elapsed time (days). Three replicates in control and highest concentration of DEET were performed with leaves discs in the absence of organisms in order to correct weight change of leaf discs due to other factors rather than feeding. Since no difference was found between leaf discs weight loss between these two treatments, the combined average of loss of weight of leaf discs of control and highest concentration of DEET was used as a correction factor in all experimental treatments.

2.4. S. vittatum biochemical experiments

After six days organisms used for feeding experiments were frozen at −80 °C and were used to assess effects of DEET on biochemical parameters. Each organism was homogenized in 1600 μL of Milli-Q water by sonication. After homogenization three

aliquots of 300 μL were removed to measure levels of lipids, proteins and carbohydrates and ETS. Also, one aliquot of 200 μL for LPO was removed and 4 μL of 4% 2,6-Di-tert-butyl-4-methylphenol in methanol were added. To the remaining (500 μL) homogenized samples we added 500 μL K-phosphate buffer (0.2 M; pH = 7.4), followed by centrifugation at 10,000*g* for 20 min at 4 °C to isolate the post-mitochondrial supernatant (PMS) that was separated in aliquots for CAT, AChE and protein quantification.

2.4.1. Lipid peroxidation

Lipid peroxidation was measured using thiobarbituric acid-reactive substances (TBARS) assay (Bird and Draper, 1984; Ohkawa et al., 1979). The reaction included a mixture of 200 μL of homogenate samples separated for LPO, 100 μL of 100% (w/v) trichloroacetic acid sodium salt, 1000 μL of 0.73% (w/v) 2-thiobarbituric acid made in 60 mM Tris–HCl with 0.1 mM diethylenetriaminepentaacetic acid. The mixture was incubated during 60 min at 100 °C, centrifuged during 5 min at 11,500 rpm and absorbance was read at 535 nm. The results were expressed as nmol TBARS per min per g of weight using $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ as molar extinction coefficient.

2.4.2. Catalase activity

Catalase activity was determined at 240 nm during 2 min by measuring consumption of the substrate hydrogen peroxide (Clairborne, 1985). The reaction included a mixture of 140 μL of K-phosphate buffer (0.05 M; pH = 7.0), 150 μL of H_2O_2 and 10 μL of PMS. Results were expressed as μmol per min per mg of protein using 40 $M^{-1}\ cm^{-1}$ as molar extinction coefficient.

2.4.3. Acetylcholinesterase activity

Acetylcholinesterase activity was measured using 50 μL of PMS that was mixed with 250 μL of reaction solution. The reaction solution was made with 30 mL of K-phosphate buffer (0.1 M; pH = 7.2), 200 μL of 0.075 M acetylthiocholine iodide solution and 1 mL of 10 mM 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman et al., 1961; Guilhermino et al., 1996). The absorbance was read at 414 nm and the enzymatic activity was expressed in nmol per min per mg of protein using $13.6 \times 10^3\ M^{-1}\ cm^{-1}$ as molar extinction coefficient.

2.4.4. Protein quantification

The protein quantification was performed according to Bradford method (Bradford, 1976) adapted from BioRad's Bradford micro-assay, using γ-globulin as a standard. For reaction 10 μL of PMS and 250 μL of BioRad solution were mixed and 15 min later the absorbance was read at 590 nm.

2.4.5. Energy available and energy consumption

Considering energy parameters, we have determine energy available (Ea) in terms of (Ea: energetic equivalents of carbohydrates, proteins and lipids contents) and also energy consumption (Ec) by measuring Electron Transport Systems (ETS). Protocols were adapted from De Coen and Janssen (1997) with slight modifications (Rodrigues et al., 2015).

In short, carbohydrates content was measured with 5% of phenol and sulfuric acid. The absorbance was read at 492 nm after 30 min of incubation at room temperature, using glucose as a standard. The total protein content was measured by following Bradford's method (Bradford, 1976), using bovine serum albumin as a standard. The absorbance was measured at 520 nm after 30 min of incubation. Total lipids was extracted with chloroform (119.38 M; ACS spectrophotometric grade, ≥99.8%) and methanol (32.04 M; ACS reagent, ≥99.8%). The lipids content was measured using tripalmitine as a standard and the absorbance was read at 375 nm.

The energy of combustion (17,500 mJ/g glycogen, 24,000 mJ/g protein and 39,500 mJ/g lipid) was used to calculate energetic values of the fractions of energy available (De Coen and Janssen, 1997).

Electron transport system activity was measured with homogenization buffer (0.3 M Tris base; 0.45% (w/v) Poly Vinyl Pyrrolidone; 459 μM $MgSO_4$; 0.6% (v/v) Triton X-100 at a pH of 8.5), buffered solution (0.13 M Tris base containing 0.27% (v/v) Triton X-100; 1.7 mM NADH; 274 μM NADPH) and INT solution (p-iodonitrotetrazolium; 8 mM). The absorbance was read at 490 nm over 3 min. The cellular oxygen consumption rate was calculated using the stoichiometric relationship (2 μmol of INT-formazan formed 1 μmol of oxygen consumed) and using formula of Lambert–Beer: $A = \varepsilon \times l \times c$ (A = absorbance; ε for INT-formazan = 15,900 M cm; l = 0.9 cm; c = oxygen consumed). The values of ETS was then transformed into caloric values using specific oxyenthalpic equivalent for an average lipid, protein and carbohydrate mixture of 480 kJ/mol O_2.

The allometric equation $Z = Y(M\text{-}0.71)$ was used to correct final values of carbohydrates, proteins, lipids and ETS to weight of organisms where Y = energetic values of each measured; M = fresh weight of the samples; Z = values correct to weight of organisms (Penttinen and Holopainen, 1995).

The Ea and Ec values were calculate as described by Verslycke et al. (2003):

Ea = Lipids + Carbohydrates + Proteins (mJ/mg org)

Ec = Electron Transport System activity (mJ/mg org/h)

2.5. Chemical analysis

After the 6-day period, three composite replicates were used in each treatment to measure DEET water concentrations. For the acute toxicity one composite replicate per concentration were analysed.

Liquid chromatography–mass spectrometry (LC-MS) grade methanol and acetonitrile (Li Chrosolv Hypergrade) were purchased from Merck (Darmstadt, Germany). Formic acid for the mobile phase acidification was purchased from Labicom (Olomouc, Czech Republic). Ultra-pure water was produced using an Aqua-MAX-Ultra System (Younglin, Kyounggi-do, Korea). All compound used were analytical standards or of high purity (>98%). DEET was purchased from Sigma–Aldrich. Diclofenac was purchased from Sigma Aldrich (UK) and it was used as internal standard. Stock solutions of compounds were prepared in methanol at a concentration of 1 mg/mL and stored at −20 °C. A spiking mixture was prepared for each compound by diluting stocks in methanol to a final concentration of 1 μg/mL and stored at −20 °C.

A triple stage quadrupole MS/MS TSQ Quantum Ultra mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) coupled with an Accela 1250 LC pump (Thermo Fisher Scientific) and an HTS XT-CTC autosampler (CTC Analytics AG, Zwingen, Switzerland) was used for analysis of DEET in water samples. Thawed water samples were filtered through a syringe filter (0.45 μm, regenerated cellulose, Labicom, Olomouc, Czech Republic), after that 10 ng of internal standard was added to 1 mL of sample.

Cogent Bidentate C18 column (50 mm × 2.1 mm i.d., 4 μm particle size from MicroSolv Technology Corporation Eatontown, NJ, USA) was used as an analytical column for chromatographic separation of the target compounds. A heated electrospray ionization (HESI-II) was used in order to ionize the target compounds. Method parameters are reported in Table S1.

The limit of quantification (LOQ) for simultaneous analysis of DEET was determined by measuring aqueous standard solutions in a concentration range from 1 mg/L to 100 mg/L. LOQ was calculated as one quarter of the lowest calibration point in the calibration curve where relative standard deviation of average response factor was <30%. Peak area corresponding to this concentration was used to calculate LOQ for DEET in each sample. Recovery of DEET from

aquaria water was evaluated by spiking water samples with the target compound. MS/MS and method performance parameters are depicted in Table S2.

Matrix-matched standard response was used as factors for correcting the response derived from the calibration curve. Matrix-matched standard was prepared from tested water blank by spiking with both internal standard and native compound at 10 mg/L and 100 mg/L, respectively.

The values of DEET concentrations at the end of 6 days are 9.00 (±0.37), 15.92 (±0.70) and 36.80 (±2.01) mg/L and the degradation of DEET in chronic toxicity tests did not exceeded 12%. In the acute assay the degradation of DEET did not exceeded 21%.

2.6. Statistical analysis

Significant effects of DEET exposure on *S. vittatum* feeding rate and biochemical data were analysed by analysis of variance (ANOVA) with multiple comparisons examined by Dunnett's *post hoc* test. For all statistical tests the significance level was set at $p < 0.05$. All variables were assessed for normality using residual probability plots while Levene's and Bartlett's tests verified the homoscedasticity of data ($p > 0.05$). The lipids and protein data were Log-transformed to correct for unequal variances. All statistical analysis were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA).

The 48 LC_{50} for DEET was calculated by the probit method using Minitab software (Minitab Inc., State College, PA, USA).

3. Results and discussion

DEET is synthetized to be used against adult and biting insects but as it reaches surface waters it can also affect non-target aquatic species and especially insects. Knowledge on the effects of this contaminant and its mechanisms of toxicity is scarce.

Results of this study suggest that DEET is moderately toxic in the aquatic environment, since estimated values of 48 h LC_{50} (95% CI) for *S. vittatum* were 80.12 mg/L (53.53–106.71). *S. vittatum* showed to be a more sensitive species when compared to the crustacean *Daphnia magna* (48 h $LC_{50} = 160$ mg/L DEET) (Seo et al., 2005) and to fish species such as *Gambusia affinis* (48 h $LC_{50} = 235$ mg/L) (Michael and Grant, 1974) and *Oncorhynchus mykiss* (96 h $LC_{50} = 71.3$ mg/L) (USEPA, 1998).

Since DEET affects gustatory and olfactory receptors of insects, our results seems to confirm that DEET can act as a feeding deterrent since *S. vittatum* larvae exposed to DEET showed feeding inhibition (Fig. 1; $F_{(3,32)} = 3.65$; $p < 0.05$). Similar effects in terms of reduced feeding had already been reported for other organisms. Schoenig et al. (1999) showed a decrease in food consumption for mice and female rats when DEET was used in their diets. Lee et al. (2010) showed that DEET, in *D. melanogaster*, is highly effective antifeedant and Sanford et al. (2013) showed that DEET and others insect repellents act as feeding deterrents for *A. aegypti*. All these studies showed that DEET acts on gustatory receptor neurons causing significant alterations of feeding behaviour (Lee et al., 2010; Sanford et al., 2013). DEET also interferes with olfactory receptors but in Trichoptera there is evidences that food choice is only related with gustatory cues and, in general, the antennae of Trichoptera are not so well developed (Crespo, 2011) compared to other aquatic insects. Spänhoff et al. (2005) showed that for *Melampophylax mucoreus* the detection of food was not related with antenna but with sensilla in maxillary palps and galea. Motyka et al. (1985) showed that in *Pycnopsy guttifer* the preference of food can be initiated by gustatory cues since the organisms chose colonized leaves after being in contact with them. For *D. melanogaster* Lee et al. (2010) showed that the strong ability of DEET to prevent feeding is only dependent of gustatory receptor neurons and do not involve olfactory receptors neurons. Nonetheless, other insect orders such as Ephemeroptera, Plecoptera and Diptera are expected to be more sensitive to DEET exposure than Trichoptera since they have antenna with a variety of types of sensilla (Crespo, 2011). Nevertheless, our investigations have shown that high concentrations of DEET can alter life history traits of aquatic insects by reducing feeding with possible consequences for growth and development which were also observed in the dipteran *Chironomus riparius* (this study, Campos et al., 2016). Moreover, because insect detritivores and shredders are common in benthic communities it is also possible that DEET can affect organic matter processing in streams through effects on other detritivore insect species with potential indirect effects (Campos et al., 2014).

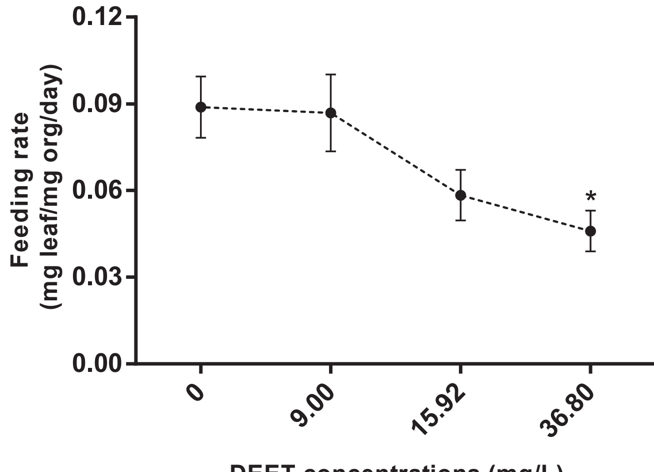


Fig. 1. Effects of DEET exposure on feeding rates of *S. vittatum* (mg leaf/mg organism/day; mean ± SE). *denotes a significant difference compared to control (0) treatment at $p < 0.05$ (Dunnett's test).

Concerning biochemical effects, DEET has been shown to cause oxidative stress in rats, (Abu-Qare and Abou-Donia, 2000) and in fish (Slaninova et al., 2014). However, we did not observe any effects of DEET exposure on *S. vittatum*' LPO (Fig. 2a; $F_{(3,33)} = 0.82$; $p > 0.05$) and also no effects were observed in CAT activity (Fig. 2b; $F_{(3,33)} = 1.07$; $p > 0.05$). This lack of effects in terms of oxidative damage has been previously observed for other aquatic insect, *C. riparius* (Campos et al., 2016). Regarding neurotoxicity effects, our results showed a reduction (although not significant) in terms of AChE activity in organisms exposed to DEET (Fig. 2c; $F_{(3,32)} = 1.31$; $p > 0.05$). In this study no distinction was made between different forms of ChE. However *S. vittatum* contains mainly AChE activity (Pestana et al., 2014) and the enzymatic activity measured in organism was considered to represent AChE activity. AChE activity inhibition, which has also been observed for *C. riparius* exposed to DEET can also lead to behavioural effects (locomotion, feeding) and consequently effects at the population level (Campos et al., 2016). DEET has been previously shown to affect AChE activity in neuronal preparations of mammals and insects since DEET binds to the active site of cholinesterase, inhibiting the hydrolysis of acetylthiocholine by AChE (Corbel et al., 2009). In fact, it is suggested that DEET might have important synergistic effects when combined with neurotoxic insecticides, but further studies should be performed. For example, the toxicity of carbamates increases in the presence of DEET which targets AChE especially when in mixtures with carbamates (Corbel et al., 2009). This calls for more ecotoxicity studies on the possible synergistic effects of mixtures of DEET with insecticides or other compounds such as UV-filters which are sometimes simultaneously

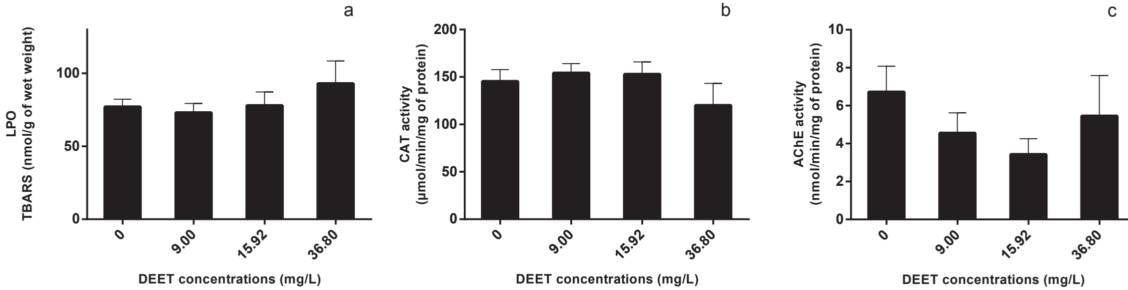


Fig. 2. Effects of DEET exposure on a) lipid peroxidation (TBARS nmol/g wet weight; mean ± SE); b) catalase activity (µmol/min/mg protein; mean ± SE); and c) acetylcholinesterase activity (nmol/min/mg protein; mean ± SE).

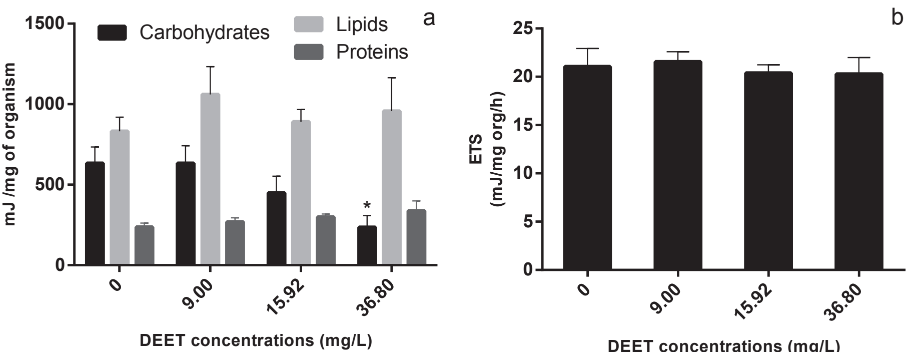


Fig. 3. Effects of DEET exposure on the energy budget of *S. vittatum* a) fractions of energy available: E_{lipids}, $E_{carbohydrates}$ and $E_{proteins}$ (mJ/mg organism; mean ± SE) and b) the energy consumption, ETS (mJ/mg organism/h; mean ± SE). *denotes significant differences compared to the control (0) treatment at $p < 0.05$ (Dunnett's test).

present in commercial sunscreens and insect repellents.

In this study we also addressed effects of DEET exposure on energetic reserves and energy consumption. Organisms use energy for growth, reproduction and basal metabolism. However, when exposed to stressful conditions (as e.g. contaminant exposure), and to maintain physiological or biochemical homeostasis, organisms can initiate compensatory adjustments in the energy metabolism (Choi et al., 2001; De Coen and Janssen, 2003). As a consequence, the consumption of sugars, lipids and proteins may increase to overcome the energetic requirements for detoxification processes with consequent effects in terms of energy available for growth and development (Choi et al., 2001). Thus effects at cellular levels can be indicative of less energy available to growth, reproduction and development processes (Smolders et al., 2004). In the present study, exposure to DEET resulted in a decrease of carbohydrates content (Fig. 3a; $F_{(3;33)} = 3.01$; $p < 0.05$), but no effects were observed for lipid (Fig. 3a; $F_{(3,33)} = 0.57$; $p > 0.05$) and protein contents (Fig. 3a; $F_{(3,33)} = 1.88$; $p > 0.05$). Consequently no effects were reported for energy available as a whole ($F_{(3,33)} = 1.091$; $p > 0.05$). Decrease observed in glycogen contents were not specific of chemical stressor, but can be due to processes involved in the reaction to stress by chemical exposure (Choi et al., 2001). De Coen and Janssen (2003) suggested that the reduction in energy reserves may be a result of decreased consumption of food or an increased metabolic activity. Since no effects were observed in neither energy consumption (Fig. 3b; $F_{(3,33)} = 0.175$; $p > 0.05$), nor biochemical parameters (LPO, CAT and AChE) probably the decrease in carbohydrates levels observed can be the result of a reduction in food consumption which can negatively affect growth and reproduction of organisms.

According with our study, DEET is slightly toxic to *S. vittatum* since effects were only observed for concentrations much higher than the ones reported in the environment. However, and taking into account that the occurrence of DEET in surface water increases during summer months (Aronson et al., 2012), further studies should monitor DEET's occurrence over seasons and possible hotspots in the aquatic environment. Also, indirect effects of exposure to insect repellents are possible in natural environments and future studies should focus on realistic exposure scenarios. Since DEET acts on gustatory and olfactory receptors, it is plausible that organisms exposed to DEET are affected in terms of chemical recognition of their surrounding environment (such as the ability to detect food and the presence of predators). Moreover, since DEET affects olfactory receptors of adult insects and have a repellent action, it can also locally reduce or alter insect's oviposition patterns (Prajapati et al., 2005; Yi et al., 2014).

Acknowledgements

This work was supported by the Portuguese Science Foundation (FCT) through CESAM: UID/AMB/50017/2013. FCT also financed a doctoral grant to Diana Campos (SFRH/BD/87370/2012), a postdoctoral research grant to João L.T. Pestana (SFRH/BPD/45342/2008) and Carla Quintaneiro (SFRH/BPD/89951/2012). We also thank FCT and POPH/FSE (Programa Operacional Potencial Humano/Fundo Social Europeu) for the contract of Carlos Gravato (IF/1401/2014). Chemical analysis was financially supported by the Ministry of Education, Youth and Sports of the Czech Republic - projects “CENAKVA” (No. CZ.1.05/2.1.00/01.0024) and “CENAKVA II” (No. LO1205 under the NPU I program) and by the Grant Agency of the University of South Bohemia in Ceske Budejovice (No. 012/2016/Z).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2016.01.098.

References

Abu-Qare, A., Abou-Donia, M., 2000. Increased 8-hydroxy-2′-deoxyguanosine, a biomarker of oxidative DNA damage in rat urine following a single dermal dose of DEET (*N*,*N*-diethyl-*m*-toluamide), and permethrin, alone and in combination. Toxicol. Lett. 117, 151–160.

Aronson, D., Weeks, J., Meylan, B., Guiney, P.D., Howard, P.H., 2012. Environmental release, environmental concentrations, and ecological risk of *N*,*N*-diethyl-*m*-toluamide (DEET). Integr. Environ. Assess. Manag. 8, 135–166.

ASTM, 1980. Standard Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians. American Standards for Testing and Materials, Philadelphia, P.A.

Bird, R.P., Draper, H.H., 1984. Comparative studies on different methods of malonaldehyde determination. Methods Enzym. 105, 299–305.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brausch, J.M., Rand, G.M., 2011. A review of personal care products in the aquatic environment: environmental concentrations and toxicity. Chemosphere 82, 1518–1532.

Calza, P., Medana, C., Raso, E., Giancotti, V., Minero, C., 2011. *N*,*N*-diethyl-*m*-toluamide transformation in river water. Sci. Total Environ. 409, 3894–3901.

Campos, D., Alves, A., Lemos, M.L., Correia, A., Soares, A.V.M., Pestana, J.T., 2014. Effects of cadmium and resource quality on freshwater detritus processing chains: a microcosm approach with two insect species. Ecotoxicology 23, 830–839.

Campos, D., Gravato, C., Quintaneiro, C., Soares, A.V.M., Pestana, J.T., 2016. Responses of the aquatic midge *Chironomus riparius* to DEET exposure. Aquat. Toxicol. http://dx.doi.org/10.1016/j.aquatox.2015.12.020.

Choi, J., Roche, H., Caquet, T., 2001. Hypoxia, hyperoxia and exposure to potassium dichromate or fenitrothion alter the energy metabolism in *Chironomus riparius* Mg. (Diptera: Chironomidae) larvae. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 130, 11–17.

Clairborne, A., 1985. Catalase activity. In: Greenwald, R.A.E. (Ed.), Handbook of Methods for Oxygen radical research. CRC Press, Boca Raton, pp. 283–284.

Corbel, V., Stankiewicz, M., Pennetier, C., Fournier, D., Stojan, J., Girard, E., Dimitrov, M., Molgo, J., Hougard, J.-M., Lapied, B., 2009. Evidence for inhibition of cholinesterases in insect and mammalian nervous systems by the insect repellent deet. BMC Biol. 7, 47.

Costanzo, S.D., Watkinson, A.J., Murby, E.J., Kolpin, D.W., Sandstrom, M.W., 2007. Is there a risk associated with the insect repellent DEET (*N*,*N*-diethyl-*m*-toluamide) commonly found in aquatic environments? Sci. Total Environ. 384, 214–220.

Crespo, J.G., 2011. A review of chemosensation and related behavior in aquatic insects. J. Insect Sci. 11, 62.

Damásio, J., Barceló, D., Brix, R., Postigo, C., Gros, M., Petrovic, M., Sabater, S., Guasch, H., de Alda, M.L., Barata, C., 2011. Are pharmaceuticals more harmful than other pollutants to aquatic invertebrate species: a hypothesis tested using multi-biomarker and multi-species responses in field collected and transplanted organisms. Chemosphere 85, 1548–1554.

De Coen, W.M., Janssen, C.R., 1997. The use of biomarkers in *Daphnia magna* toxicity testing. IV. Cellular energy allocation: a new methodology to assess the energy budget of toxicant-stressed Daphnia populations. J. Aquat. Ecosyst. Stress Recovery 6, 43–55.

De Coen, W.M., Janssen, C.R., 2003. The missing biomarker link: relationships between effects on the cellular energy allocation biomarker of toxicant-stressed *Daphnia magna* and corresponding population characteristics. Environ. Toxicol. Chem. 22, 1632–1641.

Ditzen, M., Pellegrino, M., Vosshall, L.B., 2008. Insect odorant receptors are molecular targets of the insect repellent DEET. Science 319, 1838–1842.

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

Feio, M.J., Graça, M.A.S., 2000. Food consumption by the larvae of *Sericostoma vittatum* (Trichoptera), an endemic species from the Iberian Peninsula. Hydrobiologia 439, 7–11.

Glassmeyer, S.T., Furlong, E.T., Kolpin, D.W., Cahill, J.D., Zaugg, S.D., Werner, S.L., Meyer, M.T., Kryak, D.D., 2005. Transport of chemical and microbial compounds from known wastewater discharges: potential for use as indicators of human fecal contamination. Environ. Sci. Technol. 39, 5157–5169.

Guilhermino, L., Lopes, M.C., Carvalho, A.P., Soares, A.M.V.M., 1996. Acetylcholinesterase activity in juveniles of *Daphnia magna* Straus. Bull. Environ. Contam. Toxicol. 57, 979–985.

Leal, W.S., 2014. The enigmatic reception of DEET - the gold standard of insect repellents. Curr. Opin. Insect Sci. 6, 93–98.

Lee, Y., Kim, S.H., Montell, C., 2010. Avoiding DEET through insect gustatory receptors. Neuron 67, 555–561.

Michael, A.G., Grant, G.S., 1974. Toxicity of the repellent DEET (*N*,*N*-diethyl-meta-toluamide) to *Gambusia affinis* (Baird and Girard). Mosq. News 34, 32–35.

Motyka, G.L., Merritt, R.W., Klug, M.J., Miller, J.R., 1985. Food-finding behavior of selected aquatic detritivores: direct or indirect behavioral mechanism? Can. J. Zool. 63, 1388–1394.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351–358.

Pedrouzo, M., Borrull, F., Marcé, R.M., Pocurull, E., 2011. Analytical methods for personal-care products in environmental waters. TrAC Trends Anal. Chem. 30, 749–760.

Pellegrino, M., Steinbach, N., Stensmyr, M.C., Hansson, B.S., Vosshall, L.B., 2011. A natural polymorphism alters odour and DEET sensitivity in an insect odorant receptor. Nature 478, 511–514.

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

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

Pestana, J.L.T., Novais, S.C., Lemos, M.F.L., Soares, A.M.V.M., 2014. Cholinesterase activity in the caddisfly *Sericostoma vittatum*: biochemical enzyme characterization and in vitro effects of insecticides and psychiatric drugs. Ecotoxicol. Environ. Saf. 104, 263–268.

Prajapati, V., Tripathi, A.K., Aggarwal, K.K., Khanuja, S.P.S., 2005. Insecticidal, repellent and oviposition-deterrent activity of selected essential oils against *Anopheles stephensi, Aedes aegypti* and *Culex quinquefasciatus*. Bioresour. Technol. 96, 1749–1757.

Rodrigues, A.C.M., Gravato, C., Quintaneiro, C., Golovko, O., Žlábek, V., Barata, C., Soares, A.M.V.M., Pestana, J.L.T., 2015. Life history and biochemical effects of chlorantraniliprole on *Chironomus riparius*. Sci. Total Environ. 508, 506–513.

Sanford, J., Shields, V.C., Dickens, J., 2013. Gustatory receptor neuron responds to DEET and other insect repellents in the yellow-fever mosquito, *Aedes aegypti*. Naturwissenschaften 100, 269–273.

Schoenig, G.P., Osimitz, T.G., Gabriel, K.L., Hartnagel, R., Gill, M.W., Goldenthal, E.I., 1999. Evaluation of the chronic toxicity and oncogenicity of *N*,*N*-diethyl-*m*-toluamide (DEET). Toxicol. Sci. Off. J. Soc. Toxicol. 47, 99–109.

Seo, J., Lee, Y.G., Kim, S.D., Cha, C.J., Ahn, J.H., Hur, H.G., 2005. Biodegradation of the insecticide *N*,*N*-diethyl-*m*-toluamide by fungi: identification and toxicity of metabolites. Arch. Environ. Contam. Toxicol. 48, 323–328.

Slaninova, A., Modra, H., Hostovsky, M., Sisperova, E., Blahova, J., Matejova, I., Vicenova, M., Faldyna, M., Zelnickova, L., Tichy, F., Svobodova, Z., 2014. Effects of subchronic exposure to *N*,*N*-diethyl-*m*-toluamide on selected biomarkers in common carp (*Cyprinus carpio* L.). BioMed Res. Int. 2014, 8.

Smolders, R., Bervoets, L., De Coen, W., Blust, R., 2004. Cellular energy allocation in zebra mussels exposed along a pollution gradient: linking cellular effects to higher levels of biological organization. Environ. Pollut. 129, 99–112.

Spänhoff, B., Kock, C., Meyer, A., Meyer, E.I., 2005. Do grazing caddisfly larvae of *Melampophylax mucoreus* (Limnephilidae) use their antennae for olfactory food detection? Physiol. Entomol. 30, 134–143.

Stackelberg, P.E., Furlong, E.T., Meyer, M.T., Zaugg, S.D., Henderson, A.K., Reissman, D.B., 2004. Persistence of pharmaceutical compounds and other organic wastewater contaminants in a conventional drinking-water-treatment plant. Sci. Total Environ. 329, 99–113.

USEPA, 1998. US Environmental Protection Agency. Reregistration Eligibility Decision (RED). DEET. Washington (DC): Office of Prevention, Pesticides, and Toxic Substances. EPA738-R-98–010.

Verslycke, T., Vercauteren, J., Devos, C., Moens, L., Sandra, P., Janssen, C.R., 2003. Cellular energy allocation in the estuarine mysid shrimp *Neomysis integer* (Crustacea: Mysidacea) following tributyltin exposure. J. Exp. Mar. Biol. Ecol. 288, 167–179.

Weigel, S., Berger, U., Jensen, E., Kallenborn, R., Thoresen, H., Hühnerfuss, H., 2004. Determination of selected pharmaceuticals and caffeine in sewage and seawater from Tromsø/Norway with emphasis on ibuprofen and its metabolites. Chemosphere 56, 583–592.

Yi, X., Zhao, H., Wang, P., Hu, M., Zhong, G., 2014. BdorOrco is important for oviposition-deterring behavior induced by both the volatile and non-volatile repellents in *Bactrocera dorsalis* (Diptera: Tephritidae). J. Insect Physiol. 65, 51–56.

Yoon, Y., Ryu, J., Oh, J., Choi, B.-G., Snyder, S.A., 2010. Occurrence of endocrine disrupting compounds, pharmaceuticals, and personal care products in the Han River (Seoul, South Korea). Sci. Total Environ. 408, 636–643.