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The effects of binary UV filter mixtures on the midge *Chironomus riparius*

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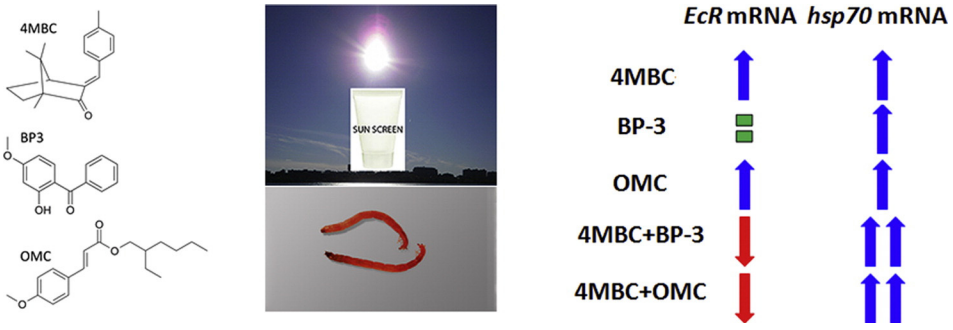
HIGHLIGHTS

- *Chironomus riparius* is sensitive to UV filter binary mixtures.
- UV filters binary mixtures show antagonism on survival of 4th instar larvae.
- BP-3 and OMC antagonize the stimulatory effect of 4MBC on *EcR* gene.
- 4MBC, OMC, and BP-3 induce *hsp70* transcriptional activity on larvae.
- *hsp70* gene expression shows different responses to UV filters binary mixtures.

GRAPHICAL ABSTRACT



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ABSTRACT

Organic ultraviolet (UV) filters are used in a wide variety of products, including cosmetics, to prevent damage from UV light in tissues and industrial materials. Their extensive use has raised concerns about potential adverse effects in human health and aquatic ecosystems that accumulate these pollutants. To increase sun radiation protection, UV filters are commonly used in mixtures. Here, we studied the toxicity of binary mixtures of 4-methylbenzylidene camphor (4MBC), octyl-methoxycinnamate (OMC), and benzophenone-3 (BP-3), by evaluating the larval mortality of *Chironomus riparius*. Also molecular endpoints have been analyzed, including alterations in the expression levels of a gene related with the endocrine system (*EcR*, ecdysone receptor) and a gene related with the stress response (*hsp70*, heat shock protein 70). The results showed that the mortality caused by binary mixtures was similar to that observed for each compound alone; however, some differences in LC50 were observed between groups. Gene expression analysis showed that *EcR* mRNA levels increased in the presence of 0.1 mg/L 4MBC but returned to normal levels after exposure to mixtures of 4MBC with 0.1, 1, and 10 mg/L of BP-3 or OMC. In contrast, the *hsp70* mRNA levels increased after exposure to the combinations tested of 4MBC and BP-3 or OMC mixtures. These data suggest that 4MBC, BP-3, and OMC may have antagonist effects on *EcR* gene transcription and a synergistic effect on *hsp70* gene activation. This is the first experimental study to show the complex patterned effects of UV filter mixtures on invertebrates. The data suggest that the interactions within these chemicals mixtures are complex and show diverse effects on various endpoints.

1. Introduction

Ultraviolet (UV) filters are compounds used in a wide variety of applications to prevent UV light-induced damage. These compounds are mainly found in personal care products (PCP) to prevent cellular damage, but they are also used in adhesives, plastics, and paints to protect

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against degradation (Gackowska et al., 2014). Although inorganic UV filters exist, most of the filters are organic and absorb UV radiation which is then dissipated by emission of higher wavelengths or relaxation by phytochemical processes (Abdelraheem et al., 2015). In parallel to the increasing use and production of these compounds, evidence shows their ubiquitous presence in multiple compartments of aquatic systems, including the biota. UV filters have been found in environmental samples (Grabicova et al., 2013; Sánchez Rodríguez et al., 2015; Tarazona et al., 2010), tap water (Díaz-Cruz et al., 2012), wastewater and treated sewage sludge (Gago-Ferrero et al., 2011), marine water (Tsui et al., 2014, 2015), and sediments (Amine et al., 2012). Filter contamination levels have been reported as high as 19,000 ng/L and 4000 ng/L in influent and effluent wastewater, respectively (Balmer et al., 2005; Gago-Ferrero et al., 2013; Kasprzyk-Hordern et al., 2009) and up to 3000 ng/L in surface water (Negreira et al., 2010; Rodil et al., 2009). Previous studies have reported the presence of UV filters in various organisms, such as fish, aquatic macroinvertebrates (Buser et al., 2006; Fent et al., 2010; Gago-Ferrero et al., 2012), and humans (Calafat et al., 2008; Zhang et al., 2013).

Using a mechanism similar to other endocrine disrupting compounds (EDCs), the majority of UV filters effect hormonal activity in vertebrates (Díaz-Cruz and Barceló, 2009). They show thyroid-disrupting activity (Axelstad et al., 2011; Schmutzler et al., 2007) and can function as antiandrogenic compounds (Ma et al., 2003; Schreurs et al., 2005); however, these compounds have attracted attention because of their disruptive effects on estrogenic activities (Kunz and Fent, 2006; Schreurs et al., 2002, 2005). Most studies have focused on the effects of individual chemicals on vertebrates despite the increasing use of mixtures in organic industrial products and cosmetics to improve effectiveness. Furthermore, various complex mixtures of UV filters have appeared as pollutants in aquatic systems (Ramos et al., 2015).

Despite the importance of invertebrates in maintaining aquatic ecosystems, scarce data are available on how UV filters affect invertebrate aquatic species. Only a few studies have been carried out and showed that these compounds can be toxic for invertebrates affecting their survival (Paredes et al., 2014) and altering the expression of endocrine related and stress genes (Ozáez et al., 2013, 2014). Chironomids are a group of Diptera with aquatic larvae that are ubiquitously distributed in freshwater streams. Several species are used in environmental toxicology but most of the studies are performed with *Chironomus riparius* Meigen, 1804, also known as *Chironomus thummi* Kieffer, 1911. *C. riparius* life cycle includes embryo, larvae, and pupae aquatic stages while adults are aerial (Armitage et al., 1995). Furthermore, this species is easily cultured in the laboratory which makes it an ideal reference species for several toxicity tests (OECD, 2010, 2011). In recent years, *C. riparius* was used in molecular studies to determine the effects of different pollutants (Martínez-Paz et al., 2012, 2014; Park and Kwak, 2010). We have previously reported the effects of six UV filters on *C. riparius*. The chemicals 4-methylbenzylidene camphor (4MBC), octyl-*p*-methoxycinnamate (OMC), also called 2-ethylhexyl-4-methoxycinnamate (EHMC), and octyldimethyl-*p*-aminobenzoate (OD-PABA) increased the expression of the ecdysone receptor gene (*EcR*), while benzophenone-3 (BP-3), 4-hidroxybenzophenone (4HB), and octocrylene (OC) did not alter the mRNA levels (Ozáez et al., 2013). Subsequent analysis demonstrated that BP-3 induced activation of the *EcR* gene after shorter exposure times that mimicked the effects of 20-hydroxyecdysone, the active form of the hormone (Ozáez et al., 2014). Additionally, heat shock protein 70 (*hsp70*) transcriptional changes were observed in presence of BP-3, which suggests the importance of this gene in the UV filter response.

Frequently, different UV filters are mixed to obtain better protection. For example, PCPs with high sun protection values use a combination of organic UVA and UVB filters and inorganic filters to enhance sun radiation blockage. These mixtures reach the environment and are distributed in the different compartments of the aquatic ecosystems. Their physicochemical properties allow for accumulation in the sediment and can affect the benthic fauna (Barón et al., 2013; Langford et al., 2015; Tsui et al., 2015). Laboratory studies are typically conducted with single UV filters; thus, there is a lack of information about the effects of mixtures of these compounds. Analyses carried out in vertebrates have shown that mixtures of UV filters can produce synergistic and antagonist effects in estrogenic and androgenic response pathways, respectively (Heneweer et al., 2005; Kunz and Fent, 2006, 2009). However, the effects of these mixtures on invertebrates are largely unknown. Therefore, to understand the effects of UV filter mixtures and to add new information about their mode of action on invertebrates binary mixtures of three UV filters have been analyzed on the aquatic larvae of *Chironomus riparius*. The single compounds 4MBC, OMC, and BP-3 as well as mixtures of 4MBC/OMC and 4MBC/BP-3 were tested. Mortality was analyzed as a toxicity endpoint, and the transcriptional activity of *EcR* and *hsp70* genes was studied as a molecular endpoint. To our knowledge, this is the first report on the effects of UV filter mixtures on invertebrates at the molecular level. Furthermore, the data suggest a complex interaction that can modulate key genes in endocrine and survival pathways.

2. Material and methods

2.1. Chemicals

The UV filters 3-(4-methylbenzylidene) camphor (CAS No.36861-47-9, purity ≥ 98%), octyl-*p*-methoxycinnamate, also called 2-ethylhexyl-4-methoxycinnamate (CAS No. 5466-77-3; purity ≥ 98%) and benzophenone-3 (2-hydroxy-4-methoxybenzophenone, CAS No. 131-57-7; purity ≥ 98%) were purchased from Sigma-Aldrich (Germany). Stock solutions were made in absolute ethanol and stored in the dark at 4 °C.

2.2. Animals

The test organisms consisted of aquatic larvae from the midge *Chironomus riparius* Meigen, 1804. The experimental animals come from a natural population in Valencia (Spain) and they were reared under standard laboratory conditions for several generations according to toxicity testing guidelines (OECD, 2010). Larvae were grown from egg masses in polyethylene tanks with culture medium (0.5 mM $CaCl_2$, 1 mM NaCl, 1 mM $MgSO_4$, 0.1 mM $NaHCO_3$, 0.025 mM KH_2PO_4, 0.01 mM $FeCl_3$) supplemented with nettle leaves, commercial fish food and cellulose tissue. Stock cultures were maintained under constant aeration at 20 ± 1 °C and under standard light-dark periods (16:8).

2.3. Treatments

For experimental treatments, four instar larvae were exposed to the chemical diluted in culture medium for up to 96 h in crystal vessels (250 mL). The culture medium was renewed every 24 h and supplemented with 3 mg of commercial fish food at 48 h. The following nominal concentrations of the UV filters were used: 0.1, 1, and 10 mg/L. UV filter-mixtures were directly added in the aqueous culture medium. Concentrations are likely to decline slightly over the 24-h exposure period as a result of the physicochemical properties of the compounds and the uptake by the larvae. To prevent losses by adsorption during treatments, no sediment, cellulose tissue or food were added during the 24-h exposure period. The treatments were carried out in the absence of light, due to their photodegradation. Non-treated control larvae were exposed to the same concentration of solvent (0.02% ethanol). Three independent experiments were performed for each set of conditions using samples from three different egg masses. In each experiment, n = 30 and n = 10 were used to analyze mortality and gene expression, respectively. For RNA extraction, surviving larvae were frozen and stored at −80 °C.