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Comparison of the behavioural effects of pharmaceuticals and pesticides on *Diamesa zernyi* larvae (Chironomidae)☆

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A B S T R A C T

Several studies have indicated the presence of contaminants in Alpine aquatic ecosystems. Even if measured concentrations are far below those that cause acute effects, continuous exposure to sub-lethal concentrations may have detrimental effects on the aquatic species present in these remote environments. This may lead to a cascade of indirect effects at higher levels of the ecological hierarchy (i.e., the community). To improve the determination of ecologically relevant risk endpoints, behavioural alterations in organisms due to pollutants are increasingly studied in ecotoxicology. In fact, behaviour links physiological function with ecological processes, and can be very sensitive to environmental stimuli and chemical exposure. This is the first study on behavioural alteration in a wild population of an Alpine species. In the present study, a video tracking system was standardized and subsequently used to identify contaminant-induced behavioural alterations in *Diamesa zernyi* larvae (Diptera, Chironomidae). *Diamesa zernyi* larvae, collected in an Italian Alpine stream (Rio Presena, Trentino Region), were acclimated for 24 h and successively exposed to several aquatic contaminants (pesticides: chlorpyrifos, metolachlor, boscalid, captan; pharmaceuticals: ibuprofen, furosemide, trimethoprim) at concentrations corresponding to their Lowest Observed Effect Concentration (LOEC). After 24, 48, 72, and 96 h of exposure, changes in the distance moved, the average speed, and the frequency of body bends were taken to reflect contaminant- and time-dependent effects on larval behaviour. In general, metolachlor, captan, and trimethoprim tended to reduce all the endpoints under consideration, whereas chlorpyrifos, boscalid, ibuprofen, and furosemide seemed to increase the distances moved by the larvae. This could be related to the different mechanisms of action of the investigated chemicals. Independently of the contaminant, after 72 h a general slowing down of all the behavioural activities occurred. Finally, we propose a behavioural stress indicator to compare the overall behavioural effects induced by the various contaminants.

1. Introduction

Currently, ecological risk assessment procedures for chemicals depend on effect characterization, which focuses mainly on the measurement of defined ecotoxicological endpoints using a battery of experimental tests on an organism that is representative of the exposed ecosystem (Hood, 2005; Stadler, 2011). For instance, the *Daphnia magna* immobilization test (ISO 6341, 2012; OECD, 2004) is one of the most frequently used tests for assessing the hazard posed by chemicals to aquatic invertebrates. However, standard tests do not take into consideration endpoints that may provide early warning signals about the health of the exposed populations, such as behavioural changes. These endpoints may be 10–100 times more sensitive than those derived from acute or chronic tests (Gerhardt, 2007), because chemicals can induce rapid behavioural responses in organisms even at very low concentrations (Amiard-Triquet, 2009). Behaviour is an organism-level effect defined as the action, reaction, or functioning of a system under a set of

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specific circumstances (Hellou, 2011). Behavioural endpoints can consist of a variety of activities such as avoidance/escape, or changes in feeding habits, locomotion, or respiration (Clotfelter et al., 2004). In the presence of contaminants, organisms may protect themselves by modifying their behaviour (i.e., avoidance), or their behaviour may be directly affected by the toxicant (i.e., locomotion, feeding rate) (Boyd et al., 2002). The behavioural responses of species have been used for decades in aquatic toxicology as a means of monitoring environments (Cairns and Gruber, 1980; Diamond et al., 1990; Gerhardt et al., 1998; Kramer et al., 1989; Van der Schalie et al., 2001). Owing to a lack of user-friendly tools to facilitate image acquisition, and because of limited scientific knowledge of the natural behaviour of many organisms, these studies have not received proper attention (Kane and Salierno, 2005; Melvin and Wilson, 2013; Scott and Sloman, 2004). However, in recent years, the development of video tracking technologies has enabled better quantification of behavioural patterns. Moreover, scientific knowledge about the importance of behaviour for the health and fitness of organisms has increased (Amiard-Triquet, 2009; Little and Brewer, 2001; Sloman and McNeil, 2012). Both aspects have led to a newfound interest in the analysis of behavioural changes of organisms (vertebrates and invertebrates) in the presence of contaminants (Anderson et al., 2004; Boyd et al., 2002; Capowiez et al., 2003; Cartlidge et al., 2017; Eissa et al., 2010; Hansen and Roslev, 2016; Melvin, 2016).

As previously stated, the characterization of ecotoxicological effects is mainly based on standardized test species. It is assumed that the responses from selected organisms will correspond to those from a larger array of species belonging to the same trophic levels (Crane, 1997). For instance, *Daphnia magna* is considered a representative of the planktonic invertebrates in aquatic ecosystems. However, this assumption does not always hold true (Maltby et al., 2005; Wiberg-Larsen et al., 2016; Wogram and Liess, 2001). According to Galic et al. (2014), the variability in the sensitivity of species to contaminants can be related to toxicokinetics (chemical uptake, biotransformation, distribution, and elimination) and toxicodynamics, which encompasses processes that occur at the target site, the generation of toxic effects, and the propagation of effects at the organism level.

It has been demonstrated that single morphological and/or physiological traits (respiration type, temperature preference, and current velocity preference) or their combinations influence the sensitivity to toxicants of macroinvertebrates (Jesus, 2008). In extremely cold ecosystems such as Alpine environments, organisms have evolved various traits that might affect the sensitivity of species towards contaminants (Chapman, 2016). For instance, in cold-adapted species, the slower uptake kinetics could be responsible of delayed toxicity response (Payne et al., 2014).

Although considered pristine, Alpine environments are often threatened by a number of stress factors at global, regional, and local levels (i.e. UV radiation, increasing temperature, acidification processes, water exploitation, and chemical pollution). Recently, measurable quantities of anthropogenic contaminants have been detected at high altitudes (Ferrario et al., 2017; Ferrey et al., 2015; Sun et al., 2017).

Even if the measured concentrations of the detected contaminants are usually far below those required to cause acute effects (Fernandes et al., 2016; Houtman, 2010), the prolonged exposure might have a detrimental effect on aquatic communities.

Chironomids (order Diptera, family Chironomidae) typically dominate the aquatic fauna in terms of individual abundance and species number of Alpine environments and for this reason have been proposed as the best macroinvertebrate bioindicators of high mountain water quality (Lencioni, 2018). In particular, species of the genus *Diamesa* are associated with pristine conditions (Lencioni et al., 2012).

To the best of our knowledge, few studies have been published on the behavioural responses of chironomid larvae following exposure to environmental stresses (Azevedo-Pereira et al., 2011; Kim et al., 2006; Nath and Gharpure, 2015), and all have been on the *Chironomus* genus from lowland freshwaters. The current study is the first on an Alpine chironomid species, *Diamesa zernyi* (Edwards). Specifically, the genus *Diamesa* prevails in kryal habitats with water temperature < 4–6 °C, and the species is one of the best adapted to colonise proglacial sites (Lencioni and Rossaro, 2005). Its autecology is well known, and the cold hardiness of *Diamesa* spp. larvae has recently been documented (Lencioni et al., 2015), but no information on its behavioural response to pesticides and emerging contaminants is available. The aim of the present work was to develop an experimental protocol to investigate behavioural changes in larvae exposed to aquatic contaminants by adapting a video tracking system that was developed for the nematode *Caenorhabditis elegans* to *Diamesa zernyi*. The video tracking system provides simultaneous responses from different endpoints. *Diamesa* larvae, collected from an Italian Alpine stream, were exposed to several plant protection products, (chlorpyrifos (CPF), metolachlor (MET), boscalid (BOS), and captan (CAP)) and pharmaceuticals (ibuprofen (IBU), furosemide (FUR), and trimethoprim (TMP)) for 96 h at concentrations corresponding to their respective lowest observed effect concentrations (LOECs). In the present study, we also developed and applied a behavioural stress indicator (BSI). Its undoubted advantage is that it enables the integration of the overall results for behavioural responses to stress caused by various chemicals over time, which allows them to be ranked and compared.

2. Materials and methods

2.1. Test species

Laboratory experiments were performed on IV-instar larvae of *Diamesa zernyi* collected in the Rio Presena (N46°13.596′, E010°34.929′) at 2685 m above sea level (Noce River catchment, Trentino Province, NE Italy) on three occasions in the late summer of 2016 (1, 7, and 14 September 2016). The larvae were collected with a 30 × 30 cm pond net (mesh size 100 μm) (Scubla SNC, Italy), sorted in the field with tweezers, transferred to plastic bottles filled with stream water, and transported to the laboratory in a cooling bag. Species confirmation was performed within 24 h of sampling using a stereomicroscope (MZ 7.5; Leica Microsystems, Germany; 50 ×) according to the method described by Rossaro and Lencioni (2015). The larvae were maintained in 1-L glass aquaria with stream water in a thermostatic chamber (ISCO, model FTD250-plus; Teledyne Isco Inc., Lincoln, Nebraska, USA) at 2 °C with aeration to maintain dissolved oxygen at higher than 80% saturation. The incubation temperature (2 °C) approximated the water temperature measured in the stream using a Hydrolab Quanta (Hydrolab Corporation®, Texas, USA) multiparametric probe at the first sampling (1 September 2016). To acclimate the larvae to exposure conditions, 24 h prior to each experiment randomly selected IV-instar larvae were removed from the rearing aquarium and transferred to a 500-mL beaker (approximately 40 larvae per beaker) containing 200 mL of hard reconstituted water (HRW) comprising: 4.36 mg/L $NaHCO_3$, 2.73 mg/L $CaSO_4 \cdot 2H_2O$, 2.73 mg/L $MgSO_4$, and 0.19 mg/L KCl (pH = 7.7), as described by Lencioni et al. (2016). During acclimatization and exposure, the larvae were maintained at 2 ± 1 °C without light or food, but the water was aerated.

2.2. Test chemicals

The larvae were exposed to seven individual chemicals at