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

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ABSTRACT

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

concentrations corresponding to their LOECs (Table 1), which had been measured previously for this organism (Miari, 2017 — Master thesis).

It should be noted that the tested concentration of CPF was close to the environmentally relevant concentration for surface waters in general, and for Alpine rivers in particular. In fact, this insecticide was found in the river Novella (Trentino Italy) at peak concentrations of above 500 ng/L, with a bulk higher than 1 μg/L in the presence of suspended solids (average concentration from a few to several tens of ng/L) (Morselli et al., 2017). Furthermore, IBU, FUR, and TMP were found in the river Noce at concentrations of 116 ng/L, 359 ng/L and 196 ng/L (Mandaric et al., 2017), respectively.

Stock solutions (stored at 4 °C until use) at the selected concentration of each chemical (with purity >95%, Sigma Aldrich Laboratories, Milan, Italy) were prepared in HRW and dimethyl sulfoxide (DMSO, analytical purity). DMSO was added to produce a final concentration of <0.1 mg/L to favour chemical solubilisation in water. Sandbacka et al. (2000) reported that at this concentration, DMSO does not cause acute toxicity and does not affect the mobility of *Daphnia magna*. Negative controls (CTRL = reconstructed water plus DMSO at the same concentration of toxicant tested solution) were carried out during the period of exposure in all experimental replicates. Test solutions were renewed every 24 h.

2.3. Experimental setup

The behavioural changes of *Diamesa* larvae were recorded at 24, 48, 72, and 96 h after exposure to the seven contaminants (Table 1). After exposure, the control and test larvae were poured, together with their exposure solutions, into 500-mL beakers on a worktable in a climate-controlled room (2.0 ± 0.1 °C). Video footage was then taken for 150—180 s using a digital high definition camera (Raspberry Pi 3 with Camera Module v2) at high resolution (1920 × 1080 pixels). The camera was positioned approximately 10 cm from the bottom of each beaker. During this time, the water temperature changed within the range +0.1 to +0.3 °C, as recorded with a portable thermometer (Koch 13211; ±1 °C). Behavioural effects were then assessed.

In organisms that are adapted to cold conditions, the expression of toxicity is delayed owing to physiological adaptation and reduced chemical reaction, both outside and within the organism (slow uptake kinetics, slow metabolic rate) (Chapman and Riddle, 2005), Therefore, a prolonged time of exposure (up to 96 h) in comparison to standardized test protocols (up to 48 h) was selected.

Data on behaviour were recorded at different exposure times (every 24 h for 4 days) because in the environment contamination is sometimes characterized by short-term peak concentrations, such as when pesticides are applied to crops. The pesticides sometimes enter the surface water in runoff resulting from rain or irrigation (Bonzini et al., 2006). In contrast, pollutants emitted by sewage treatment plants (such as pharmaceuticals and personal care products) are considered 'pseudo persistent' in the environment because they are continuously released into water bodies (Daughton, 2005). For these reasons, we decided to check the behaviour every 24 h of exposure.

2.4. Data analyses

The videos were analysed using ImageJ software (http://imagej.nih.gov/ij/), a public domain Java image processing program. The macro for tracking the larvae movements was based on the wrMTrck methodology. The plugin and a detailed description for animal tracking can be found at http://www.phage.dk/plugins/wrmtrck.html (Brooks et al., 2016; Husson et al., 2012; Selvaraj and Santhakumar, 2017). wrMTrck has been used to track the movement and speed of *Caenorhabditis elegans* crawling on agar plates or swimming in liquid. In the present study, the software was optimized/calibrated (Table 2) to monitor three different behavioural endpoints of *Diamesa* larvae:

- distances, expressed in pixels, corresponding to the distances covered by the larvae from start to finish of the analysed video;
- average speed (AvgSpeed), expressed as pixels/second; this is the average speed calculated as the ratio length/time (s), where the length is the sum of the lengths of all movement vectors between frames at a given track;
- frequency of body bends (BBps = body bends per second); this is the number of body bends, calculated using angles, during all video frames.

The ImageJ wrMTrck plugin uses ellipse fitting to count the body bends using angles, and one body bend corresponds to the movement of the head region trashing from one side to the other and back to the starting position (Lüersen et al., 2016).

Each original video was converted to an . *avi* file and was partitioned into five sections of 30 s each with FFmpeg software, an open source audio and video converter (http://www.ffmpeg.org),

Table 1
Physicochemical properties of the selected chemicals. The tested concentrations (expressed as mg L-1) for *Diamesa zernyi* larvae (Miari, 2017 - Master thesis) are indicated.

Chemical	Category of use	logKow	*Diamesa zernyi* Tested concentrations
chlorpyrifos (CPF)	insecticides	4.7[a]	1.1E-03[c] (48hrs)
metolachlor (MET)	herbicides	3.4[a]	27.4[c] (72hrs)
boscalid (BOS)	fungicides	2.96[a]	5.0E-01[c] (96hrs)
captan (CAP)	fungicides	2.5[a]	3.14[c] (24hrs)
ibuprofen (IBU)	nonsteroidal anti-inflammatory drugs (NSAID)	1.44 ($logD_{7.4}$)[*b]	100[c] (96hrs)
furosemide (FUR)	diuretics	−0.24 ($logD_{7.4}$)[*b]	500[c] (96hrs)
thrimethoprim (TMP)	antibiotics	0.63 ($logD_{7.4}$)[*b]	400[c] (96hrs)

*LogD7.4 for ionizable compounds.
[a] PPDB: Pesticide Properties DataBase.
[b] Avdeef, 2003.
[c] Miari, 2017 (Master thesis).

Table 2
Optimized parameters used to analyse the behaviour larvae of *D. zernyi*.

Parameters wrMTrck	Default	*D. zernyi*
minSize - Minimum Object Area (pixels˜2)[a]	100	10
maxSize - Maximum Object Area (pixels˜2)[a]	400	400
maxVelocity - Maximum velocity (pixels/frame)[b]	10	100
maxAreaChange - Maximum area change (%)[c]	30	75
minTrackLenght - Minimum track length (frames)	50	50
bendTreshold - Treshold for turn	2.0	2.0
Binsize - Size of bin for speed histogram (pixels/frames)	0.0	0.0
bendDetected (0 = off, 1 = Angle, 2 = AspectRatio, 3 = AR + Histogram)[d]	2	1
FPS — frames/s (0 = try to load from file)	30	30

[a] Indicate minimum and maximum larvae area in pixels.
[b] Numbers of pixels a larvae is permit to travel between two frames.
[c] % area change in area between continuous frames.
[d] For choosing the method to count body bends.

to standardize all videos to 900 frames (30 frames/second). The obtained mini-videos had a resolution of 576×324 pixels, which was appropriate for the experimental setup and the size of the larvae. Each video was then processed with ImageJ.

The behavioural results were analysed using Student's t-test to detect significant differences with respect to the control (distance, AvgSpeed, and BBps) for the various exposure times. Correlation between variables was checked using the Pearson test. When two parameters were highly correlated, one was discarded because both provided the same information. Statistical significance is indicated by asterisks: ****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; and *$p < 0.05$. All statistical analyses were performed using R-project software (R Core RDevelopment Core Team, 2016). The data for each exposure time were compared with the control data.

2.5. Behavioural stress indicator

The proposed Behavioural Stress Indicator (BSI) was designed to integrate information on various quantified behavioural endpoints to identify chemical substances that induce changes/deviation from the control conditions. To develop the BSI, deviations from the control of the three investigated endpoints were divided into six categories of behavioural response to which different scores were assigned:

1. score of 0.5 to the category of ± (0÷0.2) of deviation from CTRL;
2. score of 1 to the category of ± (0.2÷0.4) of deviation from CTRL;
3. score of 2 to the category of to ±(0.4÷0.6) of deviation from CTRL;
4. score of 3 to the category of (0.6÷0.8) of deviation from CTRL;
5. score of 4 to the category of ±(0.8÷1) of deviation from CTRL;
6. score of 5 to the category of >1 and <1 of deviations from CTRL.

The final BSI value was calculated by summing all the obtained scores for the three behavioural endpoints at the various times of exposure.

A final or partial numerical value for the BSI at a particular time of exposure was obtained for each chemical using the following algorithm (Eq. (1)):

$$BSI = \sum_{t} \left(\sum_{i,} score \right)$$ (1)

Where: t = the considered time of exposure (24, 48, 72, or 96 h)
i = the selected endpoint (distance moved, AvgSpeed, BBps)
$score$ = the individual score assigned to each endpoint.

3. Results

3.1. Behavioural endpoints

The increasing or decreasing deviations from the control of the three behavioural endpoints, measured at 24, 48, 72, and 96 h, are reported in Fig. 1. Statistical significance is indicated by asterisks: ****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; and *$p < 0.05$.

At 24 h, the behavioural endpoint with the highest deviation from the control was the distance moved. This was particularly true for MET (-59%; $p < 0.05$), FUR ($+109$; $p < 0.05$), and IBU ($+75\%$; $p < 0.05$). The other parameters varied from -58% (MET; $p < 0.05$) to $+18\%$ (FUR; $p < 0.05$) for AvgSpeed, and from -25 (MET; $p > 0.05$) to $+31\%$ (FUR; $p < 0.05$) for BBps. The AvgSpeed was particularly influenced at 24 h of exposure by MET and CAP, which induced reductions of 58 and 26% ($p < 0.05$), respectively. Finally, the BBps were not greatly affected by the presence of contaminants, with the exception of FUR, which induced an increase of 31% ($p < 0.05$).

At 48 h, the distance covered by *Diamesa* was the most affected behavioural endpoint, ranging from -62 to $+188\%$. Notably, the highest increases in term of distances covered by larvae were induced by CPF ($+188\%$; $p < 0.05$), BOS ($+158\%$; $p < 0.05$), and FUR ($+116\%$; $p < 0.05$). After 48 h, MET still induced a reduction of this endpoint of 62% ($p < 0.05$). The AvgSpeed was less relevant, but was still statistically significant, varying from -22% for MET ($p < 0.05$) to $+45\%$ for FUR ($p < 0.05$) exposure. The BBps endpoint variation was as follows: CAP (-49%; $p < 0.05$), IBU ($+42\%$; $p < 0.05$), and BOS ($+36\%$; $p < 0.05$).

After 72 h of exposure, there was a reduction in almost all the behavioural endpoints, and these data were clearly confirmed at 96 h, indicating a general slowing down of all the activities of the exposed organisms. The only exception was BOS, for which the BBps value increase proportionally with exposure time.

It is important to note that between 48 and 72 h, CPF, BOS, and FUR had the opposite effect on the distance covered by the larvae: from an initial marked increase to an evident decrease of this endpoint. BOS and CPF seemed to affect the distance endpoint in a similar way: from an initial absence of significant effects (at 24 h) to a substantial increase at 48 h, with a definite decline at 72 h and to a greater extent at 96 h.

Moreover, whereas the chemicals mentioned above induced variable responses according to exposure time, the other chemicals, particularly MET, provoked a progressive decrease of the measured endpoints over time.
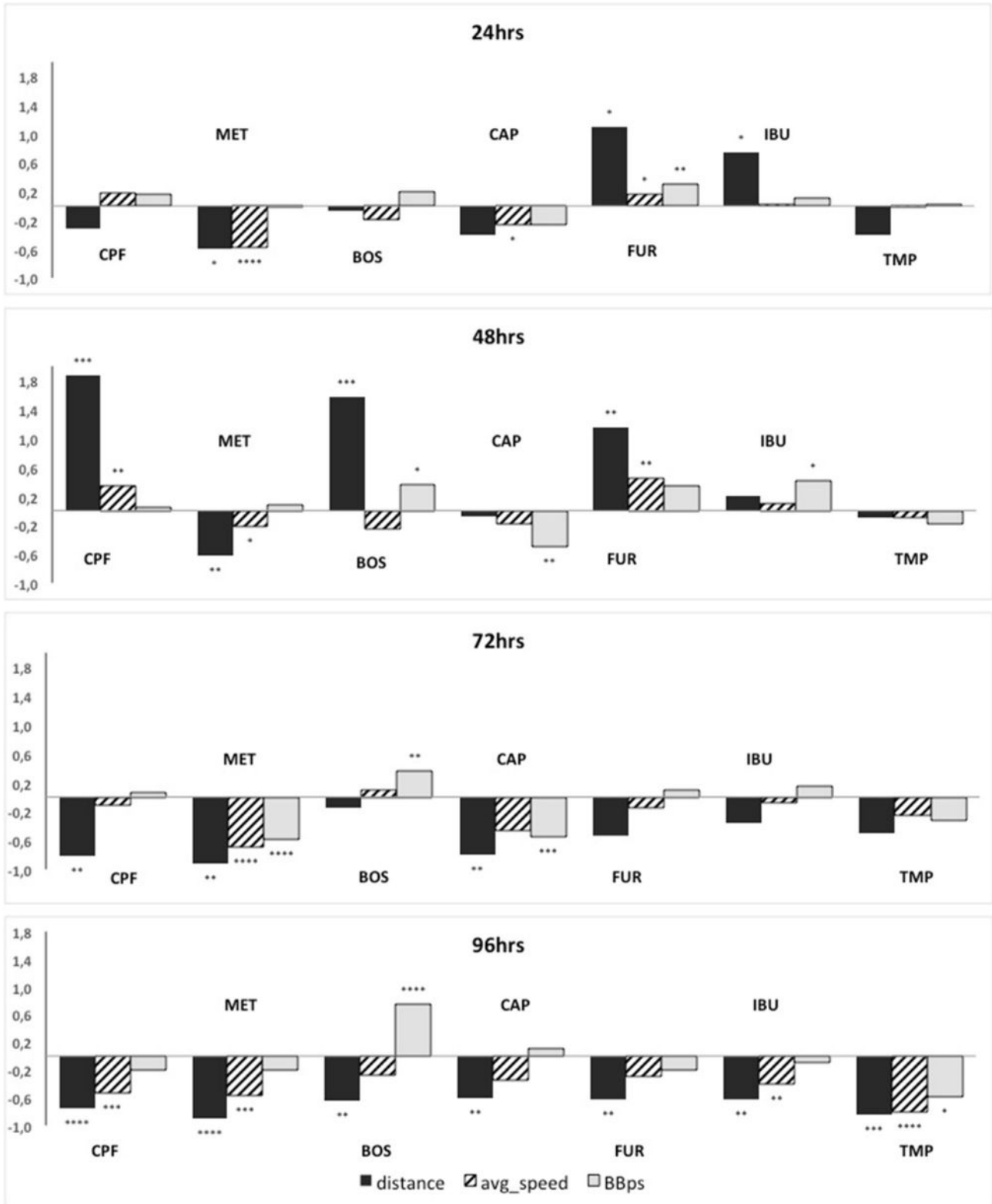
Fig. 1. The quantified behavioural endpoints (distance, AvgSpeed, and BBps) at the four exposure times are reported for all the compounds tested. The levels of statistical significance are indicated with asterisks (****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; and *$p < 0.05$). The data were normalized to the control.

3.2. Behavioural stress indicator

The results from Section 3.1 highlight deviations and fluctuations in all the behavioural endpoints investigated, suggesting that the exposed larvae experienced a general condition of stress. Information obtained from the behavioural tests may be combined by applying the proposed BSI, as reported in Fig. 2, which depicts both partial (at different times of exposure) and final BSI scores obtained

for the substances investigated.

Partial and final BSI scores were obtained by integrating the values of the three endpoints whose deviations in direction of increase or decrease were weighted in the same manner. In fact, both inhibition and stimulation of a given behavioural parameter may represent a factor of inter- or intra-specific interference of equal importance for a given ecosystem.

In the present study, the application of BSI indicated that the herbicide metolachlor had the highest BSI score, followed by the diuretic furosemide and the insecticide chlorpyrifos, whereas anti-inflammatory ibuprofen had the lowest BSI score. The fungicide captan, the antibiotic trimethoprim, and the fungicide boscalid had intermediate BSI values. The high scores attributed to MET and FUR were mainly due to the rapidity of the behavioural responses induced by these chemicals. Both compounds stimulated significant alterations in larval behaviour from the first video tracking measurement (24 h). However, in contrast to MET, which induced behavioural changes throughout the period of exposure (with a peak after 72 h), FUR mainly stimulated the changes up to 48 h. The BSI score for CPF mainly resulted from changes in the distance moved. However, CPF only had a major effect at 48 h (Fig. 1), whereas its effect was reduced at 24, 72, and 96 h.

4. Discussion

Exposure to chemicals may have very different acute effects among macroinvertebrate species (Maltby et al., 2005; Rubach et al., 2010; Wogram and Liess, 2001), and this variation can be closely related to the toxicological specificity of the mode of action of the chemical (Escher and Hermens, 2002). Species traits causing the inter-specific variation can be related to toxicokinetics (uptake, distribution, biotransformation, and elimination), or toxicodynamics (interaction with biological target sites) (Nyman et al., 2014).

There is a limited number of studies on the behavioural effects induced by contaminants on aquatic macroinvertebrates in the literature, and they mainly refer to traditional test species (i.e., *Daphnia magna* or *Chironomus tentans*) (Eissa et al., 2010; Hansen and Roslev, 2016; Hatch and Burton, 1999; Parolini et al., 2017).

In general, impaired behaviour in aquatic species may have detrimental consequences at the population level, through altered interactions with other members of the same species (i.e., change in fitness), and at higher levels of the ecological hierarchy (i.e., the community), through changes in competitive or predator/prey interactions (Duquesne and Küster, 2010; Reichmuth et al., 2009).
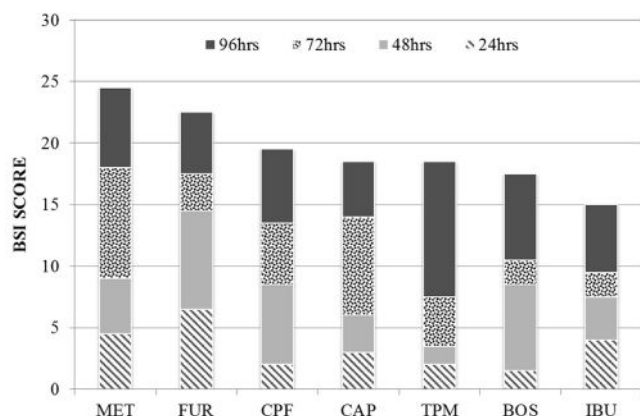

Fig. 2. Partial (for each exposure time) and final BSI scores obtained for each chemical from the behavioural tests.

4.1. Behavioural changes and toxicological mode of action

The present study represents the first attempt to standardize an experimental protocol for evaluating behavioural changes in the macroinvertebrates typically present in cold aquatic ecosystems. The optimization of video tracking software parameters (listed in Table 2) enabled the detection of even the smallest changes in the shape and position of all the single larvae monitored. This made it possible to choose the three most important parameters to consider in behavioural analysis (distances moved, average speed, and frequency of body bends).

One of the interesting results obtained in the present study related to the standardisation of the exposure time. Our results demonstrated the usefulness of following the behavioural responses of *Diamesa* larvae for 96 h. Up to this exposure time, all the tested compounds induced significant alterations in behaviour (Fig. 1). In contrast, after 96 h the larvae appear to have been completely stressed, with a strong reduction in all the parameters investigated. Our results confirm the need for long-term exposure (i.e., up to 72/96 h) to obtain toxic responses in species that are adapted to cold conditions, such as *Diamesa* spp., as reported in previous studies (Chapman, 2016).

The results obtained showed that optimization of the system makes it possible to detect significant behavioural effects (e.g., increases in swimming speed) for all the substances tested. The results reported in Fig. 1 cannot be easily understood because the effects on larval behaviour are highly variable, and depend on the contaminant and the exposure time. The highest variability was particularly noticeable in the first 24–48 h of exposure, whereas subsequently (after 48 h and up to 96 h) a generalized reduction of all the investigated parameters was evident. This decrease can be explained by the loss of fitness due to the loss of energy for muscle activity or locomotion, and the necessity of overcoming the friction of the aquatic medium during movement. In general, changes in external conditions, such as the burden imposed by toxic compounds on the habitat, induce stress so that the energy that is usually destined for normal functions (growth, reproduction, and locomotion) must be used to restore the imbalance (stress response) (Untersteiner et al., 2003; Wolf et al., 1988). For instance, organisms can react by increasing swimming/movement activity (escape or avoidance mechanisms) and/or by adaption mechanisms that involve antioxidant and detoxifying enzymes (Binelli et al., 2011; Colwill and Creton, 2011). Both mechanisms require energy, so we can hypothesize that after 48 h of exposure, and independently of the contaminant, the organisms are in a condition of energetic deficit that leads to a reduction in behavioural activity.

It is more difficult to explain the observed behavioural effects in the first 24–48 h. During this period of exposure, there is a large variation in the signals of behavioural change, which are dependent on the compound under consideration. Nevertheless, the investigated chemicals can be grouped into two broad categories: those that induced hyperstimulation of the behavioural signals, and those that produced a reduction. In fact, after 24–48 h, the pesticides CPF and BOS, and the pharmaceuticals IBU and FUR induced a statistically significant increase in most of the three parameters considered. IBU and FUR in particular seemed to increase larval activity in a shorter time compared with the pesticides. This can be attributed to the higher logKow values (Kow is the octanol/water partition coefficient) of CPF and BOS (Table 1), which may be responsible for the delayed passage of the molecules through the cell membrane (Kenakin, 2014).

In contrast, substances such as MET and CAP cause a reduction in larval behavioural activities. MET in particular induced an immediate and significant reduction in the distance moved and the average speed, whereas CAP stimulated a reduction in the average

speed and the BBps after 24 and 48 h, respectively. Larvae exposed to TMP exhibited a similar tendency. However, for up to 96 h of exposure, the reduction in activity was not statistically significant compared with the control.

It is difficult to interpret the different behavioural changes induced by the two groups of contaminants. However, a possible explanation, which should be verified by further studies, is related to the mechanism of action of the investigated chemicals. We noticed that the substances that induced hyper stimulation of larval activity exhibited a specific mode of action with regard to invertebrates, whereas those compounds that induced a slowdown in behavioural activities were narcotics.

CPF is a neurotoxic compound that induces an accumulation of acetylcholine in the synapses and a consequent disruption of normal nervous system function (Habig and Di Giulio, 1988). It has a specific mode of action with regard to invertebrates (Corbett, 1974).

BOS (formerly nicobifen or 2-chloro-*N*-(4′-chloro-2-biphenylyl) nicotinamide) has a very similar chemical structure to that of nicotine, and although it is a fungicide it behaves as a neurotoxin with regard to invertebrates (Elskus, 2014).

To the best of our knowledge, there is no available information on the potential neurotoxic effects of FUR on invertebrates, whereas some effects on the human nervous system have been reported. However, FUR has a specific mode of action with regard to insect larvae. In particular, it has been reported that it acts on the ATP-ase pump (Caruso-Neves and lopes, 2000; Maddrell and O'Donnel, 1992). Gassner and Komnick (1982) demonstrated that FUR was a non-competitive inhibitor of anion ATPase activity in the recta of larval dragonflies (*Aeshna cyanea*).

Finally, the widely used non-steroidal anti-inflammatory drug IBU works mainly by inhibiting the cyclooxygenase (COX) enzymes used for the synthesis of prostaglandins (Cashman, 1996). Prostaglandins are also widely distributed in insect tissue, where they play an important role in physiological functions other than reproduction. Dadd and Kleinjan (1979) indicated the possible role of prostaglandins in of mosquito flight capability.

In contrast, MET, CAP, and TMP caused a general trend of reduction in the behavioural endpoints, which could be related to their non-specific mode of action (narcotic-type). Such narcotic-type behaviour is typical of inert chemicals whose toxicity can largely be attributed to the hydrophobic characteristics of the molecules and not to any specific mode of action (Verhaar et al., 1992). In one of the earliest studies on the behavioural responses of fish to contaminants, McKim et al. (1987) noted that narcotic compounds induced hypoactive swimming in fish. This finding agreed with the results reported by De Lange et al. (2006). These authors, studying the behavioural responses of *Gammarus* species exposed to a cationic surfactant (CTAB: cetyltrimethylammonium bromide), reported a general reduction in the activity of the arthropods, which was attributed to a non-specific mode of action of CTAB. According to their toxicity database (MOAtox), Barron et al. (2015) recently assigned MET to the category of non-polar narcotic compounds with regard to aquatic invertebrates. Analogously, Maltby et al. (2009) included CAP among the fungicides without a specific mode of action with regard to fish and aquatic invertebrates.

It seems that the direction of the behavioural changes observed in *Diamesa zernyi* follows the broad classification in narcotic or specifically acting substances for aquatic invertebrates. This hypothesis should be verified with further investigations.

4.2. Assessment of behavioural effects

According to Melvin and Wilson (2013), behavioural studies should provide meaningful results that are relevant to the ecological consequences of exposure, by understanding the associations between specific behaviours and high-level effects on survival, health, and fitness.

In the present study, the total distance moved seemed to be the most affected/reactive signal of behavioural changes. The distance moved can be associated both to find an optimal habitat or to evasive behaviour. Indeed, larvae by finding suitable habitats (characterized by the presence of food and/or shelter) minimize mortality, accelerate development, and reduce time required to reach the adult stage and to reproduce. This behaviour results in the maximal resource use. Individuals only leave the habitat patch in response to: i) a decrease in food quality or availability, ii) competition, iii) avoid predators, or iv) a negative impact of a change in environmental conditions, such as the presence of chemical stressors.

In our experiments, the only factor that drove individual movements was the exposure to different contaminants. As previously described, there were substances which induced an increase in distance moved (at least in the first 48 h of exposure), whereas others stimulated a slowdown in this activity. Negative consequences of a hyper stimulation of the movement can be associated to a higher energetic expenditure that could exceed the available energy supply, resulting in reduced growth and moulting performances of individuals.

On the other hand, compounds that influence the evasive ability (through the reduction of the distance moved) may directly affect the ability of an organism to avoid predation. In addition, several studies showed that individuals in worse physiological condition started later their density or food dependent dispersal and moved over shorter distances than strong, healthy (thus more competitive) individuals (Bonte and de la Pena, 2009; Delgado et al., 2010).

Exposure to contaminants also modified the average speed of *D. zernyi* larvae. This was particularly relevant after 96 h of exposure when this parameter was significantly reduced. The average speed is associated to a rapid acceleration of the organisms and can be related to the presence of predators. Indeed, the faster an animal reacts, the more able it may be in evading predatory attack (Buskey et al., 2002). Thus, a reduction in the average speed can have negative consequence in the predator/prey relationship.

Finally, the frequency of body bends (BBps) was also changed during the exposure time, even with less extent if compared with the other two parameters. It is still unclear the behavioural significance of this parameters in *D. zernyi*; however, in a study of Kim and coworkers (Kim et al., 2006) studying the movement behaviour of *Chironomus samoensis* larvae exposed to anticholinesterase insecticide reported that the tested specimens repeatedly bent and stretched their bodies in a limited spatial range and this was associated to a general stress of the organisms.

On this basis, we can conclude that the exposure to different contaminants induced a marked syndrome of behavioural changes in *D. zernyi* specimens, that may have consequences both on the fitness (growth and development) of individuals and on higher hierarchical levels (predator/prey relationship).

Unfortunately, at the time being, we cannot assess which is the impact on the fitness of individuals as the consequences of increase or the decrease in one of the considered parameters; moreover, it is difficult to assess the magnitude of the consequences at higher ecological levels. For this reason, in the present study we integrated all the obtained information into a BSI. The undoubted advantage of this approach is the integration of all behavioural changes induced by contaminants over time, enabling their comparison in terms of the stress induced.

In the past, risk indicators have mostly been used in ecotoxicology to rank pesticides (Finizio et al., 2001; Sinclair et al., 2006). More recently, they have been suggested for the classification of the

ecological health of aquatic systems (Broeg et al., 2005; Hagger et al., 2008). In general, risk indicators are based on the assignment of scores from a set of physicochemical, toxicological, or ecotoxicological properties of the contaminants. For instance, Hagger et al. (2008) selected a suite of biomarkers to measure a range of biological parameters at different levels of biological organisation. Usually in risk indicators, the assigned scores are then combined through an algorithm to obtain a numerical index, which is also useful for comparative purposes.

BSI works as a simple tool that allows to get a synthetic and rapid understanding of which chemicals among the many pose the higher harm for the tested organism (at behavioural point of view) and to have an overall idea about which time of exposure provokes the greatest alteration. For instance, BSI allows us to quickly see that some chemicals induce greater alterations in *D. zernyi* since the first hours of exposure (e.g. FUR), whereas others provoke major behavioural alterations after 72/96 h (e.g. TMP). Anyway, at this level of knowledge, the BSI is proposed as an exercise tool for comparative purposes: it enables to selected chemicals, from a large set, to which further financial and research efforts could be focused. The future available scientific knowledge about the ecological significance of the behavioural changes observed on a definite species will significantly improve the application of BSI tool in a prospective of ecological risk assessment.

5. Conclusions

The present study represents the first preliminary attempt to develop a protocol for using behavioural endpoints to evaluate the overall impact of chemical contaminants on the environment. Considering the wide variability of behavioural responses over time, the test duration should be at least 72—96 h.

All the active substances studied (the plant protection products: chlorpyrifos, metolachlor, boscalid, and captan; and the pharmaceuticals: ibuprofen, furosemide, and trimethoprim) provoked behavioural alterations in *Diamesa zernyi* at concentrations at which negligible effects (i.e., LOECs) were observed for the test organism according to the classical acute test.

The total distance travelled by the larvae was the behavioural response most affected by exposure to sub-lethal concentrations of pharmaceuticals and pesticides. Within the complexity of the freshwater community, these behavioural alterations could lead to a number of consequences on different levels of the biological organisation, i.e. from individuals to community.

Considering the measured environmental concentrations of the selected active substances in surface waters, only the insecticide chlorpyrifos seems to elicit significant behavioural changes in *Diamesa zernyi*.

Author contributions

Francesco Bellamoli performed toxicological tests, implemented the video tracking system, and optimized workflows and processing parameters.

Valeria Di Nica collaborated on the toxicological and statistical analyses.

Antonio Finizio developed the BSI indicator.

Valeria Lencioni conceived and designed the experiments.

Francesco Miari performed toxicological tests and shot the video.

Tanita Pescatore performed video tracking analysis.

Sara Villa conceived and designed the experiments.

All authors contributed to writing the paper.s.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.03.029.

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