

Response of *Diamesa* spp. (Diptera: Chironomidae) from Alpine streams to newly emergent contaminants and pesticides

Valeria LENCIONI*, Francesco BELLAMOLI, Paola BERNABÒ, Francesco MIARI, Alberto SCOTTI

Department of Invertebrate Zoology and Hydrobiology, MUSE – Museo delle Scienze, Corso del Lavoro e della Scienza 3, 38122 Trento, Italy

*Corresponding author: valeria.lencioni@muse.it

ABSTRACT

Acute toxicity and genotoxic activity of 11 pollutants were investigated in wild populations of *Diamesa cinerella* and *Diamesa zernyi* (Diptera Chironomidae) from two alpine streams (Italian Alps). *D. cinerella* was collected in two sites on the non-glacial Vermigliana stream, 50 m-upstream and 5-m downstream of the Wastewater Treatment Plant (WTP) at the Tonal Pass (1799 m a.s.l.). *D. zernyi* was collected in the Presena glacial stream, close to the glacier snout (2685 m a.s.l.). IV-instar larvae were exposed for 24–96 h to increasing concentrations of three drugs (ibuprofen-IBU, furosemide-FUR, trimethoprim-TMP), three personal care products (triclocarban-TCC, tonalid-TON, sucralose-SUCR), and five pesticides (boscalid-BOS, captan-CAP, chlorpyrifos-CPS, metolachlor-MET, terbuthylazine-TER). The experimental concentrations were from one to several million times higher than the highest environmental concentration (EC) measured in the study sites. Two mixtures of pesticides were also prepared: MIX 1K = $10^3 \times$ EC of CPS, MET and TER, and MIX 10K = $10^4 \times$ EC of CPS, MET and TER. Species- and site-specific responses were observed for both tests. On the basis of survival data, both species resulted very resistant to pharmaceuticals (mainly to FUR for which no effects on survival and movement or pupation were observed), and more sensitive to pesticides (mainly to CPS, MET and CAP). Genotoxicity tests (Comet assay) highlighted a WTP effect under natural conditions and a genotoxic effect for 9 of the 11 tested compounds. Overall, a clear gradient of increasing resistance in larvae from the least (PRO) to the most polluted (TP_dw) site was highlighted by both tests, ecotoxicological and of genotoxicity, as also expected according to species autecology (*D. zernyi* is restricted to very cold and pristine habitats). *D. cinerella* living downstream of the effluent accumulates a significantly higher DNA damage than the other populations, highlighting a basal physiological stress condition in nature. It is plausible that these larvae possess chemical resistance strategies to survive already under natural conditions. *Diamesa* spp. exhibited a higher toxic resistance than any other model species tested to date under the same pollutants, probably associable to its strong cold resistance. The results emphasised that the measured concentrations of Contaminants of Emerging Concern (CECs) and pesticides seem to be far below those required to cause acute effects. However, the effects on freshwater communities of prolonged exposure to mixture of trace CECs and pesticides remain unknown.

Key words: Pollution; climate change; glacier-fed streams; sub-lethal effects; acute toxicity; genotoxicity.

Contributions: VL, experiments concept and design; FB, FM, AS, toxicological tests performing; FB, VL, data processing; PB, collaboration on data processing, performing statistical analyses; VL, manuscript writing and revision.

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INTRODUCTION

Freshwater wildlife at high altitude is threatened by climate change. Glacier retreat will alter hydrological and thermal regimes, sediment transport and biogeochemical fluxes in glacier-fed streams (kryal), with effects on biodiversity (Milner *et al.*, 2009). Glacier-fed streams are typically colonized by few animal taxa, with chironomids (Diptera, Chironomidae) dominant as individual and species number in the invertebrate community (Rossaro *et al.*, 2016).

Long-term ecological studies have highlighted changes in the invertebrate community structure and

functional diversity in glacier-fed streams due to glacier retreat (Milner *et al.*, 2017; Brown *et al.*, 2018), with loss of the most cold-stenothermal kryal inhabitants (Lencioni, 2018). In particular, some cold stenothermal *Diamesa* species (Chironomidae Diamesinae) might be seriously threatened by extinction accompanied by loss of the kryal habitat (Lencioni *et al.*, 2015). Among these, the “ice fly” *Diamesa steinboeckii* Goetghebuer, the “flagship” species of the European glacier-fed streams, already disappeared or surviving only as brachypterous populations in Alpine catchments with small feeder glaciers (with a surface $\ll 1$ km²) (Lencioni, 2018).

Climate change is not the only threat for the alpine fauna, but exposure to chemical contamination by pollutants

carried to the glaciers by atmospheric transport and released in the ice-melt waters (Guzzella *et al.*, 2016; Steinlin *et al.*, 2016) is growing. Among these, pesticides and “emerging contaminants” (CECs) (*e.g.*, PPCPs=pharmaceuticals and personal care products) (Ferrario *et al.*, 2017). PPCPs include human and veterinary drugs and many consumer chemicals used for example in fragrances, sun-screen agents, lipsticks, shampoos, cosmetic and food products (Daughton and Ternes, 1999). In Alpine environments, even if the measured concentrations of the detected contaminants may be much lower than those causing acute effects (Chiogna *et al.*, 2016), prolonged exposure to mixture of CECs may damage macroinvertebrate communities, with cascade effects on the river food webs. CECs are emitted also by Wastewater Treatment Plants (WTPs), representing an emerging environmental problem in Alpine rivers strongly affected by tourism, where pollution level peaks during holidays (Mandaric *et al.*, 2017).

While it is known that *Diamesa* spp. survives short-term heat shocks (1 h shock: LT50=32°C, LT100=35°C) by developing a Heat Shock Response (Lencioni *et al.*, 2013), to our knowledge no information is available on toxic resistance in *Diamesa* spp. or in other alpine stream invertebrates. Furthermore, ecotoxicological literature on chironomids is limited to *Chironomus* genus (Bernabò *et al.*, 2017). To evaluate the ecotoxicological and genotoxic effects of 11 pollutants were tested in two *Diamesa* species collected in two Alpine streams polluted by pharmaceuticals, personal care products and pesticides with different origin (WTPs and atmospheric transport). On the basis of species autecology (*D. zernyi* is restricted to very cold and pristine habitats, Lencioni and Rossaro, 2005) and natural level of environmental contamination, species- and site-specific responses were expected.

METHODS

Animal sampling and rearing

Laboratory experiments were performed on IV-instar larvae of *Diamesa zernyi* Edwards collected in the Rio Presena at 2685 m asl (PR0), within 20 m-downstream of the glacier snout, in late summer 2016 (1, 6, 14 September 2016) and on IV-instar larvae of *Diamesa cinerella* Meigen collected in the Vermigliana Stream at 1799 m asl, in winter 2016 (7, 9, 14 March 2016). The two sites on the Vermigliana stream were located 70 m-upstream (TP_up) and 50 m-downstream (TP_dw) of the effluent of the Tonale Pass WTP, designed to serve 10,000 equivalent inhabitants. This plant collects wastewater from a large ski resort (Mandaric *et al.*, 2017). The three sites are located in the Noce River catchment, in Trentino (NE-Italy, 46 °N, 10°E) (Fig. 1). The main physicochemical features of the three sites are reported in Tab. S1.

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, NE, 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 two streams on the first day of sampling using a multiparametric probe Hydrolab Quanta (Hydrolab Quanta, Hydrolab Corporation®, TX, USA) (Tab. S1). To acclimate the larvae to exposure conditions, 24 h prior to each experiment randomly selected larvae were removed from the rearing aquarium and transferred to a 500-mL beaker (approximately 40 larvae per beaker) containing 200 mL of reconstituted water (RW) prepared according to (Holdway, 2005): hard for larvae from the Vermigliana stream and soft-medium for larvae from the Presena stream, according to the natural conductivity (Tab. S1). During acclimatization and exposure, the larvae were maintained with aeration at 2±1°C without food, to empty the gut.

Treatment protocol

All larvae were exposed to four PPCPs selected on the basis of their environmental concentrations (EC) (referred to winter in the Vermigliana stream and to summer in the Presena stream), risk level and state of knowledge on their effects on aquatic wildlife (Tab. S2): ibuprofen-IBU (NSAID, nonsteroidal anti-inflammatory), furosemide-FUR (diuretic), trimethoprim-TMP (antibiotic) and triclocarban-TCC (antibacterial). The larvae collected in the most pristine site, PR0, were exposed to other seven pollutants, of which two PPCPs (the fragrance tonalid-TON and the food artificial sweetener sucralose-SUCR) and five pesticides: boscalid-BOS (fungicide), captan-CAP (fungicide), metolachlor-MET (herbicide), terbuthylazine-TER (herbicide), chlorpyrifos-CPS (insecticide) (Tab. S2) and to two mixtures of pesticides: i) MIX 1K=10³ x EC of CPS, MET and TER; ii) MIX 10K=10⁴ x EC of CPS, MET and TER.

The ecotoxicological effects of the 11 pollutants were evaluated by 21 acute toxicity tests (one per species-site-pollutant, from 24 to 96 h) to estimate the Lethal Concentrations (LC). The tests were stopped before 96 h when the mortality overcame 50%. Five to ten exposure concentrations for each pollutant were selected on the basis of toxicological parameters (LC50/EC50) published for other aquatic species (*Daphnia magna*, *Planorbis*

carinatus, *Hydra attenuata*, *Hydra vulgaris*, *Nitocra spinipes*, *Chironomus tentans*, *Chironomus plumosus*) (Tab. 1), the environmental concentrations and the solubility of the substances (Tab. S2). For TON, SUCR and TER, only one-two experimental concentrations were tested, corresponding to the maximum solubility in acetone (for TON), DMSO (for TER) or water (for SUCR). All pollutants (with the exception of TON and SUCR) were dissolved in DMSO to favour chemical solubilisation in water, with a final pH of 7.7. DMSO did never exceed 2% of final volume to avoid harmful effects (Dong *et al.*, 2013). At this concentration, DMSO did not cause genotoxicity in *Diamesa* spp. and other invertebrates from the Vermigliana stream (Baetidae and Rhyachophilidae) (*unpublished data*). Aliquots of stock solution were diluted with RW. The experimental concentrations ranged from once to 50

million times the predicted/measured concentrations in the two streams (Tab. S2).

The genotoxicity of the 11 pollutants was assessed with the single-cell gel electrophoresis assay (SCGE), also known as Comet assay, to detect DNA damage in individual cells in terms of strand breaks that represent one of the major damage to DNA via oxidative stress (Valverde and Rojas, 2009). The assay was performed according to Bernabò *et al.* (2017), without isolating individual cell-types (Lee *et al.*, 2009; Martínez-Paz *et al.*, 2013). Eighty-three tests were performed at sublethal concentrations of each pollutant (=LC10 estimated, LOEC, NOEC or LCx calculated where $x \ll 50$, see *Statistical analyses*; Tab. 1) in the same laboratory conditions as acute toxicity tests. Genotoxicity was evaluated also in larvae maintained in RW within 24 h from the sampling, to

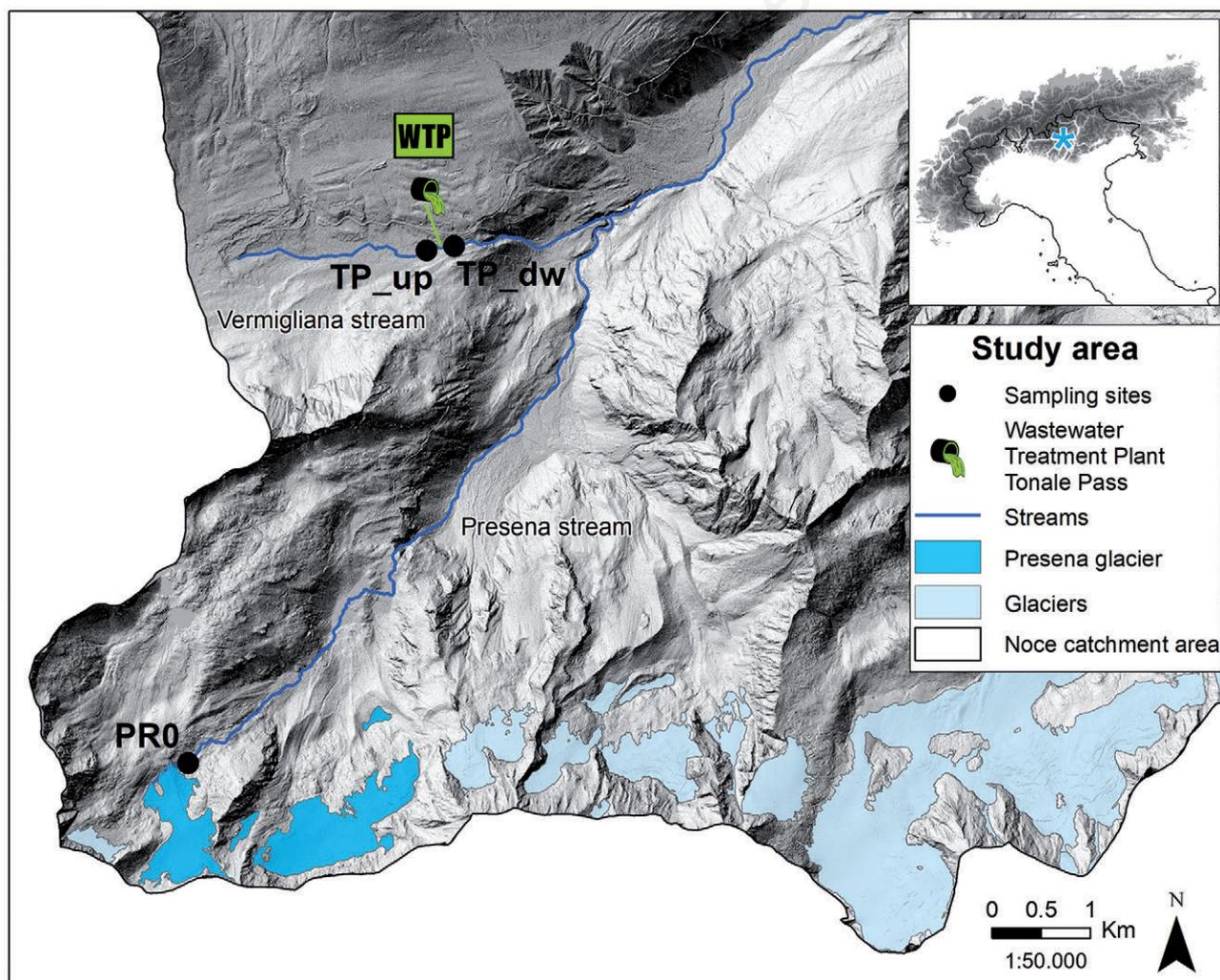


Fig. 1. Study area (Trentino, NE-Italy, 46°N, 10°E). PR0, kryal site on the Presena stream; TP_ sites on the Vermigliana stream, upstream (TP_up) and downstream (TP_dw) from the Tonale Pass (TP) WTP.

highlight any basal DNA damage under natural conditions. Cells were analyzed at the fluorescence microscope with the TriTek Comet Score™ software (TriTek Corp) and the Olive Tail Moment (OTM) was used for assessing DNA damage. OTM is a measure of both amount of DNA in the tail and distribution of DNA in the tail and it is calculated as the product of the tail length and the fraction of total DNA in the tail=(tail mean – head mean) x (tail %DNA/100) (Olive and Banáth, 2006).

For each series of experiments (acute toxicity tests and Comet assay), three biological replicates, with five larvae each, were planned for each exposure concentration and for the appropriated controls. During each test, larvae were taken in 100 mL glass bottles, containing 20 mL test solution or the solvent diluted with RW (control), with test solutions renewed daily. Negative controls (Ctrl=reconstructed water plus solvent at the same concentration of toxicant tested solution) were carried out during the period of exposure in all experimental replicates (Villa *et al.*, 2018). After each exposure of 24 h, larvae that moved spontaneously were considered active, whereas those that moved only following a tactile stimulus were considered suffering. Survival was determined by the sum of active and suffering (=alive) larvae. Immobile larvae were considered dead. Due to mortality or pupation of larvae in the genotoxicity tests (depending on the exposure concentration, *e.g.*, 10% mortality at LC10), extra replicates were added when necessary to obtain a minimum number of five live larvae for each series of experiments. In all, 2710 larvae were tested for acute toxicity (835 of *D. zernyi* and 1875 of *D. cinerella*) and 400 for genotoxicity assay (200 of *D. zernyi* and 200 of *D. cinerella*).

Statistical analyses

Toxicological parameters, *i.e.* the concentrations causing 10% and 50% of mortality (=LC10 and LC50 with 95% fiducial limits) were estimated with a Weibull distribution according to Ritz *et al.* (2015) using R (version 3.2.4) and “drc” package (version 3.0-1). In three cases it was not possible to estimate significantly the LC10 and LC50: i) when mortality was <50% after 96 h: in this case a value of LCx was calculated from survival data at the highest pollutant concentration (=Cmax); ii) when all larvae were found alive after 96 h, Cmax was considered as a NOEC (=No Observed Effect Concentration); iii) when some larvae were found suffering or pupated after 96 h, Cmax was considered as a LOEC (Lowest Observed Effect Concentration).

Comet assay data were analysed using Kruskal-Wallis test followed by Dunn test multiple comparisons. Three levels of significance are reported: *P<0.05, **P<0.01, ***P<0.001. The software GraphPad Prism 7 was used to statistically analyse and graph the Comet assay data.

RESULTS

Acute toxicity

Toxicological parameters estimated or calculated in the two target species are reported in Tab. 1. Only three pollutants exhibited a significant negative effect on survival: CAP (LC10=3.14 mg L⁻¹; LC50=81.06 mg L⁻¹; 24 h), CPS (LC10=1.06 µg L⁻¹; LC50=5.24 µg L⁻¹; 48 h) and MET (LC10=27.40 mg L⁻¹; LC50=84.12 mg L⁻¹; 72 h), all in *D. zernyi* (Tab. 1, Fig. 2). BOS and TER seemed to be harmless, with no effects by BOS (NOEC=500 µg L⁻¹, 96 h) and 20% of mortality by TER after 96 h at 25 mg L⁻¹. *D. zernyi* larvae exhibited a high resistance also to the two mixtures of pesticides, with 3% of mortality after 72 h in the mixture with the lowest concentration (MIX 1K) and 13% after 24 h in that one more concentrated (MIX 10K).

For TCC, SUCR and TON some mortality (from 7 to 33%; Tab. S2) was detected only after 96 h of exposure at the highest concentration of the product (100 µg L⁻¹ of TCC, 42.7 g L⁻¹ of SUCR and 100 mg L⁻¹ of TON). Among these, only TCC caused mortality in all *Diamesa* populations, after 96 h at 100 µg L⁻¹: LC7 in *D. zernyi*, LC13 in *D. cinerella* from TP_up and LC33 in *D. cinerella* from TP_dw). Furosemide seemed the least harmful substance for the two species in the three sites (NOEC=500 mg L⁻¹, 96h), followed by TMP (NOEC and LOEC=400 mg L⁻¹, 96 h in *D. cinerella* from TP_up and *D. zernyi* from PR0 respectively), IBU (NOEC=100 mg L⁻¹, 96 h) in *D. cinerella* from TP_dw and BOS (NOEC=500 µg L⁻¹, 96 h) in *D. zernyi* from PR0. In all other cases, a LCx was calculated, from LC3 72 h for the mixture 1K to LC33 96 h for IBU both in *D. zernyi* (Tab. 1).

Overall, some drugs were more toxic for the upstream *D. cinerella* population (*i.e.*, IBU for which a mortality of 7% was detected at 100 mg L⁻¹ after 96 h) and others for the downstream one (*i.e.*, TMP for which a mortality of 7% was detected at 400 mg L⁻¹ after 96 h). Among the drugs, only under IBU exposure, some suffering signals were detected in the other species, *D. zernyi*, with 33% of mortality after 96 h at 100 mg L⁻¹.

Genotoxic activity

A basal level of DNA damage was detected in the larvae not exposed to pollutants (Fig. 3A), with *D. cinerella* from TP_dw showing significantly more damage than the other two populations.

This population accumulated less DNA damages than that living upstream when exposed to sub-lethal concentrations of the three drugs (FUR, IBU and TMP) and the antibacterial (TCC) after 24 h (Fig. 3C). At this time of exposure, only IBU caused a significant damage in *D. cinerella* from TP_dw, while in that one living in

TP_up also FUR and TCC exhibited a significant genotoxic effect. After 96 h of exposure, a genotoxic activity remained for IBU in *D. cinerella* from TP_dw and only for TCC upstream (Fig. 3D). *D. zernyi* resulted the most sensitive species, accumulating, after 96 h of exposure, DNA damages by all four pollutants tested also in *D. cinerella* (Fig. 3D). Even the two mixtures (MIX 1K and MIX 10K) and four of the pollutants tested only in *D. zernyi* (SUCR, TON, BOS and MET) caused genotoxicity (Fig. 3B).

DISCUSSION

Species- and site-specific responses were observed for both tests, according to our hypothesis. On the basis of survival data, both species were very resistant to pharmaceuticals (mainly to FUR that did not affect survival or fitness), and more sensitive to pesticides

(mainly to CPS, MET and CAP - only for these three chemicals were estimates of significant LC10 and LC50 possible with the Weibull distribution). These results confirmed the high-hazard for the aquatic environments of the insecticide CPS (classified as very high-risk substance) and of the fungicide CAP (classified as high-risk substance) and suggest a high-hazard category for the as-yet undefined MET (Tab. S2; Lencioni *et al.*, 2017). However, 50% mortality was observed only after 72 h to an exposure concentration of MET about 18 million times higher than its EC, suggesting that the environmental concentrations are appropriate for this chironomid fauna.

Starting from these results, it was surprising that only 3% mortality was observed to mixed pesticides including also CPS and MET: after 72 h in a solution with CPS, MET and TER 1000 x higher than their EC. A non-additive toxic effect of pesticide mixtures was already been observed for other organisms, probably due to reciprocal interactions between pesticides with different chemical properties and

Tab. 1. Acute toxicity of eleven pollutants in *Diamesa cinerella* from two sites (TP_up and TP_dw) and *Diamesa zernyi* from PR0.

| Species | Toxicant | Site | Toxicological parameter (time) | Toxicological parameter (time) from literature |
|--------------------------|----------|---|--|---|
| <i>Diamesa cinerella</i> | FUR | TP_up | 500 mg L ⁻¹ (NOEC, 96h) | >100 mg L ⁻¹ (EC50, 48h) ⁽¹⁾ (<i>Hydra vulgaris</i>) |
| | | TP_dw | 500 mg L ⁻¹ (NOEC, 96h) | |
| | IBU | TP_up | 100 mg L ⁻¹ (Calc. LC7, 96h) | 20 mg L ⁻¹ (LC50, 72h) ⁽²⁾ (<i>Planorbis carinatus</i>) |
| | | TP_dw | 100 mg L ⁻¹ (NOEC, 96h) | 25 mg L ⁻¹ (LC50, 96h) ⁽³⁾ (<i>Hydra attenuata</i>) |
| | TMP | TP_up | 400 mg L ⁻¹ (NOEC, 96h) | 150 mg L ⁻¹ (EC50, 48h) ⁽⁴⁾ (<i>Daphnia magna</i>) |
| | | TP_dw | 400 mg L ⁻¹ (Calc. LC7, 96h) | |
| | TCC | TP_up | 100 µg L ⁻¹ (Calc. LC13, 96h) | 10 µg L ⁻¹ (EC50, 48h) ⁽⁵⁾ (<i>Daphnia magna</i>) |
| | | TP_dw | 100 µg L ⁻¹ (Calc. LC33, 96h) | |
| <i>Diamesa zernyi</i> | FUR | PR0 | 500 mg L ⁻¹ (NOEC, 96h) | >100 mg L ⁻¹ (EC50, 48h) ⁽¹⁾ (<i>Hydra vulgaris</i>) |
| | IBU | PR0 | 100 mg L ⁻¹ (Calc. LC33, 96h) | 20 mg L ⁻¹ (LC50, 72h) ⁽²⁾ (<i>Planorbis carinatus</i>) |
| | | | 25 mg L ⁻¹ (LC50, 96h) | ⁽³⁾ (<i>Hydra attenuata</i>) |
| | TMP | PR0 | 400 mg L ⁻¹ (LOEC, 96h) | 150 mg L ⁻¹ (EC50, 48h) ⁽⁴⁾ (<i>Daphnia magna</i>) |
| | TCC | PR0 | 100 µg L ⁻¹ (Calc. LC7, 96h) | 10 µg L ⁻¹ (EC50, 48h) ⁽⁵⁾ (<i>Daphnia magna</i>) |
| | SUCR | PR0 | 42.727 g L ⁻¹ (Calc. LC10, 96h) | >10 g L ⁻¹ (LC50, 96h) ⁽⁶⁾ (<i>Nitocra spinipes</i>) |
| | TON | PR0 | 100 mg L ⁻¹ (Calc. LC7, 96h) | 0.2 mg L ⁻¹ (EC50, 21d) ⁽⁷⁾ (<i>Daphnia magna</i>) |
| | BOS | PR0 | 500 µg L ⁻¹ (NOEC, 96h) | 50 µg L ⁻¹ (LC50, 48h) ⁽⁸⁾ (<i>Daphnia magna</i>) |
| | CAP | PR0 | 3.14 mg L ⁻¹ (LC10, 24h) | 7-10 mg L ⁻¹ (LC50, 96h) ⁽⁹⁾ (<i>Daphnia magna</i>) |
| | | | 81.06 mg L ⁻¹ (LC50, 24h) | |
| | CPS | PR0 | 1.06 µg L ⁻¹ (LC10, 48h) | 0.5 µg L ⁻¹ (LC50, 96h) ⁽¹⁰⁾ (<i>Chironomus tentans</i>) |
| | | | 5.24 µg L ⁻¹ (LC50, 48h) | |
| | MET | PR0 | 27.40 mg L ⁻¹ (LC10, 72h) | 3.8 mg L ⁻¹ (EC50, 48h) ⁽¹¹⁾ (<i>Chironomus plumosus</i>) |
| | | | 84.12 mg L ⁻¹ (LC50, 72h) | |
| | TER | PR0 | 25 mg L ⁻¹ (Calc. LC20, 96h) | >5 mg L ⁻¹ (EC50, 48h) ⁽¹²⁾ (<i>Daphnia magna</i>) |
| MIX1K | PR0 | 10 ³ x EC of CPS, MET, TER (Calc. LC3, 72h) | N.A. | |
| MIX10K | PR0 | 10 ⁴ x EC of CPS, MET, TER (Calc. LC13, 24h) | N.A. | |

FUR, furosemide; IBU, ibuprofene; TMP, trimethoprim; TCC, triclocarban; SUCR, sucralose; TON, tonalide; BOS, boscalid; CAP, captan; CPS, chlorpyrifos; MET, metolachlor; TER, terbuthylazine; MIX 1K, 10³ x EC of CPS, MET, TER; MIX 10K, 10⁴ x EC of CPS, MET, TER. References for toxicological parameters from literature: ¹(Pascoe *et al.*, 2003); ²(Pounds *et al.*, 2008); ³(Quinn *et al.*, 2008); ⁴(De Liguoro *et al.*, 2009); ⁵(Tamura *et al.*, 2013); ⁶(Wiklund *et al.*, 2012); ⁷(Grutzner, 1995); ⁸(Bundschuh *et al.*, 2016); ⁹(Kamrin, 2000); ¹⁰(Ankley *et al.*, 1994); ¹¹(Mayer and Ellersieck, 1986); ¹²(Marchini *et al.*, 1988).

mechanisms of toxic action (Lydy *et al.*, 2004; Johnson *et al.*, 2013). The other two pesticides, BOS, a medium-risk fungicide, and TER, seemed harmless, like the drug FUR, as expected on the basis of its low level of risk. The other two pharmaceuticals, IBU and TMP, both classified as medium-risk, exhibited a different effect (even if not significant) on the three populations of *Diamesa*, without overcome 33% of dead larvae after 96 h at Cmax. A high general resistance was highlighted also to the three personal care products, even to the antibacterial TCC considered as dangerous for aquatic life (Lencioni *et al.*, 2017).

A clear gradient of increasing resistance, from *D. zernyi* in PR0 to *D. cinerella* in the WTP downstream site,

was detected only for IBU, reflecting a gradient of contamination of this substance in the environment from the least (PR0) to the most polluted (TP_dw) site (Lencioni *et al.*, 2017).

Both *Diamesa* species resulted more resistant to toxicants than any model species used to estimate toxicological parameters referred to the same 11 pollutants. The ratio between the toxicological parameters we measured and the literature data ranged from 3 (for TMP, in *Diamesa* spp. LOEC, 96 h=400 mg L⁻¹ respect to *D. magna* LC50 48 h=150 mg L⁻¹, (De Liguoro *et al.*, 2009) to 21 (for MET, in *D. zernyi* LC50 72 h=84.12 mg L⁻¹ respect to *C. plumosus* LC50 48 h=3.8 (Mayer and

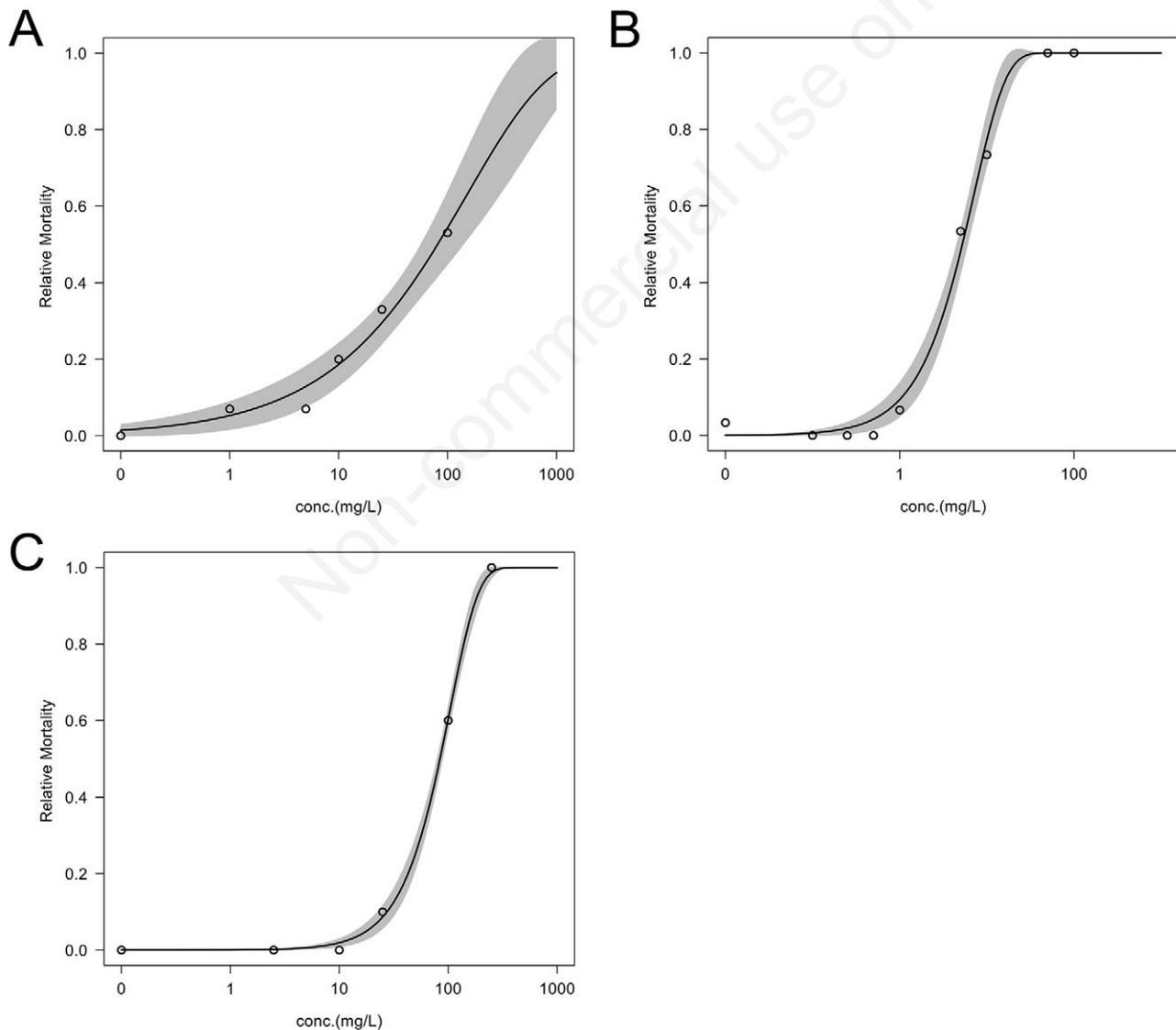


Fig. 2. Weibull distribution for (A) Captan, (B) Chlorpyrifos and (C) Metolachlor. Grey shaded areas surrounding the regression lines represent 95% confidence intervals for the regression.

Ellersieck, 1986)). For TON an extraordinary high resistance was observed in *D. zernyi* ($LC_{7\ 96\ h}=100\ mg\ L^{-1}$) respect to *D. magna* $EC_{50\ 21\ d}=0.2\ mg\ L^{-1}$ (Grutzner, 1995). For the other two pesticides with significant acute toxicity, this ratio was 8 for CAP (respect to *D. magna* (Kamrin, 2000)) and 10 for CPS (respect to *C. tentans*, Ankley *et al.*, 1994).

Basal DNA damage was detected in both *Diamesa* species under natural conditions, with, as expected according to the contamination level, a higher basal DNA damage in *D. cinerella* living downstream of the WTP effluent. This suggests that *D. cinerella* colonizes this stream reach, but survives under physiological stress. This

population accumulated less DNA damage than that living upstream when briefly exposed (24 h) to sub-lethal concentrations of the tested pollutants, highlighting a negative effect only by IBU. Conversely, the upstream population seemed more sensitive, undergoing damage also by FUR and TCC. The genotoxic activity of these pollutants decreased in the case of long-term exposure (96 h) in the upstream population, due probably to the up-regulation of the antioxidant defences and of DNA repair mechanisms as observed in other organisms (Santa-Gonzalez *et al.*, 2016). In comparison, *D. zernyi* was the most sensitive species, accumulating, after four days of exposure, DNA damage by all pollutants tested, mixtures

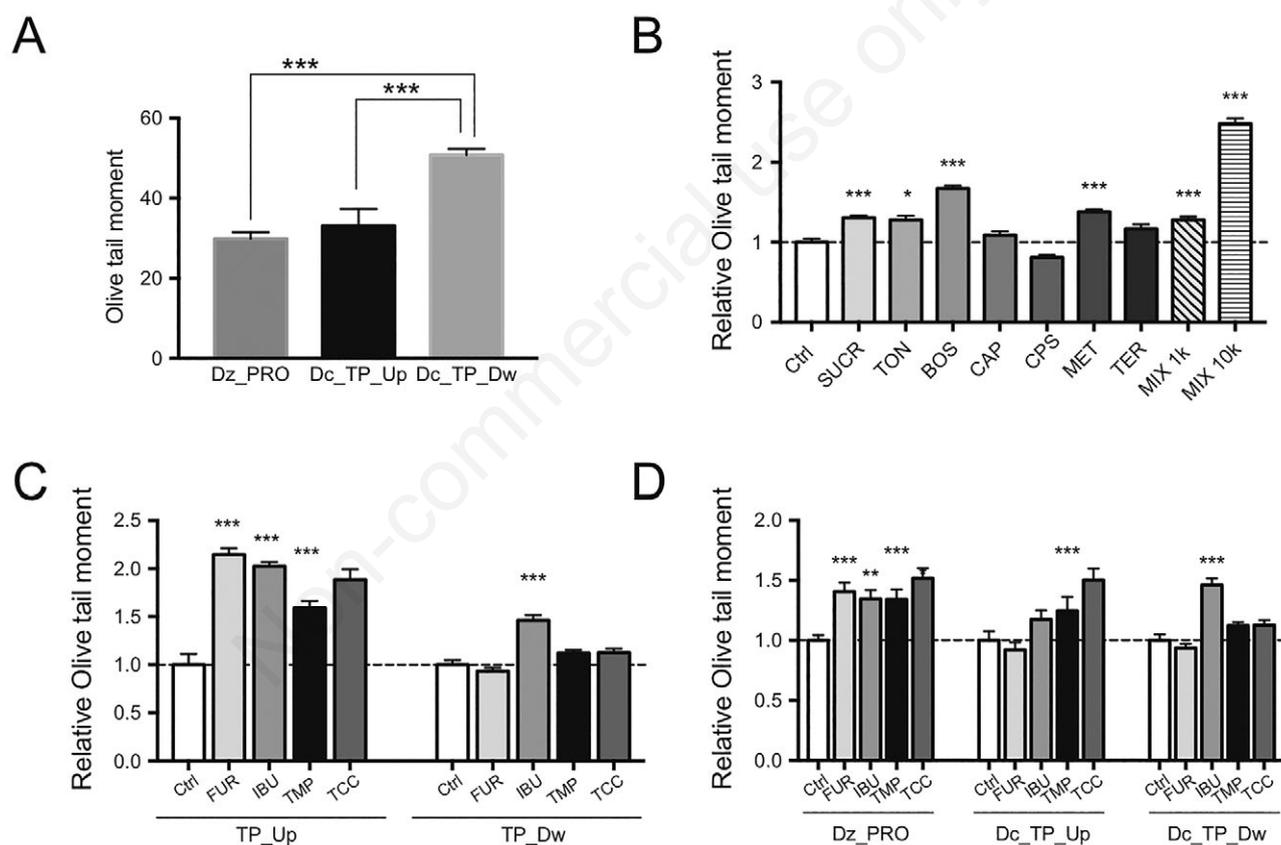


Fig. 3. A) Basal DNA damage measured by Olive Tail Moment (OTM) \pm SE in *D. zernyi* larvae collected in the Presena stream (Dz_PRO) and in *D. cinerella* larvae collected in the Vermigliana stream upstream (Dc_TP_up) and downstream (TP_dw) of the Tonale Pass WTP. B) DNA damage by emerging contaminants (PPCPs=pharmaceuticals and personal care products) and pesticides measured as Relative OTM in *D. zernyi* from PR0. C) DNA damage by short term exposure (24 h) to PPCPs measured as Relative OTM in *D. cinerella* from TP_up and TP_dw. D) DNA damage by long term exposure (96 h) to PPCPs measured as Relative OTM in *D. zernyi* from PR0 (Dz_PRO) and in *D. cinerella* larvae from TP_up and TP_dw. The histograms show the mean of the (Relative) OTM \pm SE (>100 nuclei were counted per each condition). Relative OTM: OTM values were normalized each on its corresponding control. The DNA damage of the control was fixed to 1. Significant differences (Kruskal-Wallis test and multiple comparison Dunn test) from the corresponding control (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$). In (B), (C) and (D), larvae were exposed at sublethal concentrations of each pollutant: LC_{10} estimated, LOEC, NOEC or calculated $LC \ll LC_{50}$, as reported in Tab. S2. FUR, furosemide; IBU, ibuprofene; TMP, trimethoprim; TCC, t riclocarban; SUCR, sucralose; TON, tonalide; BOS, boscalid; CAP, captan; CPS, chlorpyrifos; MET, metolachlor; TER, terbuthylazine; MIX 1K, $10^3 \times EC$ of CPS, MET, TER; MIX 10K, $10^4 \times EC$ of CPS, MET, TER.

included, with the sole exception of CAP, CPS and TER. The no-genotoxic activity of CAP and CPS was unexpected, considering their acute toxicity. Overall, as observed for survival, these results highlighted a gradient of increasing resistance from the least (PR0) to the most polluted (TP_dw) site, with demonstrated sensitivity to 9 out of 11 pollutants in *D. zernyi*, 3 out of 4 in *D. cinerella* from TP_up and only 1 in *D. cinerella* from TP_dw.

The different general resistance observed in the two species/three populations might be explained according to the different level of environmental contamination and on the basis of the autecology of the two species. *D. zernyi* might be more sensitive compared to *D. cinerella* because the species was collected in a less impacted environment, while it is plausible that *D. cinerella* from TP_dw had developed chemical resistance strategies to survive already under natural conditions and that the tested larvae were somehow pre-adapted to these pollutants. According to autecology, *D. zernyi* was hypothesised to be more sensitive than *D. cinerella* because typically restricted to very cold and pristine habitats (Lencioni and Rossaro, 2005).

Acute toxicity tests and Comet assay gave the same danger level for the 11 pollutants, some being ecotoxic but not genotoxic (*e.g.*, CPS, CAP) and *vice versa* (*e.g.*, FUR, TMP). A risk ranking might be suggested based on the results of both tests in both species, recognising as the most dangerous MET, IBU and TCC.

The observed high resistance to drugs, personal care products and pesticides observed in *Diamesa* spp. was unexpected, being considered species typical of pristine unpolluted habitats (Lencioni *et al.*, 2012). This might be a secondary effect of their strong cold resistance (*e.g.*, Supercooling Point = $-6.3 \pm 0.3^\circ\text{C}$, LLT100 = -16.2°C , thermal hysteresis >2 , accumulation of glucose and sucrose and antifreeze proteins (Lencioni and Bernabò, 2017). Furthermore, *Diamesa* spp. develops a Heat Shock Response under thermal stress (Lencioni *et al.*, 2013), and it is known that HSP70 have been involved in increasing toxic resistance (Karouna-Renier and Zehr, 2003). In fact, it is known that organisms living in extremely cold environments have evolved physiological traits that can have a major influence on the sensitivity of species towards contaminants, influencing their resistance (Chapman, 2016). Another reason of the extraordinary high resistance even to high-risk pollutants at concentrations million times higher than the EC, might be that at low water temperature toxicity of some contaminants (*e.g.* CPS) is lower than at high temperature (Patra *et al.*, 2015). Furthermore, there is increasing evidence of a delay in the manifestation of toxicity with exposure to contaminants in polar (Arctic and Antarctic) marine environments. This is probably due to multiple factors (*e.g.*, slower uptake kinetics, slower metabolic rate, higher lipid storage at low temperature (Peck, 2002;

Chapman and Riddle, 2005; Payne *et al.*, 2014). However, this phenomenon yet to be demonstrated in cold (*e.g.*, $<4^\circ\text{C}$) freshwater habitats (Chapman, 2016). In future, toxicity testing protocols should investigate this phenomenon, for example by setting a longer exposure time than 96 h to account for a delayed response, at even lower pollutant concentration and at a higher temperature within the thermal limit of the species and environmentally realistic even under global warming. In fact, possibly we underestimated the toxicity of some pollutants (due to low temperature and short time) and overestimated predicted no effect concentrations.

CONCLUSIONS

Even if measured concentrations of CECs and pesticides are far lower than those required to cause acute effects, prolonged exposure to a mixture of CECs might have damage macroinvertebrate communities, with cascade effects on the river food web. The unknown magnitude of top-down effects and trophic cascades should be considered in future experiments. Assuming that toxicants can damage or influence DNA in a variety of ways, other approaches (*e.g.*, gene expression analyses of genes involved in responding to chemical stress in natural populations) might be very useful to understand the molecular mechanisms of organismal response to human-derived ecosystem change (Bernabò *et al.*, 2017; Krosch *et al.*, 2017). Threats posed by contamination by CECs even at high altitude make it important to expand knowledge concerning heat and chemical tolerance in stenothermal insect species from freshwaters already threatened by temperature changes.

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