SCUOLA DI DOTTORATO
UNIVERSITÀ DEGLI STUDI DI MILANO-BICOCCA

Department of **Earth and Environmental Sciences**

PhD program **Chemical, Geological and Environmental Sciences**
Cycle **XXX** Curriculum in **Environmental Sciences**

Emerging contaminants: distribution, environmental fate and effects at different levels of the ecological hierarchy organization

Surname   **Ferrario**   Name   **Claudia**

Registration number **709299**

Tutor: **Prof. Antonio Finizio**

Co-tutor: **Dr. Sara Villa**

Coordinator: **Prof. Maria Luce Frezzotti**

Academic year 2016-2017

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Chapter 1

Introduction

In 1962, alarm bells of the negative impacts of human activities on the environment started to ring. Indeed, in her book "Silent Spring", Rachel Carson described the catastrophic effects of the indiscriminate use of pesticides in the 1940s and 1950s, giving life to the public awareness of environmental damages to natural ecosystems related to the exposure to potentially dangerous chemicals (Werner and Hitzfeld, 2012). Thanks to this book, from the middle of the 20th century, the environmental impacts of pollutants were recognized by public opinion, even though the emissions of chemicals produced by human activities started at the beginning of cultural development (Vighi and Villa, 2013). Consequently, environmental movement commenced to grow and develop at the popular level, and this movement also found a voice in the political legislative world (Stark et al., 2004). In this context, tools were developed to gather the information necessary to assess the environmental impact of products and create the foundation for international regulations aimed to increase the control of chemicals and improve environmental quality.

1.1 Ecological Risk Assessment

Ecological Risk Assessment (ERA) is the procedure by which the potential or actual adverse effects of pollutants and other anthropogenic activities on ecosystems and their components are

estimated with a known degree of certainty using scientific methodologies (Depledge and Fossi, 1994). The risk assessment process, such as the US EPA framework portrayed in **Fig. 1.1**, can be described as an integration of scientifically oriented risk analysis and more politically oriented risk management (van der Oost et al., 2003).
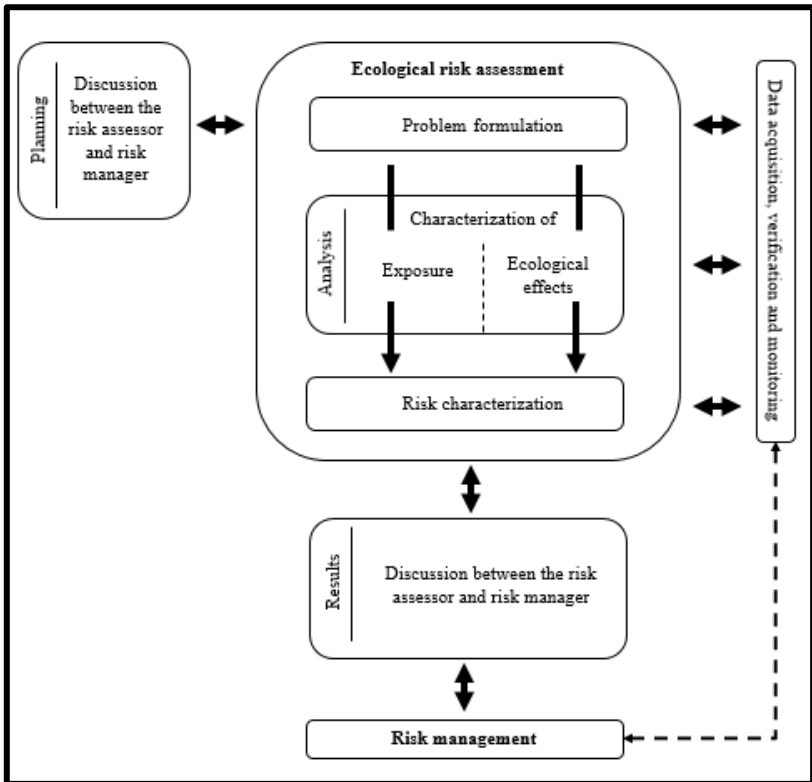
Figure 1.1 The US EPA framework for ecological risk assessment.

These two main elements of ERA are closely related and have complementary goals. Indeed, risk analysis aims to describe the risk

of a certain situation by estimating the magnitude and probability of the effects, whereas risk management examines solutions to the problem by addressing regulatory measures, choosing among alternatives and determining the acceptability of risks. This part of ERA is mainly a political process, although science is involved in the gathering of technical, social or economic information (van Leeuwen and Vermeire, 1995).

The assess procedure of the risk, defined as the probability of harmful effects due to a given hazard and the resulting consequences (De Lange et al., 2010), usually begins by collecting measurements that characterize the nature and extent of chemical contamination in the environment, as well as information needed to predict how the contaminants will behave in the future. Following a planning and scoping phase when the purpose is identified, the first step is the formulation of the problem, which aims to define an assessment endpoint to determine the ecological entity that is important to protect, e.g., a functional group of species, such as insect pollinators, or a specific habitat, such as wetlands. Once the ecological entity has been identified, its specific attributes that are potentially at risk and important to protect are chosen as a basis for the measurements carried out during the risk analysis. This following phase comprises hazard identification, effect assessment, and exposure assessment and ends with risk characterization (Van Leeuwen and Hermens, 1995). In particular, the analysis begins with the detection of adverse effects,

which a substance has an inherent capacity to cause in certain cases (De Lange et al., 2010). Indeed, a hazard is commonly defined as "a threatening event, or the probability of occurrence of a potentially damaging phenomenon within a given time period and area" (European Environmental Agency glossary). After the identification of the "potential" for producing a risk, the relationship between the dose or level of exposure to chemicals and the incidence and severity of an effect must be estimated. Most of the experiments carried out during this effect assessment phase aim to determine a no-effect level (NOEL) (van der Oost et al., 2003). Risk is distinguished from hazard because risk also considers the likelihood of exposure, thus, during the exposure assessment phase, the concentrations or doses that human populations or environmental compartments are or may be exposed to must be estimated. For existing chemicals, exposure can be assessed by measuring the concentrations, while for new chemicals, a predicted environmental concentration (PEC) can be valued (van der Oost et al., 2003). A single PEC must be calculated for each environmental compartment: water, sediment, soil and air. Ideally, this phase consists of estimating or measuring the intensity, frequency, and duration of exposure to a toxic agent and describes the sources, pathways, routes, magnitudes, durations, and patterns of exposure, the characteristics of the exposed population and the uncertainties in the assessment (European Environmental Agency). Following, the information collected during these first three steps of ERA is integrated into the

risk characterization to estimate the incidence and severity of the adverse effects likely to occur due to the actual or predicted exposure to a hazard. According to most European directives on dangerous chemicals, risk can be characterized using only the ratio between an effect indicator, such as an ecotoxicological endpoint (EC50, NOEC) or a PNEC, and an exposure indicator, such as a PEC (De Lange et al., 2010). For instance, the Technical Guidance Document (EU TGD) on Risk Assessment for new and existing chemicals from the European Commission suggests the use of the PEC/PNEC ratio (EC, 2003).
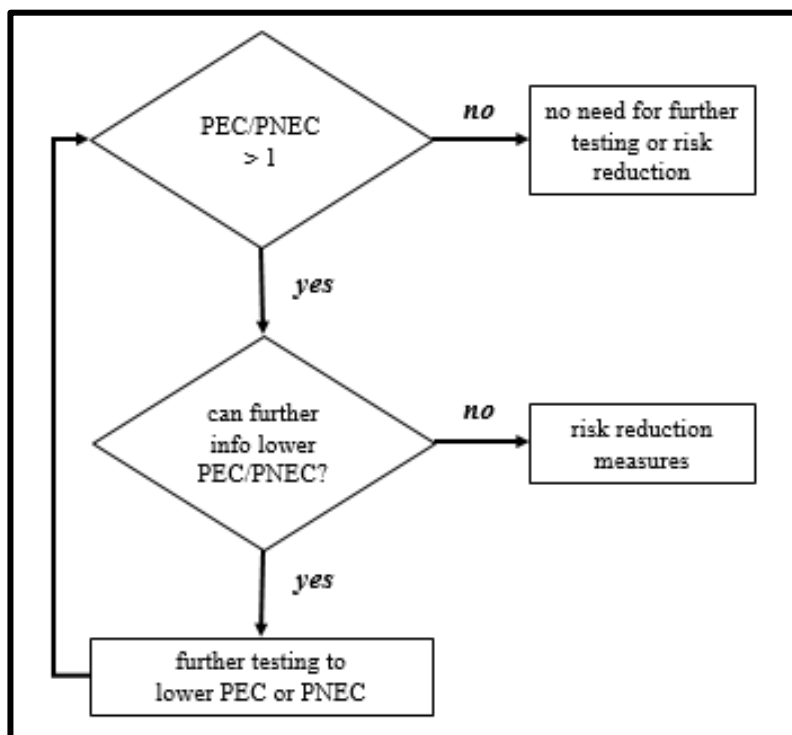
Figure 1.2 Decision tree according to the EU TGD.

According to the EU TGD, if the risk quotient is less than one, the environmental risk is deemed acceptable; while if not, further tests or risk reduction actions are necessary **(Fig. 1.2**). Indeed, this ERA procedure is a tiered process that is distinguished by levels of increasing complexity, in which the preliminary categorization step is followed by a refined assessment.

According to Van Leeuwen and Hermens (1995), the last step of the risk assessment process is monitoring, which consists of repetitive observations for defined purposes of one or more chemical or biological elements according to a prearranged schedule over time and space using comparable and standardized methods (United Nations Environmental Program definition). Monitoring is essential during several stages of the ERA process (van der Oost et al., 2003). During the formulation of a problem, observations of the chemical-physical and biological parameters of an ecosystem may indicate deviations from the normal (alarm and trend function), thus triggering problem recognition. Moreover, during the risk analysis phase, monitoring can help characterize the exposure, e.g., periodical analysis allows for the detection of seasonal variability in the emissions of contaminants such as pesticides. Finally, monitoring supports the verification of the control strategy results during the risk management phase.

Ecological risk assessment has grown and evolved since the 1980s (Hope, 2006). This procedure is commonly used during the new

registration or renewal process for contaminants. For instance, under European legislation, Directive 93/67 (EEC, 1993), Regulation 1488/94 (EC, 1994) and Directive 98/8 (EC, 1998) require that an environmental risk assessment is carried out on new notified substances before they can be offered on the market, priority existing chemicals and active substances and substances of concern in biocidal products, respectively. Currently the core of the EU chemical management policy is Regulation 1907/2006 (EC, 2006) is which consists of a single, integrated system for the Registration, Evaluation, and Authorization of Chemicals (REACH).

In this context, ecotoxicology was developed as an applied science with the aim to evolve tools that are capable of quantifying the risks from chemicals (Vighi et al., 2006). This relatively recent discipline was mainly based on testing approaches for the assessment of dose-response relationships and on a combination of monitoring and modelling approaches for exposure assessment. In particular, ecotoxicological studies allowed for the increased level of chemical control that occurred in all developed countries, even if a complete protection of ecosystems is still a distant goal and many practical and conceptual challenges remain to be resolved. Indeed, the European Commission has recently recognized that the ecological realism of exposure and effect assessments need to be improved (EC, 2013). In particular, the document "Addressing the New Challenges for Risk Assessment" (EC, 2013) reports the major objectives for promoting

the use of modelling that considers the properties and complexity of potentially exposed ecosystems, such as the spatiotemporal variability of environmental scenarios and pollutant emissions. In addition, the main goals for decreasing the large margins of uncertainty that exist in the traditional approaches used to estimate PNECs are addressed. About this issue, the necessity for a greater understanding of complex exposure patterns, indirect and endocrine disrupting effects and adverse consequences of mixtures of chemicals and stressors are highlighted. In particular, it is underlined that the protection of the structure and functions of ecosystems requires a deeper knowledge of ecological processes to improve the capacity of traditional sub-individual end-points to predict ecologically relevant effects at higher hierarchical levels by expressing them in quantitative terms. Due to the complexity of ecological processes, a greater awareness of these effects requires an integration of information from several independent sources. According to EFSA Scientific Committee, wherever more than one piece of evidence is used to support possible answers to a question, a weighing of evidence is involved. In fact, this evaluation takes into account the strengths and weaknesses of different measurement methods and highlights the nature of uncertainty associated with each of them (Bettinger et al., 1995). In particular, the weight of evidence assessment is reflected in three characteristics of measurement endpoints: a) the weight assigned to each measurement endpoint; b) the magnitude of response observed in them; and c) the

concurrence among outcomes of multiple measurement endpoints (Burton et al., 2002). As used in environmental studies, this approach allows integrating the results of multiple measurements in hazard assessment and promoting systematic analysis of risk (Wright-Walters et al., 2011).

1.2 Challenges in exposure assessment in cold areas: where and what to investigate

Until the late 1960s, the Polar Regions were considered pristine areas that were too remote to be subject to any substantial chemical contamination from human activities (Wania and Mackay, 1993). This certainty was upturned by early measurements in both the Arctic (George and Frear, 1966; Clausen and Berg, 1975) and Antarctic (Barrie, 1986) that revealed the presence of organochlorine chemicals in polar areas. In particular, during those years, detectable concentrations of organochlorine pesticides were found in different environmental matrices, such as air, water and biota (Sladen et al., 1966; Risebrough et al., 1976; Tanabe and Tatsukawa, 1980). Due to these discoveries, the comprehension of how persistent organic pollutants (POPs) migrate through the global environment has become the focus of national and international attention (Wania and Mackay, 1995).

The presence of POPs in the cold areas of the world was first explained in 1974 when Rappe (1974) suggested that these compounds migrate through the atmosphere as gases and aerosols and condense in low-

temperature regions. Further studies have underlined that warm temperatures favour the evaporation of POPs from the surface of the Earth in tropical and subtropical regions, while cool temperatures at higher latitudes favour the deposition of these compounds into soil and water (Wania and Mackay, 1993; Mackay and Wania, 1995). Currently, the general mechanisms involved in the distribution of POPs at the global level have been clarified and modelled (Wania and Mackay, 1996; Finizio et al., 1998; Gouin et al., 2004; Hageman et al., 2006; Scheringer, 2009). Based on this obtained knowledge, the environmental cycling of POPs begins in warm source areas and ends in northern latitudes, covering a distance greater than 1000 km, undergoing so-called Long Range Atmospheric Transport (LRAT) (FOCUS, FOrum for Co-ordination of pesticide fate models and their USe, 2008). In cold sites these compounds may be incorporated in snowpack and ice due to the condensation-redeposition caused by the scavenging of POPs from the atmosphere as a consequence of the capacity of precipitation to sorb gaseous and particle-bound organic contaminants. Snowpack and ice act as temporary reservoirs of pollutants until the temperature increases. Indeed, once melting begins, the re-emission of contaminants back to the atmosphere or release via meltwater to the freshwater systems occur. As a consequence, significant concentrations of these contaminants are common during snowmelt periods (Meyer et al., 2006; Meyer and Wania, 2008; Bizzotto et al., 2009; Bogdal et al., 2010; Grannas,

2011) with potential implications regarding the increased exposure of aquatic as well as terrestrial organisms.

In particular, these possible detrimental effects of POPs in cold ecosystems, such as the Arctic, have led to a series of regulations, such as the Stockholm Convention (2001), which have resulted in the ban or strong restriction of the use of many POPs worldwide. These regulations have encouraged the synthesis of new alternative products that are less persistent and bioaccumulative (Walker and Nidiry, 2002), but the environmental fates of many of these compounds are still unknown.

Meantime, several studies have demonstrated the presence of DDTs, PCBs, dioxins and HCHs on the European Alps (Villa et al., 2003, 2006a, 2006b; Nizzetto et al., 2006; Tremolada et al., 2008; Bogdal et al., 2010; Villa et al., 2011; Pavlova et al., 2016), calling attention to the importance of high-altitude monitoring. Indeed, in the frame of the growing concern for the protection of cold ecosystems, these regions are often overlooked despite the fact that these ecosystems are very specific and characterized by low biodiversity (Vighi et al. in Castro-Jiménez, 2007). In this context, particular attention should be devoted to temperate-zone mountain regions mainly because these areas tend to receive high levels of precipitation and are near pollutant sources and, thus, are particularly susceptible to the accumulation of contaminants (Blais et al., 1998).

1.2.1 Medium Range Atmospheric Transport

In 1991, Calamari and co-workers were among the first scientists to indicate mountains as potential "cold condensers" that could interfere with the global cycles of POPs, favouring the accumulation of these compounds in glaciers at mid-latitudes (Finizio et al., 2006) and the eventual storage of these compounds in ice (Villa et al., 2003). Based on this, mountains can act as cold traps and sinks for these substances, exactly like "remote cold areas", such as the Arctic and Antarctic regions. More recently, in 2005, Daly and Wania highlighted the potential of mountains to behave as regional convergence zones for selected organic pollutants through a type of "altitudinal fractionation". The effectiveness of mountains to act as cold condensers is dependent on several factors such as meteorological conditions (e.g., rain and snow precipitation regimes, wind direction, temperature) (Daly and Wania, 2005; Vighi et al. in Castro-Jiménez, 2007), orographic effects (i.e., the influence of mountain slopes on air flow) (Daly and Wania, 2005), the presence of vegetation (Nizzetto et al., 2010) and post-depositional processes (Villa et al., 2003; 2006a, 2006b; Bogdal et al., 2009). For instance, the extent to which contaminants are transported into mountain regions is clearly dependent on the meteorological conditions and orography. In particular, the presence of the Planetary Boundary Layer (PBL) can have a strong influence on the atmospheric transport of contaminants. Areas located below the PBL throughout the year are mainly affected

by regional or Medium Range Atmospheric Transport (MRAT) of contaminants that originate from local sources, while mountain sites located above the PBL are more prone to contamination from distant sources through LRAT.

In addition to the abovementioned factors, mountains are in close proximities to emission sources. In fact, whereas the Arctic and especially the Antarctic are located thousands of kilometres from the main industrial and agricultural areas, the most important mountain systems on Earth are quite close to densely populated and/or agricultural areas. For instance, the European Alps lie across the most populated and highly industrialized regions of Europe. The relative proximity of mountains to source regions has the potential to increase the concentrations of pollutants in the air that enters mountain systems to levels that are higher than those entering the Arctic. Therefore, this increase is dependent on the presence of regional or MRAT processes, which can act contemporaneously with LRAT processes. As a consequence, these ecosystems represent potential condensation sites for contaminants, such as POPs (Carrera et al., 2001; Villa et al., 2003; Wang et al., 2006), which, during summer melting, are able to reach surface waters and become a threat for the highly vulnerable biological communities in cold environments (Villa et al., 2003). Fortunately, there is some evidence that the ban of POPs is leading to the decline of the concentrations of these compounds in several compartments (Holoubek et al., 2007; Brun et al., 2008) and,

consequently, in the atmospheric transport towards mountains. On the other hand, closer distances can increase the potential of glacier contamination from other typologies of contaminants not included in the POP list because of MRAT processes, which represent a largely unknown threat for high-altitude regions.

1.2.2 Emerging Contaminants (ECs)

The findings of POPs on the European Alps (Villa et al., 2003, 2006a, 2006b; Nizzetto et al., 2006; Tremolada et al., 2008; Bogdal et al., 2010; Villa et al., 2011; Pavlova et al., 2016) confirmed that even less persistent compounds could potentially reach isolated cold areas because of MRAT processes. Indeed, unlike latitudinal LRAT, the altitudinal transport to high mountains can occur over relatively short distances from potential source regions. This means that compounds with atmospheric lifetimes not long enough to be transported to Polar Regions can reach the remote, cold areas at temperate latitudes (Blais et al., 1998; Daly and Wania, 2005; Grimalt et al., 2009). For example, Current Use Pesticides (CUPs) tend to have much greater polarity and water solubility than POPs and are much more biodegradable. However, some studies underlined the presence of CUPs, as well as polycyclic musk fragrances (PCMs), in regions isolated from their use and production such as in the Arctic (Hoferkamp et al., 2010; Zhang et al., 2013) or high mountain areas (Zabik and Seiber, 1993; Aston and Seiber, 1997; LeNoir et al., 1999; Hageman et al., 2006; Gouin et

al., 2008; Kurt-Karakus et al., 2011; Santolaria et al., 2015). These two categories of compounds belong to the so-called Emerging Contaminants (ECs), which can be broadly defined as any synthetic or naturally occurring chemical or any microorganism that is not commonly ruled and monitored in the environment but has the potential to enter the environment and cause known or suspected adverse ecological and (or) human health effects (U.S. Geological Survey, USGS, definition). Because of the vast number of possible compounds, many studies have researched different classes of emerging pollutants; however, the environmental impact of these pollutants is substantially still widely unknown. Indeed, ECs are a heterogeneous group of chemicals that includes veterinary and human pharmaceuticals, Personal Cara Products (PCPs), nanomaterials, additive feedings, and biocides (Picò and Barcelò, 2015). In some cases, the release of emerging chemicals to the environment has likely occurred for a long time but may not have been recognized until new detection methods were developed. This is what occurred in the case of PCPs that are now commonly detected in surface water systems (Peck, 2006). In addition, the synthesis of new chemicals or changes in the use and disposal of existing chemicals produces more emerging chemicals that consequently increase the number of compounds that are identified to possess potential environmental threats to ecosystems (Bolong et al., 2009).

Based on this, the occurrence of some ECs in aquatic environments has been recognized as one of the emerging issues in ecotoxicology (Richardson and Ternes, 2005).

1.3 Challenges in effect assessment: towards "Stress Ecology"

To improve the protection of ecosystems and the ERA procedure, it appears to be fundamental to produce more efficient tools that are capable of supplementing the currently limited information on a large number of the chemicals introduced into the environment. Based on this premise, ecotoxicology has evolved as an applied science, with the aim of developing methods for quantifying the risks deriving from chemicals (Vighi et al., 2006). Ecotoxicology emerged as the environmental branch of toxicology (Van den Brink, 2008), of which it maintains the basic principles, namely: experimental testing, analysis of dose-effect relationships and estimation of effect concentrations, such as EC50. This "testing-based" approach offers a solid basis for deriving maximum acceptable chemical concentrations, which are often taken as limit values by environmental regulations (Van Straalen, 2003). Indeed, the simplified approach adopted in the traditional ERA is based on the estimation of chemical concentration below which unacceptable impacts to organisms will most likely not occur (EC, 2003). Ideally, this concentration would not induce adverse effects on any element of the biological communities after any length of exposure time. In practice, this value can be estimated from limited

ecotoxicological data gathered from selected organisms, which are taken as representatives of different levels of the ecological hierarchy organization. For example, the different trophic roles in aquatic ecosystems are represented by algae, *Daphnia* and fish, for which ecotoxicological data are derived (EC, 2003). The degree of uncertainty due to the extrapolation of toxicity data from only three species from the laboratory to the real environment is covered by applying assessment factors, and the use of these factors decreases with the increase in available information. Indeed, safety factors vary from 10 to 1000 as a function of the number of species tested and if the endpoint is based on acute mortality or effects (LC50 or EC50), or chronic no-observed-effect concentrations (NOEC) (Fent, 2004). The use of these assessment factors conceals the discrepancy between the question posed in the ERA, which is focused on the protection of populations and communities in the field, and the answer given by single-species tests. As a result of the efforts devoted to the standardization of risk assessment methodologies, the current approach allows for extensive amounts of comparable data to be obtained but contemporarily reveals that ecological theory has limited integration in the fields of ecotoxicology and ERA (Van den Brink, 2008). In this context, several scientists (e.g., Chapman, 2002; Slooff et al., 2003; Relyea and Hoverman, 2006; Van den Brink, 2008; Clements and Rohr, 2009) have argued that the inclusion of the basic principles of ecology into ecotoxicology is essential for shifting from

purely descriptive to more predictive science. In particular, in his paper, Van Straalen (2003) hypnotized the evolution of the ecological part of ecotoxicology into the subdiscipline called "stress ecology". This term was occasionally used in the 1970s and 1980s (Barrett et al., 1976; Rapport et al., 1985), but became institutionalized in the field of ecotoxicology and ERA in 2003 (Van den Brink, 2008). Stress ecology can be described as the study of the consequences induced by changes in an ecosystem and of the resultant effects on the organisms, and it also considers the interactions with other organisms and the environment. However, since its introduction into ecology, the definition of stress has been debated (Grime, 1989), and pollutants are undoubtedly agents of stress that often interact with "natural" stress factors such as extreme temperature, humidity or pH values. The relations between the effects of toxic compounds and other environmental stressors depend on the specific chemical, the organisms in question, as well as the natural factors.

In terms of ecosystem conditions, the effects of toxicants are generally more severe when the organisms are far from their *optimum* or are close to the boundaries of their ecological niche (Heugens et al., 2001). However, variations in abiotic factors can significantly influence the toxicity of chemicals, even within the normal tolerance range of an organism (Abdel-Lateif et al. 1998; Martikainen and Krogh 1999; Bednarska et al. 2009). For instance, this is the case for changes in temperature, which can modify the acute toxicity of a

compound (Bao et al., 2008) or its toxicokinetics for different organisms (Janssen and Bergema, 1991). Likewise, pH plays an important role in the bioavailability of a toxicant and can interfere with the physiology of organisms (Bednarska et al., 2013). Based on this, natural factors should be included in experimental setups. Efforts should be devoted to standardization, which is indispensable to the generation of comparable data (Jafarzadeh, 2011) from "ecological experiments". Indeed, these tests do not often have wide validity because the conditions under which they are performed can be characterized by significant spatial and temporal variability. Due to the instability of environmental conditions, the results of these experiments can be different from those of the previous or successive tests. Because of their low reproducibility, it is very difficult to utilize ecological experiments for regulatory acts at the international level.

Other than the abovementioned natural factors, the effect of a toxic chemical depends on the organism considered. In particular, intrinsic species sensitivity has been recognized as a key to understanding how species and ecosystems react to chemical stress (Van den Brink, 2008). Indeed, it is largely unknown why effects on one species that are induced by a chemical can be more or less severe than those on another. This phenomenon was also observed within target species groups in relation to a specific toxicological mode of action of pollutants, such as pesticides (Maltby et al., 2005). One method that takes into account the differences in sensitivity is the application of

the Species Sensitivity Distribution (SSD) concept (Kooijman, 1987; Van Straalen and Denneman, 1989). SSD is estimated from a sample of toxicity data and visualized as a cumulative distribution function (Posthuma, et al., 2002), which permits the assessment of the probability of a species to be potentially affected by a given exposure level to toxic chemicals. More recently, Baird and Van den Brink (2007) formulated an alternative approach aimed at developing the so-called Trait-based Ecological Risk Assessment (TERA). According to this approach, the attention of risk analysis should be focused on toxicokinetic and toxicodynamic processes that are responsible for the sensitivity of an organism to stress. In particular, these authors suggested that the biology of individuals partially drives the action mechanisms of toxic chemicals that, therefore, can be predicted by studying the specific traits of organisms, such as morphology, life history, physiology, and feeding ecology. In more detail, the results of Baird and Van den Brink (2007) showed that the traits related to respiration, taxonomy, lifespan, and size explained significant proportions of the sensitivity differences between species. In this context, the development of TERA could invoke a significant shift from ecotoxicology to the stress ecology approach because the consequences on ecosystems should be described as changes in the combinations of functional traits rather than as effects on single species. However, the implementation of TERA faces some challenges, since problems related to trait definitions, their

intercorrelations, and intraspecific variation first have to be overcome (Baird et al., 2008). Indeed, ERA is currently based on experimental tests aimed to evaluate traditional endpoints that allow for the determination of only quantitative aspects, e.g., the inhibition of the growth of a population, and does not consider ecosystem quality, which is represented by species composition and richness, or stability in terms of resistance to environmental changes or resilience after disturbance (Schmitt-Jansen et al., 2008). For example, a typical endpoint of the traditional ecotoxicological tests on microorganisms (e.g., algal tests) is the population growth rate. Studies based on this endpoint usually investigate the effects on the potential growth rate (r), which is an intrinsic property of the population, and not the carrying capacity (K), which depends on the interactions between organisms and environmental factors such as resource availability, and interactions with other populations. In addition, these experimental approaches are limited by the probable repression of several biochemical and physiological processes because traditional tests do not allow organisms to cope with contaminants in the same way as they do in the field (Amiard-Triquet, 2012). For instance, at the suborganismal level, some functional mechanisms that are frequently involved in building tolerance against chemical stressors respond on the scale of days or weeks (Amiard-Triquet et al., 2011). These mechanisms, such as the adaptive ones, require a lot of energy that is taken away from other activities, and there are potential

consequences to the success of reproduction and growth of individuals (Sokolova et al., 2012). Therefore, the lack of consideration of the subindividual effects in the regulatory ERA approach may have significant negative impacts in the long term and could explain the alterations at higher biological levels. For example, at the organismal level, the presence of toxicants can lead to behavioural changes (Boyd et al., 2002), such as an increase in the average speed (i.e., escape from contamination through chemical avoidance) or a decrease in activity (protection reaction) (Wolf et al., 1998), which can affect the ecosystem structure itself (Reichmuth et al., 2009; Duquesne and Küster, 2010). Indeed, at higher ecological levels, impaired behaviour can have detrimental consequences at both the population level - through altered interactions with other members of the same species - and at the community level - through changes in competitive or predator/prey interactions (Faimali et al., 2017). Moreover, it must be emphasized that the results obtained from studies at the biochemical, molecular, cellular and even organismal levels do not automatically allow for predictions of stress responses at higher biological levels. For these reasons, developing new tools and focusing the attention on more advanced endpoints, such as the improvement of video tracking technologies for better quantify behavioural patterns, are necessary to investigate how responses to chemical stress are spread through the different levels of the ecological hierarchy.

According to the considerations above, the application of ecological principles to ecotoxicological approaches should involve shifts in the design and implementation of ecotoxicological research, overcoming the limitations of data that are obtainable by means of traditional tests, which are less useful for regulatory authorities (Slooff et al., 2003). In this sense, in the 1990s, many attempts were made to integrate ecological issues into ecotoxicology, such as the acceptance of multispecies tests as valid regulatory instruments and the use of functional endpoints (e.g., primary production) in addition to survival, growth and reproduction of single species. However, the impact on regulation was very low, as demonstrated by the tests that were once again carried out under laboratory conditions on individuals of only standard species (Van Straalen, 2003) and aimed to detect traditional endpoints.

1.3.1 Behavioural ecotoxicology

Stress is the nonspecific response of an organism to any demand made upon it (Selye, 1973). When exposed to an environmental contaminant, an organism reacts differently in function on its ability to perceive the presence of this compound. Indeed, in the absence of contaminant detection or appropriate behavioural responses, organisms endure maximal exposure that, even at concentrations lower than the lethal one, can induce a reduction in longevity. In contrast, once the toxic agent is detected, an organism can enter a

phase of adaptation, resistance (Selye, 1973) or active "alarm reaction", such as the mechanisms of avoidance (e.g., escape, valve closure in bivalves) that allow for a reduction of exposure and thus may limit potential adverse effects (Amiard-Triquet et al., 2012). All of the mechanisms involved in tolerance to chemical stressors are mediated by biochemical and physiological processes (Ren et al., 2007), and the alterations of these processes, such as endocrine disruption or changes in energy allocations, are the origins of behavioural disturbances (Amiard-Triquet et al., 2012).

Based on these premises, Peterson and co-authors (2017) highlighted the relevance and importance of behavioural studies and underlined the main benefits given by incorporating these kinds of assessments into ecotoxicology. First, behaviour can be considered an indicator of multiple levels of biological outcomes (Little, 1990; Weis et al., 2001; Scott and Sloman, 2004; Kane et al., 2005; Weis, 2013). For example, the reduced swimming capability of a fish may reduce its ability to capture prey or avoid predators, which are essential for individual growth and survival, and thus necessary for the maintenance of the size and size structure of a population (Vieira et al., 2009; Weis, 2013). In this sense, reduced feeding, a common response to contaminants, can lead to reduced energy intake, which can be worked into energy budget models to predict consequences at the population level (Maltby, 1999).

As the second benefit, behaviour is among the most sensitive indicators of the impact of exposure, since significant changes in behaviour can also be induced by concentrations that are considered sublethal (Little, 1990; Dell'Omo, 2002; Zala and Penn, 2004; Gerhardt, 2007; Hellou, 2011; Weis, 2013). This aspect was clearly underlined by the work of Melvin and Wilson (2013) which utilized a literature meta-analysis that compared the relative sensitivity of aquatic behavioural studies to those assessing traditional endpoints to demonstrate that behavioural endpoints are generally more sensitive than those assessing organism development or reproduction.

Finally, behaviour can be considered an "early warning tool" (Hellou, 2011). This is because behaviour is a rapidly changing, flexible trait and responses in behaviour can be recorded before those in other kinds of phenotypes, or in the genome (Peterson et al., 2017).

For these reasons, several authors have suggested an integration of behavioural ecology, toxicology, and conservation into a new field called behavioural ecotoxicology (Dell'Omo 2002; Chapman 2007; Gerhardt 2007; Peeters et al. 2009; Hellou 2011). However, despite the advantages mentioned, behaviour has been recognized only recently as an assay of fitness (Scott and Sloman, 2004) and an adaptive response to environmental stimuli (Gerhardt, 2007). Indeed, even if behavioural responses of aquatic organisms have been used since the 1980s as a method for environmental monitoring (Cairns and Gruber, 1980; Kramer et al., 1989; Diamond et al., 1990; Gerhardt et

al., 1998; van der Schalie et al., 2001), these types of outcomes have previously received much less attention than traditional endpoints (Scott and Sloman, 2004). Thus, ecotoxicological studies have conventionally focused on evaluating acutely lethal concentrations (e.g., EC50 or LC50) and chronic sublethal effects on developmental or reproductive endpoints (Hood, 2005; Stadler, 2011) mainly because these approaches provide results that can be directly linked to organismal health and fitness (Melvin and Wilson., 2013). In contrast, behavioural endpoints are rarely incorporated as a fitness consequence or as a trait to measure toxicity (Dell'Omo 2002; Clotfelter et al. 2004) largely due to the limited understanding of the natural behaviours of many organisms as well as the relevance of behavioural responses for inferring higher-level effects (Kane et al., 2005). Nevertheless, the recent progresses made in the technological tools used to quantify behaviour (Lv et al., 2013), such as upgraded video tracking technologies, allow for the improvement of the knowledge of the relationships between organism behaviours and physiological and ecological consequences (Little and Brewer, 2001; Amiard-Triquet, 2009; Sloman and McNeil, 2012). In particular, the knowledge of the links between the responses measured at a particular hierarchical level and those measured at the adjacent levels would be very effective in risk assessment procedures, mostly for improving the use of biomarkers, such as behavioural changes, as early warning indicators of adverse effects of pollutants on populations and communities.

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Chapter 2

Outline of the thesis

The Ph.D. activities have been carried out to highlight the emerging issue about well-known contaminants, i.e. situations where new information is jostling our understanding of environmental and human health risks related to such contaminants. In general, the persistence, bioaccumulation and toxicity of legacy contaminants, such as DDT and other organochlorine pesticides, led to a series of regulations on many of them, such as the Stockholm Convention (2001), which aims at the ban of or the strongly restriction on the use of POPs worldwide. As a direct consequence, the continuing need for new products resulted in the development of alternatives, which were intended to be less persistent, less bioaccumulative and generally much more toxic (Walker and Nidiry, 2002). However, the environmental impact of these so-called ECs is substantially still unknown.

Based on these premises, in the present work two aspects of the ECs environmental distribution have been deepened. In particular, the environmental fate of some of these chemicals in temperate-zone mountain regions and their sub-lethal effects on no-target organisms have been studied in order to improve knowledge and propose new approaches that would be useful in the risk assessment procedures, principally for the development of more realistic ecological modelling.

2.1 Environmental fate of ECs in temperate-zone mountain regions

In this thesis, the role of Medium Range Atmospheric Transport (MRAT) of contaminants towards glaciers was deepened. It has been largely recognized that atmospheric transport is a major pathway for the distribution of anthropogenic contaminants (Harriss et al., 1984). This phenomenon mainly affects POPs that, due to the combination of their physico-chemical properties and persistence, are ubiquitous and have been detected even in remote regions far from emission sources began in the early 1960s (Sladen et al., 1966, Tanabe et al., 1982). However, high mountains, acting as potential "cold condensers" (Calamari et al., 1991), could interfere with the Long Range Atmospheric Transport (LRAT) and global cycle of POPs, favouring accumulation of these chemicals in glaciers at the different latitudes (Finizio et al., 2006). Indeed mountains can be a cold trap and sink for these substances (Carrera et al., 2001; Villa et al., 2003; Wang et al., 2006) and act as regional convergence zones for selected organic pollutants through a type of "altitudinal fractionation" (Daly and Wania, 2005). In contrast to latitudinal atmospheric long range transport, the altitudinal transport into high mountains can take place over relative short distance from potential sources (Vighi et al. in Castro-Jiménez, 2007). As a consequence, relatively "short living" compounds, with atmospheric lifetimes too short to be transported to Polar Regions, can reach the remote cold areas of temperate latitudes

(Blais et al., 1998; Daly and Wania, 2005; Grimalt et al., 2009), thus increasing the importance of MRAT of these contaminants. This phenomenon is especially significant in the frame of the growing concern on the protection of cold ecosystems, because high mountain regions are often disregarded despite their ecosystems are very specific and characterized by low biodiversity, aspect that may lead to lower stability and higher vulnerability in comparison with more complex communities (Vighi et al, 2007). In this context, Italian Alps represent an interesting area to be investigated. In fact, there are several studies showing the presence and the accumulation of POPs in these environments (Villa et al., 2003; 2006a, 2006b; Bogdal et al., 2009; Pavlova et al., 2016). Moreover, Italian Alps lie close to industrialized and agricultural areas, which represent significant emission sources of Emerging Contaminants (ECs), such as Current Use Pesticides (CUPs) and Personal Care Products (PCPs).

For the above mentioned criticalities, in **Chapter 3** the potential release of legacy POPs and emerging pollutants from the melting of Italian Alpine glaciers is described with the aim of highlighting the presence of these compounds in a remote high-altitude cold site as a consequence of MRAT processes.

By analysing glacial meltwater samples, collected on three Italian alpine glaciers (Lys, Forni and Giogo Alto), two contrasting processes leading to glaciers contamination have been underlined. On one hand,

the results suggest a declining trend of the considered POPs (HCHs, HCB, DDTs, PCBs). On the other hand, the accumulation processes of ECs in the three glaciers has been highlighted. Indeed, the obtained data confirmed the presence of some of the selected CUPs (chlorpyrifos and terbuthylazine) and musk fragrances (HHCB, AHTN) in glacial meltwater.

Based on these results, a study of the potential degradation of chlorpyrifos (CPF) in a remote high-altitude cold site was carried out and reported in **Chapter 4**. The aim of this work is to describe the role of cryoconite in the accumulation of organic pollutants and biodegradative microbial communities in order to include its contribution to the removal of organic pollutants in models predicting the environmental fate of these compounds in cold areas. For this purpose, *in situ* microcosm experiment was carried out on Forni Glacier by testing the degradation of CPF in light and dark conditions, as well as in abiotic and biotic environments. The results highlight not only that this insecticide can be degraded on glaciers but also that biodegradation contributes to the removal of CPF from the glacier surface more than photo- and chemical degradation. In particular, this study suggests that organic contaminants can represent a source of nutrient for microbial communities living on glaciers. Therefore, microbial degradation can contrast the accumulation of pollutants transported on glaciers and the possible re-emission of contaminants back to the atmosphere or to the freshwater systems. Finally, the

relative extent of biodegradation documented in this study implies that environmental fate models of pesticides in glacierized areas should account for biodegradative processes.

2.2 Sub-lethal effects induced by ECs environmental concentrations on no-target organisms

In order to propose new approaches that would be useful for the development of more realistic ecological modelling, the potential sub-lethal effects induced on aquatic invertebrates by environmental concentrations of widespread ECs were investigated.

Indeed, currently the ecotoxicological effects of chemical compounds are evaluated by means of standardised toxicity tests, which are performed on target organisms considered representative of the exposed ecosystems (Hood, 2005; Stadler, 2011). For the aquatic compartment, they comprise tests on algae, *Daphnia* and fish, which are mainly focused on acute exposure and short-term effects. However, these tests should completely overcome a number of biochemical and physiological processes because they do not allow organisms to cope with contaminants as they do in the field (Amiard-Triquet, 2012). This should be particularly true at sub-lethal concentrations (commonly measured in the aquatic environments) since these mechanisms are functional, and many of them respond on the scale of days or weeks (Amiard-Triquet et al., 2011). Especially not lethal effects, including changes in behaviour, could affect fitness and consequently population dynamics (Brodin and Johansson, 2004;

Smith et al., 2008), thus contrasting to the protection goals of ERA, which includes also the protection of populations and communities.

These criticalities are particularly true for the ECs, whose adverse effects towards non-target organisms have been only recently highlighted, for instance effects of drugs of abuse (Binelli et al., 2013) or pharmaceuticals and personal care products (Canesi et al., 2007, Nassef et al., 2010). Moreover, increasing laboratory evidences show that the exposure to "environmentally relevant" concentrations of different ECs may induce several adverse effects to organisms (Duquesne and Küster, 2010; Brodin et al., 2013; Bean et al., 2014). Nevertheless, it is largely unknown how the responses to chemical stress are spread through the different levels of the ecological hierarchy (Amiard-Triquet, 2009). Unveiling this kind of information would be very effective for improving the use of biomarkers as early warning indicators of risk.

In order to understand how and if the stress signals measured at a given ecological level are transmitted through the other hierarchical levels and the capability of sub-individual endpoints to predict ecologically relevant effects, such as change in behaviour, *Daphnia magna* individuals were exposed to environmentally relevant concentrations of two widespread ECs, namely CPF and benzoylecgonine (BE), for 96 hrs. At sub-organismal level, the amount of reactive oxygen species, and the activity of antioxidant and detoxifying enzymes were measured to assess the alteration of the oxidative status, while the lipid

peroxidation was investigated as a marker of oxidative damage. Finally, the acetylcholinesterase (AChE) activity was considered because it is strictly involved in crucial functions for the survival, growth and reproduction, in both invertebrate and vertebrate species (Rosenberry, 2006). At the same time, a video tracking analysis were performed to describe changes in swimming behaviour while the consequences on reproduction were assessed by a chronic toxicity test. In particular, the results of the study reported in **Chapter 5** show that BE concentrations similar to those found in aquatic ecosystems induced oxidative stress and inhibited AChE activity, thus affecting swimming behaviour and the reproduction of *D. magna* individuals. In the same way, the data obtained in the work presented in **Chapter 6** show that daphnids exposed to environmental levels of CPF were in a condition of stress, which was highlighted by changes in both sub- and supra-organismal biomarkers. In addition, testing two different concentrations has allowed to notice that the activation -induced only by the higher considered concentration- or non-activation in some enzymes activities can lead to different modifications of the swimming behaviour in *D. magna*, suggesting the existence of a link between sub- and supra-organismal levels.

Both these studies (**Chapter 5 and 6**) have been performed on *D. magna,* which is a planktonic invertebrate commonly used as a model organism in ecotoxicological tests because it is easily cultured in

laboratory (ten Berge, 1978) and organism variability is ideally low due to parthenogenetic asexual reproduction in which females asexually produce genetically identical female offspring. Furthermore, test animals of any desired age are available throughout the year, and tests of chronic toxicity can be extended to several generations (Adema, 1978). Moreover, daphnids are of ubiquitous occurrence and form an important link in food chains (Gulati, 1978). Despite these advantages, the use of *Daphnia* species may penalize the ecological realism because these individuals are bred in laboratory and thus their sensitivity may not be representative of the one of organisms adapted to extreme conditions.

Based on this, the behavioural responses of daphnids and *Diamesa cinerella gr* induced by different dilutions of treated sewage effluents were compared. Contrarily of *D. magna*, *D. cinerella* is well adapted to glacial habitats (Lencioni and Rossaro, 2005) and, for this reason, it is considered a key-species in the Alpine aquatic ecosystems (Lencioni and Rossaro, 2010). The comparison of these two species is embedded in the work aiming to improve knowledge in order to contribute to the wider purpose of protection and safeguard of water quality of Alpine river streams. Indeed, according to current ERA procedures adopted for sewage effluents and water, the treated effluents from Sewage Treatment Plants (STP) are recognized as an important route of release of regulated and emerging contaminants in Alpine rivers. In particular, even if STP effluents could be considered

safe for aquatic ecosystems, the results obtained in the present study, reported in **Chapter 7**, highlight that water samples collected at Passo del Tonale area (TN) induced significant alteration on different swimming behavioural parameters in both species.

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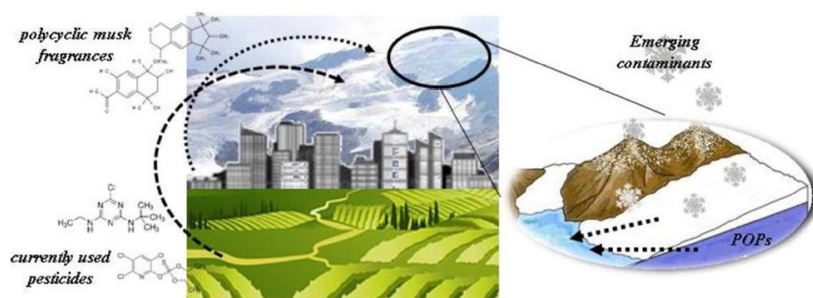
Chapter 3

Legacy and emerging contaminants in meltwater of three Alpine glaciers

Ferrario C.[1], Finizio A.[1], Villa S.[1]

Graphical abstract

[1] Dept. of Earth and Environmental Sciences, University of Milano Bicocca, Piazza della Scienza 1, 20126 Milano.

Abstract

Meltwater samples collected in early and late summer from three Alpine glaciers were analysed to determine the occurrence of POPs (Persistent Organic Pollutants: DDTs, HCHs and PCBs) and emerging contaminants (current used pesticides and polycyclic musk fragrances). For legacy POPs, we reconstructed a concentration time series using data from previous surveys in the same areas (starting from 2000). The results suggest a declining tendency of these compounds, probably related to the introduction of international regulations, which has led the strong use reduction and ban of these compounds.

Among the analysed current used pesticides the terbuthylazine and chlorpyrifos were found in all the analysed samples. The experimental results were in line with the prediction of the OECD tool screening model, which was applied to estimate the potential of these substances to undergo regional-scale atmospheric transport processes. Temporal and spatial differences in concentrations for these compounds were related to the timing of applications, weather conditions and crop distribution along the adjacent Po River Plain. Despite model predictions, the herbicide pendimethalin was never detected, probably due to the lower use of this compound in the agricultural practices. Conversely, concentrations of polycyclic musk fragrances galaxolide and tonalide were more homogeneous both temporally and spatially, in agreement with their continuous release from emission sources.

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Keywords: chlorpyrifos, terbuthylazine polycyclic musks, POPs, glacial meltwater

3.1 Introduction

It is well recognized that contaminants are widely distributed all over the globe and that atmospheric transport is a leading pathway for their diffusion (Harriss et al., 1984). The mechanisms underlying the global atmospheric transport of contaminants were exhaustively explained by Wania and Mackay (1996) and other authors (Finizio et al., 1998; Gouin et al., 2004; Hageman et al., 2006; Scheringer, 2009). As a result of long range as well as local/regional atmospheric transport, contaminants can be transported in polar regions and mountain glaciers, which are hundreds to thousands km far from their emissions sources (Carrera et al., 2001; AMAP, 2004, 2009, 2011; Wang et al., 2006; Bidleman et al., 2010; Daly and Wania, 2005; Kallenborn et al., 2007; Hung et al., 2010; Zhao et al., 2015). According to Grannas et al. (2013), organic contaminants may enter snowpack through wet (snow, rain) and dry (gaseous and particle) deposition processes. The effectiveness of both processes in scavenging contaminants from the atmosphere greatly varies in function of the differences in physical chemical properties among contaminants; this has been largely investigated by means of models (Daly and Wania, 2004; Stocker et al., 2007).

Incorporation of contaminants in snowpack and ice creates temporary reservoir of pollutants. However, once the melting starts due to

increased temperature, a re-emission of contaminants back to the atmosphere or release with meltwater to the freshwater systems occurs. Consequently, pronounced concentration peaks of contaminants are common during snowmelt periods (Meyer et al., 2006; Meyer and Wania, 2008; Bizzotto et al., 2009; Bogdal et al., 2010; Grannas, 2011) and this can have strong implication regarding increased exposure of aquatic as well as terrestrial organisms.

According to Blais et al. (1988), temperate-zone mountain regions, which tend to receive high levels of precipitations while being close to pollutant sources, are particularly susceptible to the accumulation of contaminants. In this, the European Alps, which lie across the most populated and highly anthropized regions of Europe, can be particularly at risk. Historically studies on European Alps have been mainly focused on monitoring the presence of persistent organic pollutants (POPs) such as DDTs, PCBs, dioxins, HCHs in glaciers (Villa et al., 2003, 2006a, 2006b; Bogdal et al., 2010; Pavlova et al., 2016) as well as in other environmental matrices (Nizzetto et al., 2006; Tremolada et al., 2008; Villa et al., 2011). However, closer distances of European Alps to local/regional sources of pollutants increase the potential of glacier contamination from other categories of contaminants. For instance, Current Use Pesticides (CUPs), as well as many other emerging contaminants (e.g., PCMs: Polycyclic Musk Fragrances) tend to have much greater polarity and water solubility than POPs and are much more biodegradable. This has led researchers

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to consider the potential for atmospheric transport of these substances to be quite negligible. In spite of this, CUPs and PCMs have been found in regions isolated from their use and production such as in the Arctic (Hoferkamp et al., 2010; Zhang et al., 2013) or high mountain areas (Zabik and Seiber, 1993; Aston and Seiber, 1997; LeNoir et al., 1999; Hageman et al., 2006; Gouin et al., 2008; Kurt-Karakus et al., 2011; Santolaria et al., 2015). In addition, a few studies highlighted the presence of CUPs and PCMs in snowpack samples (Hageman et al., 2006 and 2010; Villa et al., 2014).

In this context, the aim of this study is to investigate the release of legacy POPs and emerging pollutants (CUPs and PCMs) from the melting of three Alpine glaciers (Lys, Forni and Giogo Alto). This was completed by measuring the concentration levels of the selected compounds in glacial meltwater that was collected in two different monitoring campaigns (summer and late summer/autumn). For legacy POPs, we reconstructed a concentration time series using data obtained from previous monitoring campaigns (Villa et al., 2003; Villa et al., 2006a, 2006b). For CUPs, in consideration of the high number of active ingredients utilized in agricultural practices on the Po River plain, a preliminary screening was performed to select those compounds with a higher potential to reach the glaciers. This was completed by considering both the volume of use (sales data) and the results of the "OECD P_{OV} and LRTP screening tool model" (OECD, 2002, 2004; Wegmann et al., 2009). Finally, the decision to include

the synthetic musk fragrances in the study was made to widen the evidence of their presence in Alpine glaciers and confirm the data obtained in previous studies (Villa et al., 2014).

3.2 Materials and methods

3.2.1 Description of the sampling area and meteorological conditions

The selected sampling sites are located in the Italian Alps: the Lys Glacier in Monte Rosa massif, the Forni Glacier in the Ortles-Cevedale group and the Giogo Alto Glacier in the Palla Bianca-Similaun group (**Fig. 3.1**). The Lys Glacier is close to France and Switzerland and extends from 4282 m a.s.l. to 2600 m a.s.l., covering an area of approximately 9.58 km^2 (~81% of the total catchment area of the sampling point) (Smiraglia and Diolaiuti, 2015). Further east, the Forni Glacier, in the Ortles-Cevedale group, belongs to the Stelvio National Park. This glacier extends from 3673 m a.s.l. to 2501 m a.s.l. and covers approximately 11.34 km^2 (~88% of the total catchment area of the sampling point) (Smiraglia and Diolaiuti, 2015). Finally, the Giogo Alto Glacier, in the Palla Bianca-Similaun group, is located near the Italian and Austrian borders. This glacier extends from 3260 m a.s.l. to 2760 m a.s.l., covering an area of 0.95 km^2 (~92% of the total catchment area of the sampling point) (Smiraglia and Diolaiuti, 2015), and is located in the area characterized by the lowest

precipitation rate of the entire Italian Alpine system (Gabrieli et al., 2011).
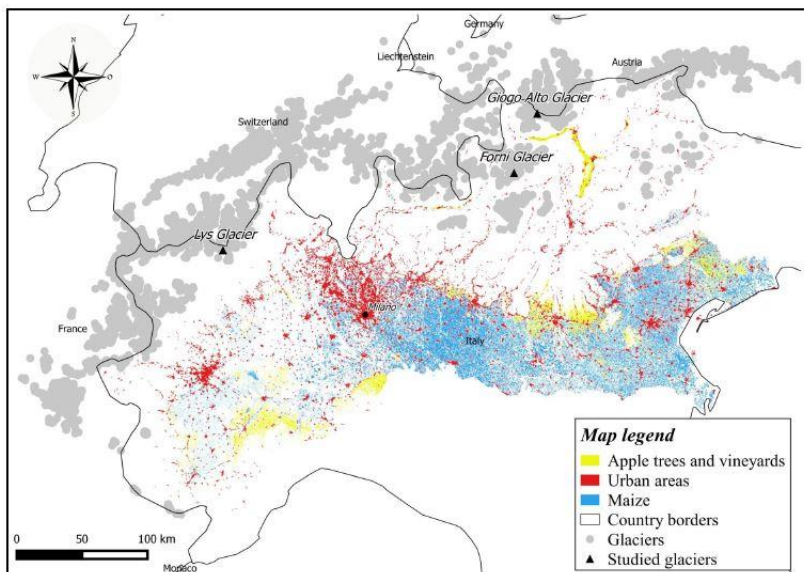

Figure 3.1 Location of the sampling sites on the Lys Glacier, the Forni Glacier and the Giogo Alto Glacier and land use in the surrounding areas in Northern Italy

The criteria for site selection were based on differences in geographic positions, meteorological regimes (Gabrieli et al., 2010, 2011) and land uses. Forni and Lys Glaciers are directly influenced by human activities to a small extent, whereas Giogo Alto Glacier is exploited as a skiing area. During spring and summer, all of the glaciers receive air masses coming from both the surrounding agricultural and urbanized areas (Gabrieli et al., 2011; Villa et al., 2014). These authors reported a strong link between Po River plain emissions and atmospheric

depositions over high-altitude Alpine glaciers during spring and summer seasons. In that period, Maggi et al. (2006) indicated significant vertical exchanges between the boundary layer and the free troposphere due to the presence of convective storm systems. This phenomenon produces snowfalls that are highly loaded with chemical substances, which deposit on glaciers (Largiuni et al., 2003). In addition, even though the air mixing within the Po river plain is very efficient (APAT, 2008), the presence of local hot-spot emissions may cause heterogeneous depositions of pollutants over the Alps.

The monitoring design was defined on the basis of previous experimental works in the same areas (Villa et al., 2001; Villa et al., 2003; Villa et al., 2006a, 2006b; Bizzotto et al., 2009; Villa et al., 2014). Lys Glacier meltwater samples were collected on 25th July 2014 and 3rd October 2014, from the proglacial lake situated in front of the glacier at 2350 m a.s.l. The samples from the Frodolfo stream (that originates from the Forni Glacier) were gathered on 2nd July 2014 and 9th September 2014 near stream source approximately at 2200 m s.l.m. Finally, the Giogo Alto Glacier meltwater samples were collected at 3000 m on 24th July 2014 and 10th September 2014. All samples were collected using aluminium cans (5 and 2.5 L) pre-rinsed with acetone and hexane. The water volume collected for each sample was approximately 10 L. They were kept refrigerated during the transport and stored at -20 °C until analysis.

3.2.2 Chemical analysis

Analytes included in this study were selected on the basis of the following variables: i) previous experiences of this research group in the studied areas (Villa et al., 2001; Villa et al., 2003; Villa et al., 2006a, 2006b; Villa et al., 2014); ii) pesticide sales data from the Po river plain area; and iii) output of the screening model (see paragraph 2.2.4). The studied analytes are organic pollutants belonging to three different categories:

- Persistent Organic Pollutants (POPs): DDTs (including DDE, and DDD), α- and γ-HCHs, HCB, PCBs (28, 52, 101, 118, 138, 153, 180 congeners);
- PolyCyclicMusk fragrances (PCMs): galaxolide (HHCB) and tonalide (AHTN);
- Current Use Pesticides (CUPs): terbuthylazine (TBZ), chlorpyrifos (CPF) and pendimethalin (PEN).

Analytical standards were purchased from Dr. Ehrenstofer, GmBH (Augsburg, Germany) and Sigma-Aldrich (St. Louis, USA). All solvents (residue analysis grade; Merck Darmstadt, Germany) were checked by gas chromatography (GC) before use.

To separate particulate matter, water samples were filtered on a glass fibre filter (GFF, 0.45 μm, Whatman, Maidstone, England) before the SPE procedure. Prior to the extraction of suspended solids of the samples, the glass fibre filters were cleaned with hexane.

Filtered water samples were extracted with SPE cartridges (Oasis HLB, 6 cm^3/500 mg, LP Extraction cartridge, 60 μm; Waters Corporation Milford, Massachusetts, USA). One cartridge was used for each liter of sample (about 10 L meltwater for sample). The cartridges were precleaned with 5 mL of hexane and conditioned with 3 mL of methanol and 3 mL of deionized water (Milli-Q). Samples were then drawn through the cartridges using a vacuum manifold at a flow rate of 10 mL/min. To assess the recovery efficiency, 10 μL of recovery standard (mixture of PCB 40 and PCB 128 at concentration of 400 pg/μL), previously diluted in 3 mL of methanol, were added to each sample. After the extraction, the cartridges were dried under vacuum pressure and subsequently eluted (under gravity) with 6 mL of hexane and 3 mL of ethyl acetate.

Extraction of GFFs was performed via a Soxhlet extractor with n-hexane for 24 h. The extracts were then concentrated to 2 mL by rotoevaporation. Then, 50 μL of recovery standard (mixture of PCB 40 and PCB 128 at concentration of 400 pg/μL), previously diluted in 3 mL of methanol, was added to each sample. The extracts were then concentrated to 0.5-0.7 mL under a gentle stream of nitrogen. Finally, purification of the extracts was achieved using Supelclean ENVI-carb (0.5 g Supelclean ENVI-carb SPE tubes Supelco, Bellefonte, PA, USA) cartridges that were pre-cleaned with 10 mL of hexane. Samples were drawn through the cartridges under vacuum pressure at a regulated flow rate of 10 mL/min. After the extraction, the cartridges

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were dried using a vacuum pump and subsequently eluted (under gravity) with 10 mL of n-hexane, 10 mL of a mixture of n-hexane/ethyl acetate (60:40) and 5 mL of ethyl acetate.

All the extracts were finally concentrated to 0.2-0.3 mL under a gentle stream of nitrogen and transferred into GC micro-vials with 25 μL of dodecane as a keeper.

Finally, the internal standard (PCB 30) was added for subsequent analysis by GC-MS (Agilent Technologies, Santa Clara, CA, USA) using SIM (Single Ion Monitoring).

The GC identification and quantification was performed using an Agilent Technologies 6890 N Series gas chromatograph equipped with a 30-m long, 0.25-mm internal diameter capillary column (Zebron Capillary GC Column, ZB-SemiVolatiles Guardian). Samples were injected by an Agilent Technologies 7683 Series autoinjector with the injection port at 250 °C in splitless mode. Samples were run in splitless mode using helium as a carrier gas (flow = 1 mL/min). The transfer line and detector temperature were maintained at 280 °C.

Details on the oven program, the MS setting and the limit of detection are reported in Section 3.1 of the Supporting Information (SI) (**Chapter 9**).

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3.2.3 Quality control

The obtained recoveries of five water and suspended solids surrogate samples (spiked with the analysed compounds) were satisfactory, averaging ≥ 80% (**Table SI-3.2**). Meltwater blanks were generated by filling a pre-cleaned bottle in the field with 1 L of Milli-Q water. The Milli-Q was treated like a meltwater sample and extracted through the GFF and HLB cartridges. No CUPs were found in the blanks, while HCB, PCB p,p′-DDT and musk fragrances were present occasionally. Reported values were then corrected by subtracting the average blank values of HCB (73 pg/L ± 11), p,p′-DDT (631 pg/L ± 63), PCB 52 (429 pg/L ± 38), PCB 101 (185 pg/L ± 5), HHCB (380 pg/L ± 76) and AHTN (128 pg/L ± 23).

3.2.4 The OECD P_{OV} and LRTP screening tool model

In this study, the potential atmospheric transport of 12 CUPs frequently used in the agricultural practices of the Po River plain (**Table SI-3.3**) was estimated using the OECD P_{OV} and LRTP Screening Tool software (ver. 2.2, downloadable at http://www.oecd.org/env/ehs/riskassessment/oecdpovandlrtpscreenin gtool.htm). A more detailed description of the model is reported in Section 3.2 of the Supporting Information (**Chapter 9**) and in the original papers (OECD, 2002, 2004; Wegmann et al., 2009). It is currently applied as a screening tool for making comparative assessments of environmental hazard properties of non-ionizing

chemicals using metrics of overall persistence (P_{OV}) and long-range transport potential (LRTP) (Öberg and Iqbal, 2012). Very recently, it has also been utilized to evaluate the potential for the Medium Range Atmospheric Transport (MRAT) of the musk fragrances AHTN and HHCB (Villa et al., 2014). As substance-specific inputs, the software requires the air-water partition coefficient (K_{AW}) of the substance of interest and the octanol-water partition coefficient (K_{OW}), as well as the degradation half-lives (DT_{50}) in soil, water and air (**Table SI-3.3**).

3.2.5 Data analyses

The statistical analysis was performed using R 3.1.2 software (R core team 2015). Paired t-test was performed to investigate differences in contaminant concentrations between the two sampling seasons (*p*-values > 0.05).

3.3 Results and discussion

The application of the OECD P_{OV} and LRTP screening tool allowed the identification of the CUPs to be included in the monitoring campaigns. Specifically, CPF, PEN and TBZ were selected. Further information on the obtained results is reported in Section 3.2 of the Supporting Information (**Chapter 9**).

3.3.1 Contamination patterns in meltwater samples of the investigated glaciers

An overall picture of the analysed chemical residues found in the meltwater samples collected in the three glaciers is reported in **Fig. 3.2** (details in **Table SI-3.4**). It is emphasized that the data here presented refer to individual samples, as replicates were not available. For this reason, a certain degree of uncertainty is associated. In **Fig. 3.2**, data are referred to single as the sum of concentrations measured in both the dissolved and sorbed phases and are grouped according the three broad categories of POPs, CUPs and PCMs. Some compounds (o,p′-DDD, p,p′-DDD, o,p′-DDT, o,p′-DDE and PEN) were always below the detection limits.
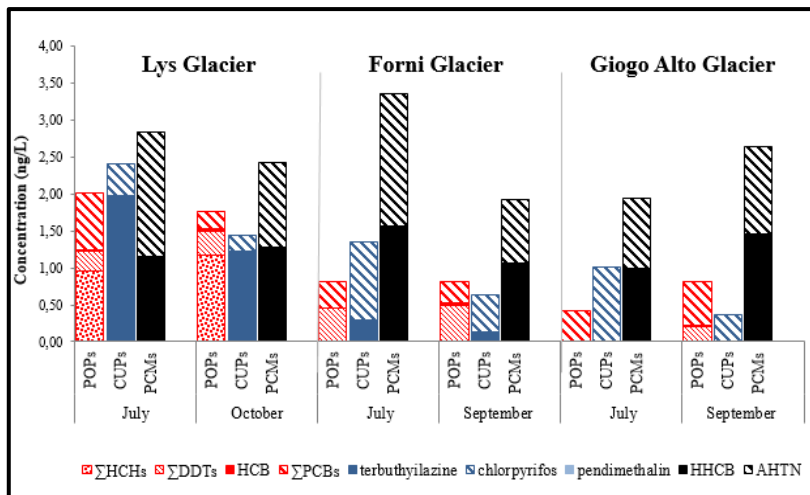

Figure 3.2 Concentrations in Lys Glacier, Forni Glacier and Giogo Alto Glacier meltwater.

In all sampled sites, he observed trend was PCMs > CUPs > POPs and PCMs > POPs > CUPs in early and late summer monitoring campaigns, respectively. Changes in the seasonal trends were mainly due to a decrease in CUPs concentrations in the second sampling period. Yet, no statistically significant differences were found among samples collected in July and September (paired t-test $p > 0.05$).

However, going into deeper detail, it becomes evident that there were some qualitative differences in contaminants among the sites starting from the West (Lys Glacier) and moving East (Giogo Alto Glacier). For instance, among POPs, HCHs isomers were exclusively present in Lys meltwater samples. This site was also characterized by the highest levels of TBZ. On the contrary, both compounds were not detected or were present in very small concentrations (TBZ in Forni Glacier) in the other two glaciers.

In the paragraphs below, a deeper discussion for each contaminant category of the investigated chemicals is made.

3.3.1.1 Current use pesticides

As previously described, in this study, attention was focused on those CUPs (TBZ, CPF and PEN) that showed the highest Characteristic Travel Distance (CTD) and Transfer Efficiency (TE). Two of the CUPs (TBZ and CPF) were frequently present in the analysed samples, whereas PEN was always below the detection limit (**Fig. 3.2**).

TBZ seemed to show a decreasing profile from West to East, with the highest measured concentrations in the melting waters of Lys Glacier (1.98 ng/L and 1.23 ng/L in July and September, respectively). TBZ has been frequently measured in air and rainwater samples (Dubus et al., 2000; Mai et al., 2013); nevertheless, there is no evidence of LRAT of this substance, or of its presence in snow or glaciers. To the best of our knowledge, the data presented in this study are the first record of TBZ in alpine glaciers. However, it must be noted that very recently Santolaria and co-workers (2015) reported the presence of TBZ in a Pyrenean cirque glacial lake (Sabocos tarn) located in the Tena Valley (Spain). In the surface lake water samples, these authors found TBZ concentrations ranging from 2.27 ng/L (July 2011) to 0.32 ng/L (August 2014). This could be an indirect confirmation of the potential accumulation of this substance in mountain glaciers.

The potential LRAT of TBZ could be limited by rain scavenging at the lower latitudes. In fact, this compound has a low value for Henry's law constant, and this could favour its partition into rain. In the presence of natural barriers, such as the Alps mountain ranges, the higher precipitation rate could provide an effective sink for this substance, limiting the LRAT process but enhancing the accumulation of TBZ in mountain glaciers.

In our study, CPF was detected in all the three investigated glaciers, with concentrations ranging from 0.21 ng/L (Lys Glacier in September) to 1.06 ng/L (Forni Glacier in July). In contrast to the case

for TBZ, there are a number of studies showing that CPF can undergo long-range and regional-scale atmospheric transport (Muir et al., 2004). In fact, CPF has been frequently detected in Artic media (air, water, sediment and biota) (Hageman et al., 2006; Landers et al., 2008; Hoferkamp et al., 2010). The historical reconstructions of CPF concentrations in Artic ice cores (Hermanson et al., 2005; Ruggirello et al., 2010) suggested its constant presence since 1953.

Highly variable concentrations of CPF, ranging from 0.02 ng/L (Hageman et al., 2006) in the Rocky Mountains to 80 ng/L in Arctic samples (Hoferkamp et al., 2010), are reported for snow samples. Finally, the only available data on the presence of CPF in European mountains are those reported in the study by Santolaria et al. (2015). These authors reported CPF concentrations ranging from 0.62 ng/L (July 2011 and April 2014) to 0.12 ng/L (August 2014). Although these data refer to lake water samples rather than glacial melt samples, it must be noted that they are on the same order of magnitude as our findings.

Despite the model prediction, PEN residues were not measured in any of the analysed samples. The usage of this substance could be a possible explanation for this discrepancy. In fact, in Northern Italy, the purchased volume of PEN is two to three times less than the purchased volumes of CPF and TBZ, respectively (**Table SI-3.3**). In the available literature, there are few and contrasting data about the atmospheric transport of this substance. In fact, in two different ice

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cores drilled in the Svalbard Islands (Norway), PEN residues were either not detected or measured sporadically at a very high concentration (18 ng/L) in the Holtedahl Fionna and Austfonna ice core samples, respectively (Ruggirello et al., 2010).

From the analyses of **Fig. 3.2** and **Table SI-3.4**, temporal differences can also be observed. In all the glaciers, the highest CUP concentrations were measured in samples collected in early summer, and this can be ascribed to the release of CUPs buried in snow fallen the winter season, and lying above or in proximity the glacier tongue. In fact, as shown by Villa et al. (2014), seasonal variations in contaminant concentrations in glacial meltwater is a function of the different origins of the sampled waters: in late spring, the predominant source of meltwater feeding glacial streams is the last season's snowpack, whereas in late summer, the glacial melt is more pronounced. Concentrations measured in meltwater deriving from seasonal snowpacks are the joint result of two factors: the timing of pesticide applications and weather conditions. In fact, the spring and summer months are the growing seasons and periods of intensive pesticide use. In addition, the transport of ground-emitted pollutants to high altitude locations is enhanced by the behaviour of the PBL allowing for vertical exchanges (Maggi et al., 2006). At high elevations, the spring/summer snow efficiently scavenges the atmosphere (Lei and Wania, 2004) and deposits the contaminants on

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glaciers, giving rise to highly concentrated spring/summer layers (Largiuni et al., 2003).

Lastly, TBZ and CPF concentrations show different geographic profiles along the Alps (**Fig. 3.2** and **Table SI-3.3**). The differences in land uses, the presence of natural geographical barriers and/or differences in precipitation regimes could help to explain these results. In **Fig. 3.1**, the agricultural land use of the Po River plain is depicted in relation to the geographic positions of the three investigated glaciers. Historically, maize is the main cultivated crop in northern Italy, and TBZ is applied on this crop as a pre- and post-emergence herbicide. On the contrary, CPF is widely used on vine and fruit trees (primarily apple trees). These crops are prevalently present in valleys and foothills of the Alps; the latter are mainly located on the southern edge of the Po River plain. The Lys Glacier faces directly to the West side of the Po River plain in an area without geographical barriers. In contrast, Forni Glacier is separated from the Po River plain by the presence of the Alps foothills (reaching maximum altitudes of 2500 m a.s.l.), and the Dolomite (highest altitude 3050 m a.s.l.). Adamello (max elevation 3560 m a.s.l.) mountain groups lie between the Giogo Alto Glacier and the Po River plain. Moreover, the Giogo Alto Glacier is located at the boundary between the central and southern European climate regions and differs from the glacial areas of the Western Alps, especially in precipitation rates and seasonality (Davis et al., 2003).

The lack of geographical barriers and the presence of higher precipitation rates (1100-1300 mm yr^{-1}) in Lys area favour the atmospheric transport and wet deposition on the glacier, leading to an increase of TBZ concentration in snowpack and afterword in meltwater. Alternatively, high-elevation mountainous groups (Dolomites and Adamello) represent an area of high scavenging rates (1300 mm yr^{-1} of rain). This area acts as a barrier and prevents the herbicide from reaching the inner part of the Italian Alps: the Giogo Alto Glacier. Finally, among the three investigated sites, Forni Glacier is the closest to the area of high intensive maize cultivation. However, the presence of the Alps foothills and lower precipitation rates (750-850 mm yr^{-1}) could reduce the transfer of TBZ. Therefore, the combination of these factors leads to the observed TBZ concentrations, which were in between those measured in Lys and Giogo Alto Glaciers.

The different trend shown by CPF could be ascribed to the spatial distribution of vine and fruit trees. In contrast to maize, which is widespread, these crops show a patchy distribution along the Po River plain. Particularly, they are present in the Alps valleys in an area that is very close to Forni and Giogo Alto Glaciers (< 40 km in linear distance). Consequently, the higher concentrations of CPF in both site could be due to the very short travelling distance.

3.3.1.2 Polycyclic musk fragrances

The potential for HHCB and AHTN to undergo regional atmospheric transport was assessed in a previous study by Villa et al. (2014). Furthermore, these authors confirmed the presence of both fragrances in freshly fallen snow samples and water sampled from the area of the Forni Glacier. They hypothesized that there is the potential for these substances to accumulate in cold environments near densely anthropized areas.

The present data support this hypothesis; indeed, both compounds were detected at comparable concentrations in all the analysed sites; the average concentrations for HHCB and AHTN were 1.25 ng/L (0.99 - 1.57 ng/L) and 1.27 ng/L (0.86 - 1.79 ng/L), respectively, which are on the same order of magnitude as those previously reported in the study by Villa and co-workers (2014) (HHCB and AHTN mean concentrations: 3.14 ng/L and 3.88 ng/L, respectively). The two investigated fragrances are characterized by high and constant releases in the urbanized areas present in the Po River plain. This can explain the widespread level of contamination of the Alps. Finally, the HHCB/AHTN ratio is much lower than the value of 4:1 that was estimated from the market volumes of the two substances (OSPAR Commission, 2004). However, the calculated ratio of approximately 1 substantially confirms those reported by Villa et al. (2014) in fresh fallen snow samples collected in the area of the Forni glacier (HHCB/AHTN ratio=1.24) Xie et al. (2007) also reported similar

HHCB/AHTN ratio values in air samples collected along the North Sea (HHCB/AHTN mean ratio = 1.4). The different fate of two musks are probably driven by their half-lives in the atmosphere. As Aschmann et al. (2001) reported, AHTN is slowly removed by atmospheric degradation because its atmospheric lifetime is almost twice that of HHCB, which reacts faster with ·OH.

3.3.1.3 Persistent organic pollutants

Table 3.1 presents a summary of the available data of POP concentrations measured in glacial meltwaters collected in Lys and Forni Glaciers in different monitoring campaigns (starting from 2000). More detailed information on individual compounds is reported in **Table SI-3.3** of the Supporting Information section (**Chapter 9**).

The reported data, expressed as the mean concentrations, refer to previous studies conducted by this research group (Villa et al., 2006a, 2006b). To the best of our knowledge, the POP concentrations measured in the glacial meltwater of the Giogo Alto Glacier are the first published data reporting the presence of POPs in this glacier.

From **Table 3.1** the following considerations can be made:

- HCB: In this study, the concentration values measured for this compound are on the same order of magnitude as those of previous studies (Villa et al., 2006; Villa et al., 2006; Bizzotto et al., 2009). In addition, there are no particular differences among

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the considered glaciers (both in the historical and present data). Our findings are in agreement with the most recent studies on HCB in mountains. In the already reported study of Santolaria and co-workers (2015), the HCB concentrations ranged from not detected (April 2014) to 0.05 ng/L (August 2014). In a previous study, a reconstruction of the seasonal deposition trend of HCB was made through the analysis of samples of a firn core drilled into the Lys Glacier (Villa et al., 2003). According to these authors, the concentrations of HCB measured in the Lys ice core for the time period between the 1960s to late 1970s were below the detection limit. After an increasing trend during the 1980s, there was a decline in concentration starting in the early 1990s.

- HCHs: In the considered period (2000-2014), the detection of α and γ-HCH isomers seemed to show a declining temporal trend. This is particularly evident in the meltwater samples from the Forni Glacier. In 2014, both compounds were below their relative detection limits. In general, γ-HCH showed a greater declining rate, and this can be observed from the α/γ-HCH ratio. Historically, the α/γ-HCH ratio largely has been used to identify the possible HCH source. A ratio >3, generally indicates input due to the use of technical mixtures of HCHs (55-80 % α-HCH, 5-14 % β-HCH, 8-15 % γ-HCH, and 2-16 % δ-HCH) (Breivik et al., 1999) and LRAT (Lane et al., 1992). On the contrary, a ratio near or below 1 indicates the local/regional use of lindane (γ-HCH) and

MRAT (Iwata et al., 1993; Willett et al., 1998). During the considered period, the ratio seemed to be shifted from values indicating a prevalence of lindane and local/regional sources towards values suggesting a prevalence of α-HCH and a higher contribution of LRAT. A possible explanation of this trend can be ascribed to the introduction of new EU regulations concerning γ-HCH. In 2004, lindane uses with crop protection purposes were prohibited in many EU countries (EU, 2004), and since 2009, γ-HCH (together with α-HCH and β-HCH) has been listed as a POP in the Stockholm Convention (UNEP, 2015). Therefore, the lower accumulation rates of lindane in glaciers may be a result of the decline in the use of this substance in areas surrounding the glaciers.

- DDTs: In this study, only p,p'-DDE and p,p'-DDT were detected. In 2014, the measured concentrations of p,p'-DDT were on the same order of magnitude along the monitored sites, with Giogo Alto showing the lowest concentrations in both sampling periods. p,p'-DDT showed a fluctuating temporal trend, which becomes particularly evident when the Forni Glacier is considered (however, p,p'-DDT concentrations in Lys Glacier seemed to follow the same trend). In the Forni Glacier, starting from 2000, there was clear decreasing trend of p,p'-DDT up to 2006, followed by a sudden increase in 2014. In contrast to p,p'-DDT, its metabolite p,p′-DDE seemed to have a different spatial pattern of

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contamination. In Lys Glacier meltwater samples from 2014, the levels of this compound were near or below the detection limit, whereas appreciable concentrations of this compound were found in the other two glaciers, at least during the second sampling campaign. In addition, the concentration of p,p'-DDE seemed to be quite constant during the considered period (however, a seasonal variability was noticed).

The differences shown by the parent compound and its metabolite (in both concentrations and temporal trends) led to a variability of the p,p′-DDE/p,p′-DDT ratio trends. In Lys Glacier samples, this ratio was always ≤1, whereas in the Forni Glacier samples, this ratio had higher variability, reaching a peak in 2006. The only available data for the Giogo Alto Glacier indicated similar concentrations of the parent compound and its metabolite (p,p'-DDE/ p,p'-DDT = 1). In general, the p,p′- DDE/p,p′-DDT ratio is used to estimate the age of DDT contamination because DDE is the main product of DDT degradation under aerobic conditions in the environment (Wang et al., 2006). A higher ratio reflects an aged DDT source and is mainly related to LRAT of DDT (due to the transformation of p,p'-DDT into its metabolite). Conversely, a ratio < 1 indicates a fresh input of p,p'-DDT and usually is associated with local emissions of DDT. In a previous work, (Villa et al., 2003) analysed an ice core that was sampled at the Lys Glacier (4240 m a.s.l.) and found an increasing temporal trend of p,p'-DDT, which was clearly in contrast with the

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declining trends highlighted by other authors after the ban of this substance. In the Lys Glacier ice core samples, the concentrations of p,p'-DDT were nearly constant up to the late 1970s, and then curved slightly upward to a sharp increase during the mid-1990s. The authors explained this trend as the consequence of continuous emissions from an industry located near the Toce River (an effluent of Lake Maggiore) that used DDT as a precursor for dicofol. The accidental release of p,p'-DDT can help to explain the presence and the different in concentration of this compound in the three glaciers (Bettinetti et al., 2005). In fact the Giogo Alto Glacier, which is the farthest glacier from the Lake Maggiore area, showed the lowest level of contamination in 2014).

- PCBs: The ∑PCBs concentrations ranged from 0.24 to 0.76 ng/L (**Table SI-3.4**). PCB congener No. 180, the heaviest considered, was never found above detection limit or background values. On the contrary, PCB congener No. 52 was present in all samples. The concentrations found in Forni meltwater samples, ranging between 0.28 - 0.35 ng/L, were close to the levels found by Bizzotto and co-workers (2009) in the same area. In 2006, the concentrations of ∑PCBs in Frodolfo (Forni Glacier stream) ranged from <0.01 ng/L to 0.3 ng/L. However, in June 2006, these compounds reached extremely high concentrations (12.6-31.7 ng/L). The unexpected pulse in PCBs was explained by the fact that seasonal snow melting was coming to an end during June.

Therefore, the peaks in concentrations of these compounds were due to a short but heavy precipitation event that washed out substantial amounts of highly hydrophobic particle-sorbed chemicals (Bizzotto et al., 2009). In glacial streams of remote areas, the reported concentrations of PBBs ranged from 0.04-0.10 ng/L (e.g. for the Himalayan areas) (Sharma et al., 2015; Guzzella et al., 2011) to 0.48 ng/L (e.g. for the Canadian Rocky Mountains) (Lafrenière et al., 2006). Very recently, Pavlova and coauthors (2016), investigated the presence of iPCBs in meltwater of Silvretta Glacier (European Alps). These authors reported concentrations of these contaminants ranging from 0.05 - 0.1 ng/L with an average value of 0.07 ng/L. The data of the selected PCBs were available also for Alpine ice cores. In particular, in firn cores from the Lys Glacier, the $\sum$PCBs concentration ranged from 0.21 to 0.68 ng/L in the period from 1997 to 2000 (Villa et al., 2006). These levels were comparable to concentrations determined in Fiescherhorn Glacier (Switzerland) in the same years, highlighting a homogeneous distribution of these compounds in the Alpine region (Pavlova et al., 2014). Based on the Fiescherhorn ice core, which covered the entire time period of the industrial use of PCBs, the concentration in 2002 is comparable to the concentration in the 1940s, when PCBs were introduced to the market (Pavlova et al., 2014). Another deposition record of PCBs that covers a long time period was available from

Lomonosovfonna Glacier in the Artic (Svalbard) (Garmash et al., 2013). Generally, PCBs concentrations in the Arctic are lower by a factor of approximately 100 than in the Alps due to their further distance from the sources of these compounds. In addition, the heavier congener concentrations are lower in the Arctic ice core because the long-range atmospheric transport to the Arctic is more hampered for these compounds, which are more susceptible to dry and wet particle deposition (Pavlova et al., 2014).

Table 3.1 Comparison of measured concentrations of POPs in glacial meltwaters (ng/L).

	Lys glacier					Forni glacier						Giogo Alto glacier	
	2000[a]	2001[a]	2002[a]	2014[b] (July)	2014[b] (Oct.)	2000[a]	2001[a]	2002[a]	2006[c]	2014[b] (July)	2014[b] (Sept.)	2014[b] (July)	2014[b] (Sept.)
HCB	0.03	0.03	0.02	0.03	0.03	0.02	0.02	0.02	0.04	n.d.	0.04	n.d.	0.02
α-HCH	1.28	0.82	0.47	0.62	0.74	0.53	0.26	0.21	0.29	n.d.	n.d.	n.d.	n.d.
γ-HCH	1.41	1.33	0.98	0.34	0.44	1.55	0.93	0.71	0.24	n.d.	n.d.	n.d.	n.d.
α/ γ ratio	0.91	0.62	0.48	1.8	1.67	0.34	0.28	0.29	1.23	-	-	-	-
p,p'-DDT	0.14	0.05	0.03	0.23	0.33	0.15	0.01	0.03	0.01	0.46	0.23	n.d.	0.10
p,p'-DDE	0.01	0.02	0.03	0.04	n.d.	0.04	0.01	0.03	0.09	n.d.	0.27	n.d.	0.10
p,p'-DDE/ p,p'-DDT ratio	0.07	0.4	1	0.17	<<1	0.27	1	1	9	<<1	1.17	-	1
ΣPCBs	n.a.	n.a.	n.a.	0.76	0.24	n.a.	n.a.	n.a.	0.212 - 0.006	0.35	0.28	0.43	0.60

n.a. = not available; n.d. = not detected;
Data from: [a] Villa et al., 2006, [b] present study, [c] Σ7-ICES Bizzotto et al., 2009

3.4 Conclusions

This study examined the occurrence in meltwater samples collected in three Alpine glaciers of a large number of airborne organic pollutants. For legacy POPs, the results seemed to confirm a declining trend of POPs measured in meltwater of Alpine glaciers. Although no alarming amounts of CUPs and PCMs were recorded in meltwater samples, the results are of undoubtful interest. In fact, for the first time, the presence of TBZ and CPF in melt water from Alpine glaciers has been highlighted with a spatial and temporal variability, which is clearly related to the agricultural practices in the adjacent Po River plain. Finally, the study has also highlighted the presence of AHTN and HHCB in the meltwater samples. This is clearly related to the high and constant releases in the urbanized areas present in the Po River plain, which explain the widespread contamination of the Alps found for these compounds.

Acknowledgments

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Chapter 4

Bacteria contribute to pesticide degradation in cryoconite holes in an Alpine glacier

Ferrario C.[1], Pittino F. [1], Tagliaferri I. [1], Gandolfi I. [1], Bestetti G. [1], Azzoni R.S. [2], Diolaiuti G. [2], Franzetti A. [1], Ambrosini R. [1], Villa S. [1]

Graphical abstract

[1] Dept. of Earth and Environmental Sciences, University of Milano Bicocca, Piazza della Scienza 1, 20126 Milano.
[2] Dept. of Environmental Science and Policy, University of Milano, Milano, Italy.

4. Bacteria contribute to pesticide degradation in cryoconite holes in an Alpine glacier
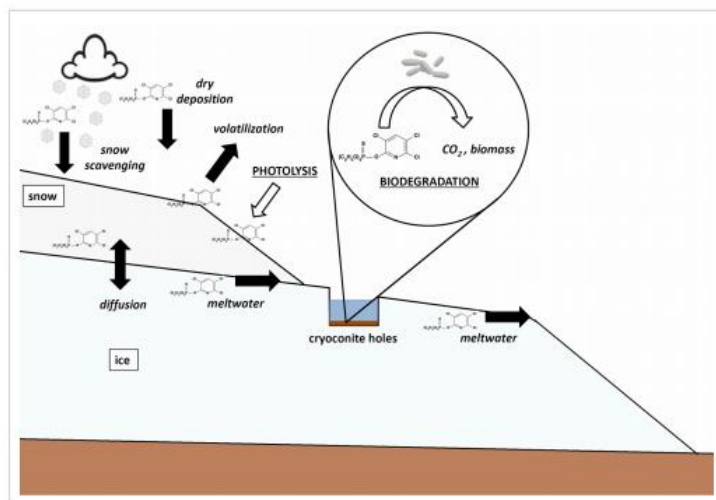
Abstract

Organic contaminants deposited on glacier snow and ice are subject to partitioning and degradation processes that determine their environmental fate and, consequently, their accumulation in ice bodies. Among these processes, organic compound degradation by supraglacial bacteria has been investigated to a lesser extent than photo- and chemical degradation. We investigated biodegradation of the organophosphorus insecticide chlorpyrifos (CPF), a xenobiotic tracer that accumulates on glaciers after atmospheric medium- and long-range transport, by installing in situ microcosms on an Alpine glacier to simulate cryoconite hole systems. We found that biodegradation contributed to the removal of CPF from the glacier surface more than photo- and chemical degradation. The high concentration of CPF (2-3 µg g^{-1} w.w.) detected in cryoconite holes and the estimated half-life of this compound (35-69 days in glacier environment) indicated that biodegradation can significantly reduce CPF concentrations on glaciers and its runoff to downstream ecosystems. The metabolic versatility of cryoconite bacteria suggests that these habitats might contribute to the degradation of a wide class of pollutants. We therefore propose that cryoconite acts as a "biofilter" by accumulating both pollutants and biodegradative microbial communities. The contribution of cryoconite to the removal of organic pollutants should be included in models predicting the environmental fate of these compounds in cold areas.

Keywords: cryoconite, glaciers, chlorpyrifos, biodegradation, microcosm

4.1 Introduction

It is widely documented that organic contaminants are present in polar and mountain regions far from their emission sources (Bidleman et al., 2010; Carrera et al., 2001). High mountains, acting as cold condensers (Calamari et al., 1991), interfere with the atmospheric transport and global cycling of semi volatile organic compounds (SVOCs) (Carrera et al., 2001; Villa et al., 2003). These organic pollutants can be efficiently scavenged from the atmosphere by snow (Grannas et al., 2013), along with aerosols, microorganisms and nutrients (Price et al., 2009). When deposited on glaciers, pollutants undergo partitioning among different environmental matrices (e.g. snow, ice, water, interstitial atmospheric gases and supraglacial sediments) and post-depositional alteration processes.

Among those environmental matrices, cryoconite, a wind-borne fine debris deposited on glacier surfaces, represents a potential sink for organic pollutants because of its high content of organic matter (OM). Indeed, recent studies showed that cryoconite can accumulate organic aromatic pollutants like polycyclic aromatic hydrocarbons, polychlorinated biphenyls (PCBs) and organochlorine pesticides (Li et al., 2017; Weiland-Bräuer et al., 2017). This occurs particularly in cryoconite holes, small depressions on glacier surfaces filled with water and added with a layer of cryoconite at the bottom, which are

considered the most biologically active habitats on glaciers (Cook et al., 2015).

Currently, studies on the post-depositional alteration of pollutants in glacier environments have considered mostly physicalchemical processes, such as photodegradation, hydrolysis and revolatilization of contaminant burdens in snowpack and ice (Grannas et al., 2007; Herbert et al., 2006). Conversely, although the pollutant biodegradation by microbial communities has been proved in different cold environments (Margesin, 2007), their microbial degradation on glaciers has received less attention (Hodson, 2014). For instance, Cappa and colleagues investigated the effect of pollutants on microbial communities on glaciers (Cappa et al., 2014). Moreover, the microbial potential to degrade PCBs on a glacier has been assessed by a laboratory microcosm study (Weiland-Bräuer et al., 2017) and a metagenomics approach (Hauptmann et al., 2017) whereas pesticide biodegradation has been quantified in laboratory by microcosms simulating glacier surface (Stibal et al., 2012a), followed by a comparison with the results of *in situ* metabolomic analyses (Cook et al., 2016). However, to the best of our knowledge, no studies have been conducted to quantitatively evaluate pollutant biodegradation rate in cryoconite holes through in situ experiments.

In this work, we tested the hypothesis that cryoconite might act as a "biofilter" for organic pollutants on glaciers by both accumulating them and promoting their biodegradation, thus significantly

contributing to their removal. To this end, we conducted in situ microcosm experiments on an Alpine glacier simulating a cryoconite hole system exposed to the organophosphorus insecticide chlorpyrifos (CPF). CPF is one of the most widely used pesticides (George et al., 2014) and represents an ideal model compound because it is frequently detected in matrices from both Arctic (air, water, sediment and biota; Hageman et al., 2010; Hoferkamp et al., 2010; Landers et al., 2008) and Alpine areas (glacial meltwater; Ferrario et al., 2017, **Chapter 3**). Although not officially classified as a Persistent Organic Pollutant (POP) or Persistent, Bioaccumulative and Toxic Substance (PBT), CPF shows great potential to undergo long-range atmospheric transport and to reach cold areas (Mackay et al., 2014). The experiments were carried out on the Forni Glacier, one of the largest Italian valley glaciers, where CPF was found in meltwater in the order of ng L^{-1} (Ferrario et al., 2017, **Chapter 3**).

4.2 Methods

4.2.1 Study area and field methods

The experiments were carried out on the Forni Glacier (Italian Alps, coordinates of the approximate centre of the Glacier: 46°12'30'' N, 10°13'50'' E), one of the largest Italian valley glaciers. It is about 3 km long, stretches over an elevation range of 2600 to 3670 m a.s.l. (Senese et al., 2012b) and its surface area was 11.34 km^2 in 2007 (Smiraglia et al., 2015). The microcosms were placed on the Eastern

ablation tongue of the Forni Glacier at about 2700 m a.s.l. near an Automatic Weather Station (named AWS1 Forni; 46°23'51.96'' N, 10°35'39.16'' E) (Azzoni et al., 2016) in a flat area with no crevasses. Microcosms were prepared using cryoconite and water collected on the Forni Glacier on 17 July 2015. Sediment was taken from cryoconite holes with a spoon sterilized with alcohol and flame and transferred into sterile Falcon tubes (50 mL). The meltwater was gathered from a supraglacial stream in a sterilized bottle. After collection, 42 transparent Pyrex bottles (50 mL) were filled with 2 g of sediment and 36 mL of meltwater.

Eighteen bottles were covered with tinfoil ("dark" condition, hereafter) while the other ones were kept transparent ("light" condition, hereafter). Nine of these dark and nine of these light bottles were also sterilized in a pressure-cooker. In each bottle, 8 µg of CPF, dissolved in DMSO, were spiked. We stress that CPF was added after bottle sterilization, while taking care to avoid sterilized microcosm contamination. Six light bottles were immediately brought to the lab for the analyses, which occurred within 4 h of CPF injection, and served as a control at time zero (t_0). The remaining 36 bottles were then placed in three Plexiglas racks, covered with a plastic net to avoid bottle overturning and dispersal and placed on the glacier surface bound to a wooden stake drilled in the ice (**Fig. SI-4.1**). Thus we placed on the glacier microcosms belonging to four experimental groups ("light biotic", "dark biotic", "light sterile", "dark sterile")

including nine microcosms each. Three bottles from each experimental group were then collected on July 27, August 10, August 26, and brought to the lab within 4 h for analyses. At each visit, all non-collected bottles were aerated by quickly opening the lid to assure aerobic conditions. CPF mass present in natural cryoconite was determined taking sediment from cryoconite holes on 2 July 2015 and on 5 July 2016. Samples were collected with a precleaned spoon, transferred into Falcon tubes (50 mL) and brought to the lab within 4 h for analyses.

4.2.2 Chemical analyses

Chlorpyrifos (IUPAC name: O,O-diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate) was purchased from Sigma-Aldrich (product number: 45395-100 MG, Saint Louis, MO, purity > 99.7%). All solvents were checked by gas chromatography (GC) before use.

Water samples were filtered on a glass fiber filter (0.45 µm; Whatman, Maidstone, UK). Prior to the extraction of suspended solid samples, glass fiber filters were cleaned with n-hexane (Carlo Erba, Milan, Italy; purity N99.8%). 10 mL aliquots of filtered water of each sample were separately extracted using OASIS HLB cartridges (Oasis HLB, 6 cc/500 mg, LP Extraction cartridge, 60 µm; Waters Corporation Milford, MA). Cartridges were conditioned with 5 mL of methanol (J.T. Baker, Center Valley, PA; purity N99.8%) and 5 mL of deionized water. Samples were drawn under vacuum through the cartridges at a

regulated flow rate of 10 mL min^{-1}. After extraction, cartridges were dried using a vacuum pump and subsequently eluted (under gravity) with 3 + 3 mL of ethyl acetate (Carlo Erba, Milan, Italy; purity N99.8%) and 4 mL of acetone (Carlo Erba, Milan, Italy; purity N99.8%). The extracts were then concentrated to 0.5 mL under a gentle stream of nitrogen.

The extracted suspended solids were dried by adding anhydrous sodium sulfate (Granular 12-60 Mesh; J.T. Baker Center Valley, PA; purity Ultra Resi-Analyzed), transferred into cellulose extraction thimbles (19x90 mm; Albet Labscience, Seville, Spain) and extracted with n-hexane for 24 h. The extracts were then concentrated to 0.5 mL under a gentle stream of nitrogen.

Both water and suspended solid extracts were transferred into GC micro-vials. An internal standard (PCB 40, lot: 40714; Dr. Ehrenstorfer GmbH, Augsburg, Germany; purity 99.0%) was added for subsequent analysis by GC-MS (Agilent Technologies, Santa Clara, CA), in SIM (Single Ion Monitoring) mode. Identification and quantification ions were 314 and 316 for CPF, and 290 and 291 for PCB 40. The GC analysis was performed with an Agilent Technologies 6890N Series gas chromatograph equipped with a 30-m long, 0.25-mm internal diameter capillary column (Zebron™ Capillary GC Column, ZB-SemiVolatiles Guardian; Phenomenex, Torrance, CA). Samples were injected by an Agilent Technologies (Santa Clara, CA) 7683 Series autoinjector, with the injection port at

250 °C in splitless mode. Samples were run in splitless mode using helium as a carrier gas (flow = 1 mL min^{-1}). The oven program started with a temperature of 70 °C and increased of 15 °C per min to 280 °C, hold for 2 min.

Procedural blanks were included during analyses, generated and handled in a manner identical to that of water samples, with no CPF detected.

Two different fortification levels were used to validate the analytical procedure, and average recoveries were of 107% for 0.4 µg mL^{-1}, and 96% for 0.004 µg mL^{-1}. The method detection limit was determined as the instrument detection limit of the lowest concentration standard of CPF, which was 3 ng mL^{-1}. In order to test for repeatability in CPF measures, we replicated the analyses of 50 samples and calculated ANOVA-based repeatability (Nakagawa and Schielzeth, 2010), which was highly significant (R = 0.919, F $_{49,50}$ = 23.78, P < 0.001). The operator was unaware of measurements collected on the previous measure. We note that the nine dark sterile microcosms served also as controls of mass change under field conditions. Indeed, neither biodegradation nor photodegradation can occur in them (but hydrolysis can occur, see below) and these samples were kept on the glacier and collected at different time along with the other samples.

4.2.3 Microbiological analyses

Total DNA was extracted from cryoconite using the FastDNA® Spin kit for Soil (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instructions.

To characterize the bacterial community, the V5-V6 hypervariable regions of 16S rRNA gene were sequenced by MiSeq (Illumina Inc., San Diego, CA, USA) with a 2 x 250 bp paired-end protocol as previously reported (Gandolfi et al., 2017).

Different 6-bp barcodes were included at the 5' end of each primer to allow sample pooling and sequence sorting. Groups of 9 amplicons bearing different barcode pairs were pooled together to build libraries. Further library preparation with the addition of standard Nextera indexes (Illumina, Inc., San Diego, CA, USA) and sequencing were carried out at Parco Tecnologico Padano (Lodi, Italy). Sequence analysis was performed as previously reported (Ambrosini et al., 2017).

16S rRNA and *mpd* genes, coding for Methyl Parathion Hydrolases, were quantified with quantitative PCR (qPCR) to estimate, respectively, the total number of bacteria and the putative populations with degradative abilities towards CPF. 16S rRNA gene copies were quantified as previously reported (Gandolfi et al., 2015). No primer pairs were available in literature for the quantification of the *mpd* gene and were therefore designed with the primer design tool from NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer sequences

were: *mpd*F 5'-AGTTCAAGCCTTTCTCGGGG- 3' and *mpd*R 5'-CACTTGGGGTTACGACCGAG- 3', giving a product size of 360 bp. A synthetic 400-bp fragment of *mpd* gene, containing the region of interest, was obtained from Eurofins Genomics and cloned in pGEM®-T Vector System (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Serial dilutions of the plasmid were used to build standard concentration curves for qPCR after measuring the concentration of plasmid DNA with a NanoDrop ND-1000 spectrophotometer (NanoDrop™, Thermo Scientific, Wilmington, DE, USA). Each qPCR reaction was carried out in a total volume of 10 mL using the FluoCycleII Sybr reaction mix (Euroclone, Pero, Italy) with 0.3 µM forward and reverse primer. The amplification was carried out with the Eco Real-Time PCR system (Illumina, San Diego, CA, USA) under the following conditions: initial denaturation at 95 °C for 4 min; 40 cycles at 95 °C for 15 s, 58 °C for 30 s and 72 °C for 40 s, with acquisition of the fluorescence on the FAM channel at the end of each 72 °C elongation step. The standards and the samples were included in triplicate in each run.

4.2.4 Analysis of metagenomic data

Whole metagenome sequences of 6 cryoconite samples on Forni glacier were retrieved from a previous study (Franzetti et al., 2016b) and analysed as follows. Paired-end reads were quality-trimmed (minimum length: 80 bp; minimum average quality score: 30) using

Sickle (https://github.com/najoshi/sickle). Filtered reads were co-assembled using IDBA-UD (Peng et al., 2012). Contigs were binned in putative reconstructed genomes using MaxBin (Wu et al., 2014). The presence of genes related to CPF biodegradation (Chishti et al., 2013) into the reconstructed genome was evaluated as follows. First, sequences of methyl parathion hydrolases (*mpd*), organophosphorus acid anyhydrolase (*opaA*), and organophosphorus hydrolases (*opd*) genes were retrieved from the NCBI-nr database. The reconstructed genomes were annotated with Prokka (Seemann, 2014) using these retrieved sequences as preferential proteins to first annotate from. This resulted in the annotation of 81 predicted genes as *mpd*. However, due to the high similarity of these proteins with β-lactamases, we checked the annotation by aligning the nucleotidic sequence of these predicted genes to the nt database with Blastn and we conservatively onsidered as *mpd* those genes showing at least one *mpd* gene entry from nt database in the first ten best-matching entries. Genome annotation was visualized with CGView (Grant and Stothard, 2008).

4.2.5 Estimation of cryoconite mass in the ablation area and of meltwater discharge

We estimated the cryoconite mass and the surface of the cryoconite holes from remote-sensing data and field investigations. High-resolution images obtained by an unmanned aerial vehicle flight allowed for quantification of the cryoconite coverage: cryoconite holes were detected and mapped by selecting those clustered pixels

with brightness values below a specified grayscale threshold level using *ImageJ* software (Azzoni et al., 2016). This method allows separating the cryoconite holes from sparse debris/dust and bare ice and evaluating the cryoconite surface. We then estimated the total mass of the sediment in the cryoconite holes on the whole ablation area of the Forni Glacier by assuming an average thickness of the sediment layer of 2.5 mm (R. Ambrosini and R.S. Azzoni, unpublished data) and a density of 4.75 g cm^{-3} (Cook et al., 2015). The estimation of the amount of meltwater from the Forni Glacier during 2015 was performed from the data collected from the network of ablation stakes installed on the ablation tongue of the Glacier (Senese et al., 2012a).

4.2.6. Statistical analyses

Analyses were conducted by linear models assuming a Gaussian error distribution on log-transformed contaminant mass. Experimental treatment was included as a four-level factor and time (in days) as a covariate. Since contaminant measures at t_0 were the same for all experimental groups, we constrained regression lines to have a common intercept. Since data were log-transformed before the analyses, slopes indicated first-order decay rates of CPF under each experimental condition. Analyses were followed by post-hoc comparisons of regression slopes. All tests were two-tailed. All the

analyses were performed in R 3.1.2 (R Core Team, 2013) with the lsmeans package.

4.3 Results and discussion

4.3.1 Chlorpyrifos in cryoconite

CFP concentrations were determined in nine cryoconite samples collected at the beginning of the ablating season (early July). CPF concentrations ranged from 2 µg g^{-1} to 3 µg g^{-1} w.w. Very few information about contaminant concentrations (both organic and inorganic) in cryoconite is available (Li et al., 2017; Łokas et al., 2016) and, to the best of our knowledge, data presented in this study are the first experimental evidence of the presence of CPF in this environmental matrix.

4.3.2 Decay rates of chlorpyrifos in cryoconite

Microcosms simulating cryoconite holes were prepared by using cryoconite and water collected on the Forni Glacier. They were spiked with CPF to a concentration of 4 µg per gram of cryoconite, a concentration in the same order of magnitude of the environmental background detected in the same area. Residual mass of CPF in microcosms over time in the four experimental conditions (see Methods for details) is reported in **Fig. 4.1**, while CPF decay rates and half-life in the different conditions, calculated assuming first-order

kinetics (Hui et al., 2010), are reported in **Table 4.1**. Results showed that CPF decay rates differed significantly among the four experimental groups ($F_{3,26}$ = 9.771, P < 0.001) and post-hoc comparisons indicated that decay was significantly faster under light-biotic conditions than under other conditions ($t_{26} \geq 3.055$, P $\leq$ 0.026), which, in turn, did not differ significantly from one another (($|t_{26}| \leq$ 2.044, P $\geq$ 0.198). We stress that the different experimental conditions accounted for different combinations of degradation processes: in light biotic (Lb) conditions, degradation occurred due to hydrolysis, photolysis and biodegradation; under dark biotic (Db) conditions, degradation occurred by hydrolysis and biodegradation; under light sterile (Ls) conditions, degradation occurred by hydrolysis and photolysis; under dark sterile (Ds) conditions, degradation was due to hydrolysis only. We also found that the amount of CPF did not decline significantly in the Ds conditions, while it did in all the other ones, as indicated by the fact that confidence limits of decay rate included zero in the Ds group only (**Table 4.1**). This result suggests that CPF was not degraded by hydrolysis in cryoconite holes. Since CPF can be degraded by hydrolysis at higher temperatures in water environments (European Commission, 2005; Mackay et al., 2014; Williams et al., 2014), we speculate that low temperatures and the presence of suspended solids decreased hydrolysis rates in cryoconite. To estimate decay rates due to other individual processes (e.g. decay rate due to photolysis only), we calculated the differences in decay

rates observed under different experimental conditions (**Table 4.2**). Photodegradation rates were estimated from the difference between Ls and Ds conditions. We estimated a half-life of 115.52 days under photodegradative conditions (**Table 4.2**), much larger than the 29.6 days reported in the Pesticide Properties Database for CPF. On the glacier, microcosms were irradiated by more intense solar radiation (50-184 kW m^{-2} of shortwave radiation, depending on exposure time; data from an Automatic Weather Station on Forni Glacier, see Methods), than in another study where CPF photodegradation was estimated in various types of water (603-729 W m^{-2} in the wavelength range 285-2800 nm) (Muhamad, 2010). Biodegradation rates under dark and light conditions were estimated from the difference between Ds and Db conditions and between Ls and Lb conditions, respectively (**Table 4.2**). The lower decay rate estimated in our study was due to the effect of the lower temperature, since in the other work the photodegradation experiments were carried out at 30 °C (Muhamad, 2010).
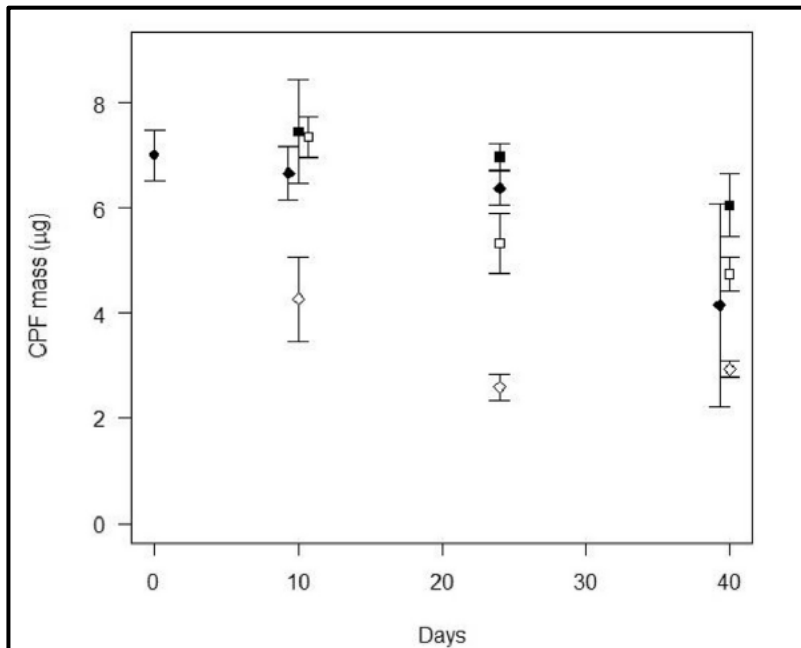
Figure 4.1 Chlorpyrifos mass in microcosm experiments (squares: abiotic controls, diamond: biotic experiments, filled symbols: dark conditions, open symbols: light conditions, dot: time zero). Bars represent standard errors (n = 3). The position of some points on the x-axis are slightly offset to avoid bar overlap.

Biodegradation rates were higher than those of photolysis and hydrolysis (**Table 4.2**), indicating that in cryoconite biological processes largely contributed to the removal of CPF. Comparison between biodegradation rates in dark and light conditions showed no significant difference between them (t_{26} = -1.311, P = 0.201). The significant role of biodegradation in the fate of CPF in a cold environment represents a novel and interesting result. Indeed,

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although it is well known that a wide variety of bacterial and fungal populations are able to metabolize CPF up to a concentration of 1000 mg L^{-1} (Yadav et al., 2016), previous research on CPF biodegradation focused on the ability of isolated bacterial strains or enriched consortia in liquid cultures under mesophilic laboratory conditions (Yadav et al., 2016) and, to the best of our knowledge, no previous study assessed CPF biodegradation at temperatures below 15 °C like those in our microcosms.

Table 4.1 Decay rate of CPF and half-life estimated according to first order kinetics in the four experimental groups

Condition	Decay rate (d^{-1})	SE	95% CL		DT50 (d)
			Lower	Upper	
Dark-sterile	-0.003	0.004	-0.011	0.005	230.05
Light -sterile	-0.010	0.004	-0.019	-0.001	69.31
Dark-biotic	-0.013	0.004	-0.022	-0.004	53.32
Light-biotic	-0.028	0.004	-0.037	-0.019	24.76

Table 4.2 Decay rates due to individual chemical-physical or biological processes estimated as the differences in decay rates observed under different conditions. Ds: dark-sterile conditions; Db: dark-biotic conditions; Ls: light-sterile conditions; Lb: light-biotic conditions.

Degradation process	Groups compared	Decay rate (d^{-1})	SE	DT50 (d)
Hydrolysis	Ds	-0.003	0.004	231.05
Photodegradation	Ls-Ds	-0.006	0.005	115.52
Biodegradation dark	Db-Ds	-0.010	0.005	69.31
Biodegradation light	Lb-Ls	-0.020	0.005	34.66

4.3.3 Microbial populations involved in chlorpyrifos biodegradation in cryoconite

To gain more insight into the biodegradation processes contributing to the removal of CPF, we described the structure of the bacterial communities by high-throughput sequencing (HTS) of 16S rRNA gene amplicons. Particularly, we analysed the cryoconite used for microcosm set up (one t_0 sample; see Methods) and that collected from one bottle from each of the two non-sterile experimental groups at each exposure time. Copy number of 16S rRNA and *mpd* genes (methyl parathion hydrolase, known to be involved in CPF metabolism) (Yadav et al., 2016) were monitored with quantitative

PCR (qPCR) to estimate, respectively, the total number of bacteria and the number of bacteria putatively able to degrade CPF.

Overall, HTS data enumerated 284 Operational Taxonomic Units (OTUs) across all experimental conditions, with the number of OTUs in each sample ranging from 125 to 192. The structure of the bacterial communities based on OTU abundance classified at order level, except for the class *Cyanobacteria*, is reported in **Fig. 4.2**. *Cyanobacteria* was the dominant taxon (>50% of the sequences) in t_0 cryoconite, with *Burkholderiales* and *Sphingobacteriales* accounting for 14.9% and 8.9% of the sequences, respectively. In microcosms, the relative abundance of Cyanobacteria decreased under both light and dark conditions, while *Burkholderiales* and *Sphingobacteriales* increased. Surprisingly, Cyanobacteria were less abundant in light than in dark microcosms. This result is difficult to explain. We can speculate that light microcosm conditions may be less favourable for *Cyanobacteria* than those of natural holes. For example, *Cyanobacteria* abundance is known to decrease with sediment thickness in cryoconite holes, and these bacterial populations can affect cryoconite hole shape, particularly enlarging holes and thus reducing sediment thickness and promoting light penetration in the sediment (Telling et al., 2012; Gokul et al., 2016). Clearly, these processes would be impossible in microcosm bottles. Except for this, bacterial community structures in microcosms were generally similar to those of natural cryoconite of Forni Glacier collected in previous

years (Franzetti et al., 2016a). This suggests that the experimental systems did not strongly affect the bacterial community structure. Temporal changes in microbial community structure observed in microcosms were also similar to those described for natural cryoconite holes during the ablation season on the Forni Glacier (Franzetti et al., 2016a). The absolute number of 16S rRNA copies ranged from 0.6×10^8 to 1.1×10^8, with no qualitative difference between dark and light conditions nor over time. Comparison of bacterial community structures and of the 16S rRNA gene copy abundance between t0 and subsequent samples suggested that spiked CPF did not lead either to a selection of CPF degrading populations or to an increase in bacterial biomass, with no significant differences between dark and light conditions.

Quantitative PCR analyses were performed also to investigate the abundance of *mpd* gene (methyl parathion hydrolase, known to be involved in CPF metabolism) (Yadav et al., 2016), but the *mpd* gene was never detected above the detection limit (10^2 copy number per gram). To better characterize the microbial communities involved in CPF biodegradation, we investigated metagenomics data of natural cryoconite from Forni Glacier collected and analyzed in a previous study (Franzetti et al., 2016b). These data allowed for the reconstruction and annotation of 65 partial bacterial genomes (**Table SI-3.1**), and the detection of genes involved in CPF biodegradation. Among them, the reconstructed genome "bin 6", one of the most

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abundant in the metagenome, with a relative coverage in the Forni metagenome of 6% (**Table SI-3.1**), harbored the *mpd* gene (the complete annotation of bin 6 genome is provided as Supporting Information (**Chapter 9)** whereas the contig sequences are available at http://www.ebi.ac.uk/ena/data/view/PRJEB21726). Bin 6 is phylogenetically related to *Burkholderiales* according to RAST annotation service (Overbeek et al., 2014), and also harbored *puf*ML genes, which code for photosynthetic reaction center L and M subunits and have been used as marker genes for the detection of aerobic anoxygenic phototrophs (AAPs) (Caliz and Casamayor, 2014). AAPs are obligate aerobic bacteria (Koblížek, 2015) which use light to supplement their metabolic demands and organic molecules as carbon source, suggesting that AAPs may be involved in CPF biodegradation. Under light and aerobic conditions, they replace oxidative respiration with photophosphorylation, thus saving carbon, which is used in anabolic reactions for building cell biomass (Caliz and Casamayor, 2014; Cuperová et al., 2013). To the best of our knowledge, studies regarding pollutant degradation by AAPs are not available in the literature, although central and peripheral pathways for aromatic compound metabolism have been described in many members of *Burkolderiales* (Hristova et al., 2007; Mattes et al., 2008; Parnell et al., 2006; Providenti et al., 2006). Conversely, pesticide biodegradation by anoxygenic Purple Phototrophic Bacteria (PPB) has been extensively reported (Idi et al., 2014). PPB are metabolically

versatile and are able to switch between phototrophic, aerobic, and anaerobic modes of metabolism, differing from the obligate aerobic AAPs (Koblížek, 2015). Previous studies showed that PPB are able to biodegrade aromatic compounds very efficiently both in dark/anaerobic (van der Woude et al., 1994), light/anaerobic (Frigaard, 2015) and aerobic conditions (Harwood and Gibson, 1988). Since bin 6 was the only reconstructed genome harboring the *mpd* gene, our findings suggest that AAPs might be involved in CPF biodegradation, although microcosm experiments indicated that biodegradation was not significantly enhanced under phototrophic conditions. It can be speculated that AAPs, being highly versatile in their catabolic abilities, might degrade CPF in dark conditions, similarly to what has been reported for PPB (van der Woude et al., 1994). Annotation revealed that bin 6 also harbored genes coding for carbon monoxide dehydrogenase (*cox*LM), suggesting that this population can also use CO as energy source. CO-oxidizers are a phylogenetically diverse group of bacteria inhabiting a diversity of environments (King and Weber, 2007) that can feed on CO, which might form rapidly from organic carbon (OC) in melting snow exposed to light (Franzetti et al., 2016b; Haan et al., 2001; Xie and Zafiriou, 2009). The bin 6 genome also carries genes involved in biodegradation of aromatic hydrocarbons such as benzoic acid (See Supporting Information, **Chapter 9**).
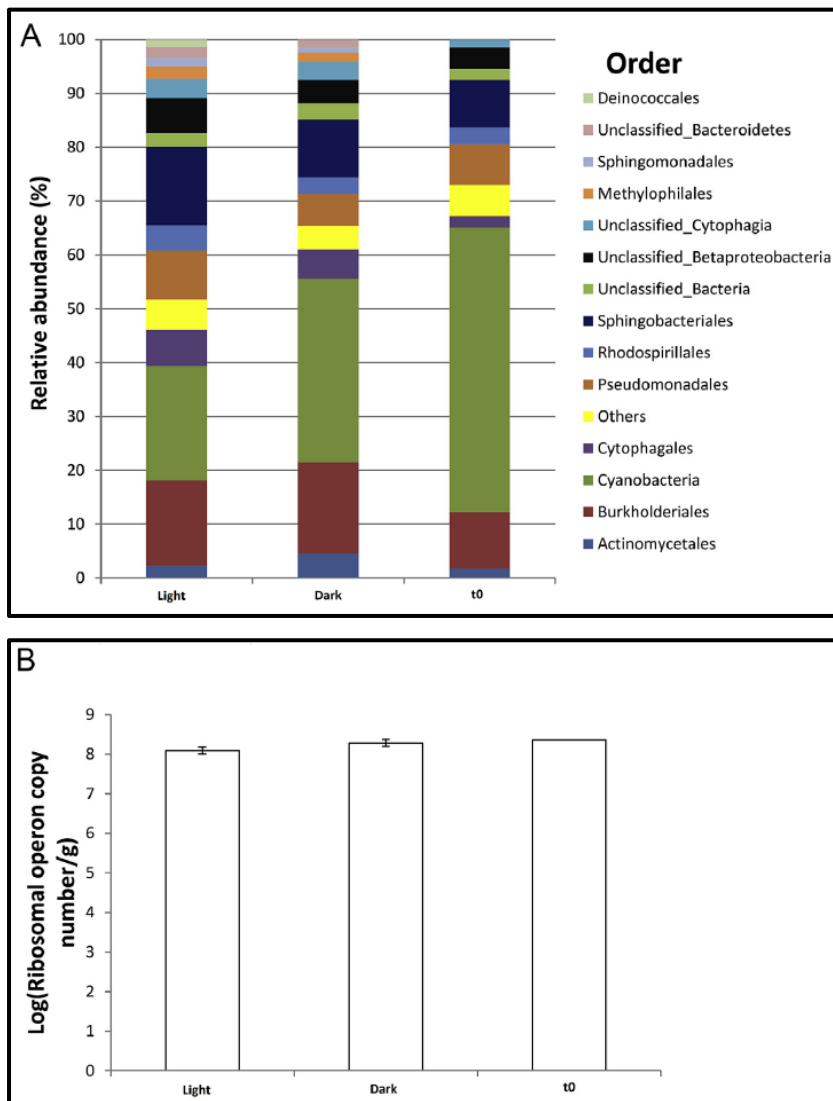
Figure 4.2 Composition of bacterial communities in t_0 cryoconite and microcosms (A), and abundance of 16S rRNA gene copies (B).

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4.3.4 Extent of CPF biodegradation in cryoconite

Our data indicate that biodegradation was the most efficient process
for CPF degradation in cryoconite holes (**Table 4.2**) while the other
post-depositional processes investigated (hydrolysis and photo-
degradation) were less relevant. The environmental relevance of
biodegradation is supported by the fact that the biologically active area
of a glacier might cover about 5% (0.1-10%) of the surface, with 10^{14}
to 10^{17} microbial cells per km^2 (Stibal et al., 2012b). It is reasonable to
suppose that this percentage might increase as a result of the increase
of debris on glacier surfaces that is occurring in recent years (Reid et
al., 2012).

Here we quantified the role of cryoconite in the fate of CPF on the
Forni Glacier. We estimated that the total amount of sediment in
cryoconite holes (not including sparse debris) in the ablation area of
the Forni Glacier (see Methods) is 20 kg. Given a concentration of
CPF in cryoconite at the beginning of the ablation season of 2-3 µg
g^{-1} w.w., we estimated that the total mass of CPF in the cryoconite
should be about 40-60 mg. Since microbial activity can decrease CPF
concentrations by 75% during one melting season (**Table 4.2**), about
30-45 mg of CPF can be biodegraded. The total water discharge from
the ablation area of Forni Glacier during 2015 was estimated at
3,000,000 m^3 (see Methods). The concentration of CPF adsorbed to
suspended particles in meltwater (0.07-0.13 ng L^{-1}) (Ferrario et al.,
2017, **Chapter 3**) translates to a total mass of 200-400 mg of CPF

discharged from the glacier during one ablation season. Hence, the amount of CFP removed from the glacier by biodegradation within cryoconite holes might account for more than 10% of the absorbed CPF released to surrounding freshwaters. This value should be considered as a conservative estimate since we did not account for the potential biodegradation activities in supraglacial sediments outside the cryoconite holes, which might also contribute to CPF biological removal.

Biodegradation in the cryoconite hole ecosystem is therefore a relevant process affecting CPF fate in the cryosphere. Indeed, cryoconite may promote the absorption and subsequent biodegradation of a relevant fraction of pesticides deposited on glacier ablation areas. In addition, in situ biodegradation might significantly limit the accumulation of contaminants transported to glaciers, their reemission to the atmosphere or freshwater, and their impact on surrounding ecosystems (Bizzotto et al., 2009). This is particularly important since cryoconite holes are present on glaciers during the same times of the year when most insecticides are applied in lowland agricultural areas (late spring to late summer) and atmospheric conditions favour their transport to Alpine summits (Ferrario et al., 2017, **Chapter 3**). The extent of biodegradation we documented implies that environmental fate models of pesticides in glacierized areas should account for these processes.

Acknowledgements

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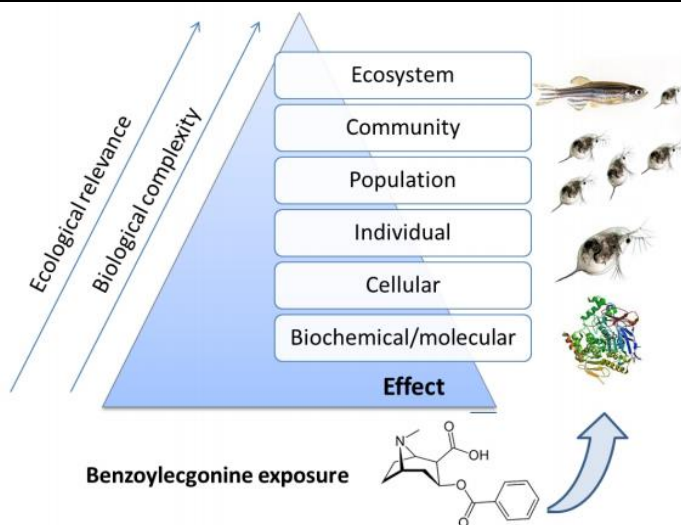
Chapter 5

Benzoylecgonine exposure induced oxidative stress and altered swimming behavior and reproduction in *Daphnia magna*

Parolini M.[1], De Felice B. [1], Ferrario C. [3], Salgueiro-González N. [2], Castiglioni S. [2], Finizio A. [3], Tremolada P. [1]

Graphical abstract

[1] Dept. of Environmental Science and Policy, University of Milano, Milano; [2] IRCCS e Istituto di Ricerche Farmacologiche "Mario Negri", Department of Environmental Health Sciences, Via La Masa 19, I-20156, Milano; [3] Dept. of Earth and Environmental Sciences, University of Milano Bicocca, Piazza della Scienza 1, 20126 Milano.

Abstract

Several monitoring studies have shown that benzoylecgonine (BE) is
the main illicit drug residue commonly measured in the aquatic system
worldwide. Few studies have investigated the potential toxicity of this
molecule towards invertebrate and vertebrate aquatic non-target
organisms focusing on effects at low levels of the biological
organization, but no one has assessed the consequences at higher ones.
Thus, the present study was aimed at investigating the toxicity of a 48
h exposure to two concentrations of BE, similar to those found in
aquatic ecosystems (0.5 µg/L and 1.0 µg/L), on the cladoceran
Daphnia magna at different levels of the ecological hierarchy. We
relied on a multi-level approach focusing on the effects at
biochemical/biomolecular (biomarkers), individual (swimming
activity) and population (reproduction) levels. We measured the
amount of reactive oxygen species and of the activity of antioxidant
(SOD, CAT, and GPx) and detoxifying (GST) enzymes to assess if
BE exposure can alter the oxidative status of *D. magna* specimens,
while the lipid peroxidation (TBARS) was measured as a marker of
oxidative damage. Moreover, we also measured the
acetylcholinesterase (AChE) activity because it is strictly related to
behavioural changes in aquatic organisms. Changes in swimming
behaviour were investigated by a video tracking analysis, while the
consequences on reproduction were assessed by a chronic toxicity test.
Our results showed that BE concentrations similar to those found in

aquatic ecosystems induced oxidative stress and inhibited AChE activity, affecting swimming behaviour and the reproduction of *Daphnia magna* individuals.

Keywords: benzoylecgonine, biomarkers, behavioural effects, chronic toxicity, *Daphnia magna*

5.1 Introduction

Cocaine (COC) is a psychostimulant that affects behaviour and brain physiology by altering dopamine release from dopaminergic neurons (Jeon et al., 2008). Differently from other illicit drugs, COC use declined worldwide as a result of the consumption trends in North America and Europe, but it has been estimated that globally 18.3 million people aged 15-64 is still a cocaine user (UNODC, 2016). However, COC remains the most used illicit stimulant in Europe, and its market accounts for about one half of the global COC market (UNODC, 2016). As consequence of its use, COC and its metabolites are the most abundant illicit drugs found in surface waters (Pal et al., 2013 and references therein). After a dose consumption, COC is metabolized by the liver and excreted through the urine as two main metabolites, the benzoylecgonine (BE, 45% of the swallowed dose) and the ecgonine methyl ester (EME, 40%), while only a limited amount (1-9%) is eliminated unchanged (Baselt, 2004). Considering human metabolism, BE is the main COC-related molecule measured

in freshwater, reaching concentrations up to 7500 ng/L and 3425 ng/L in inlet and outlet of wastewater treatment plants (WWTPs; Pal et al., 2013 and references therein; Mendoza et al., 2014). Since WWTP efficiency in removing BE is incomplete (Zuccato et al., 2008), this molecule enters the surface water, where it was measured at concentrations up to 316 ng/L (Pal et al., 2013 and references therein). Even though the current BE levels in freshwaters are quite low, the risks for the aquatic community cannot be neglected. Because of its pseudo-persistency and molecular activity, BE may exert different adverse effects towards aquatic non-target organisms. For instance, a 14-day exposure to 1 µg/L of BE imbalanced the antioxidant activity and caused oxidative and genetic damage in the zebra mussel (Parolini et al., 2013). Results from a companion study of functional proteomics showed that a 14-day exposure to BE altered the protein profile of gills from the zebra mussel, modulating the expression of proteins involved in diverse functions, including energy and amino acidic metabolism, stress response, and protein biosynthesis (Binelli et al., 2013). Moreover, a redox-proteomics approach showed that BE caused oxidative modifications in different classes of gill proteins involved in cytoskeleton, energetic metabolism and stress response (Pedriali et al., 2014). A recent study showed that the exposure of zebrafish embryos to increasing BE concentrations (0.01 µg/L -10 µg/L range) caused the overproduction of reactive oxygen species (ROS) and altered the gene expression and the activity of antioxidant enzymes, leading to

cytogenetic damage in 96 h post fertilization larvae (Parolini et al., 2017). Lastly, Spasiano et al. (2016) investigated the potential adverse effects induced by BE and its transformation by-products due to UV_{254}/H_2O_2 process in four different model species. BE and its by-products did not affect the growth of *Raphidocelis subcapitata* and the viability of *D. magna* individuals, even if an increase of lipid droplets within the body of cladocerans were noted. Differently, the viability of *Caenorabditis elegans* was seriously influenced by the exposure to both BE and its by-products, while a marked genotoxicity was found in *Vicia faba* individuals, showing an increase of cytogenetic damage during the cell mitosis of primary roots.

All these studies highlighted the potential sub-lethal toxicity of BE towards aquatic non-target organisms and suggested a central role of oxidative stress in the mechanism of action of this molecule. However, they were only focused at biochemical and/or cellular levels of the bio-ecological organization, while no investigations concerning the potential consequences at higher hierarchical levels have been performed. The first effect induced by the exposure to a toxicant appears at the sub-organism level and then it tends to propagate to the higher hierarchical levels of the bio-ecological organization through a bottom-up mechanism. The propagation of that signal can lead to a plethora of adverse effects that can influence the eco-ethological performances of exposed individuals and, consequently, populations. In addition, this effect can propagate at community level, impairing

ecological relationships (e.g. the prey-predator relationship). The investigation on the linkage between responses at different levels of the ecological hierarchy remains a challenge in ecotoxicology (Amiard-Triquet, 2009). Some recent studies of aquatic organisms have related biomarkers endpoints involved in crucial physiological responses with behavioural responses (e.g., Castro et al., 2004; Wallace and Estephan, 2004; Sandahl et al., 2005; Kennedy and Farrell, 2006; Ballesteros et al., 2009; Gravato and Guilhermino, 2009). In fact, behaviour is linked to diverse contaminant-induced stress responses, and alterations in some behavioural endpoints have been associated with biochemical and/or physiological changes (e.g., Weis et al., 2001; Peakall et al., 2002; Moreira et al., 2006; Gravato and Guilhermino, 2009). For instance, the impairment in locomotion has been related to changes of neural, metabolic and endocrine processes in aquatic animals (Baatrup, 2009). Locomotor alterations can induce detrimental consequences also at higher levels of the biological organization causing direct or indirect effects on the population growth rate and changes in the intra- and inter-specific relationships. In spite of these findings, the effect of an illicit drug at different levels of the ecological hierarchy in an aquatic non-target species has never been investigated so far.

The present study was aimed at evaluating the adverse effects induced by the main cocaine metabolite, the benzoylecgonine (BE) at two environmentally relevant concentrations (0.5 µg/L and 1.0 µg/L) in

the cladoceran *Daphnia magna*. We decided to test the toxicity of these concentrations because they both fall in the range of concentrations found in aquatic system worldwide. In detail, the lowest tested concentration was close to the highest BE concentration found in surface waters, while the highest one was similar to the mean concentration of BE measured in the influents of wastewater treatment plants worldwide (see Pal et al., 2013 and references therein). In addition, in our previous studies we assessed the toxicity of the same BE concentrations on the zebra mussel *Dreissena polymorpha* (Parolini et al., 2013) and on zebrafish (*Danio rerio*) embryos (Parolini et al., 2017). BE-induced adverse effects were studied by a multi-level approach at biochemical/biomolecular (biomarkers), individual (swimming activity) and population (reproduction) levels. Regarding biomarkers, we mainly focused on oxidative stress-related endpoints because previous studies, conducted on aquatic organisms treated with BE, showed an overproduction of ROS, the impairment of antioxidant defenses and the occurrence of oxidative damage (Parolini et al., 2013, 2017). Thus, we measured the amount of ROS, the activity of antioxidant (SOD, CAT, and GPx) and detoxifying (GST) enzymes, as well as the lipid peroxidation (TBARS). In addition, we also measured the acetylcholinesterase (AChE) inhibition because it is directly/indirectly involved in crucial functions for the survival, growth and reproduction, in both invertebrate and vertebrate species (Rosenberry, 2006). For instance, contaminant-induced

changes in AChE activity may affects behavioural endpoints related to locomotion and feeding activity in aquatic species, including *D. magna*, which may results in reduced growth and reproduction, as well as in changes of predator avoidance behaviour (e.g., Lovern et al., 2007). At individual level, the swimming activity of *D. magna* was investigated by a video tracking approach, while a chronic toxicity test was performed to assess the potential effects of BE on reproduction. Effects of BE on biomarker and swimming behaviour were investigated in *D. magna* individuals (8-day old at the beginning of the exposure) after a 48 h of exposure, while effects on reproduction were evaluated following the reproductive cycle of single daphnids (younger than 24 h old at the beginning of the exposure) for 21 days. We investigated sub-individual and individual effects *D. magna* specimens after 48 h of exposure because we would like to evaluate the capability of BE to induce oxidative stress, to modulate AChE activity and to alter the swimming behaviour by excluding any potential confounding effects of reproduction, which were then investigated by a standard 21-d reproduction test (OECD, 2004). Our choice was also due to experimental constraints because for video-tracking analyses we had to use 8-day old *D. magna* individuals, which were sufficiently large to be recorded and their movements tracked. So, to avoid effects of reproduction we could not expose *D. magna* specimens more than 48 h because after the tenth day of life the most of individuals begins parthenogenic reproduction. Our multi-

level approach allowed to investigate and to follow the propagation of BE-induced effects at different levels of the ecological hierarchy, as well as to interpret these effects on individuals in a broader ecological context.

5.2 Material and methods

The analytical standards of benzoylecgonine (BE) and benzoylecgonine-d3 (BE-d3) were purchased from Cerilliant Corporation (Round Rock, Texas, USA) as liquid solutions in methanol. Methanol for pesticide analysis, and hydrochloric acid (37%) were from Carlo Erba (Italy); ammonium hydroxide solution (25%) and acetic acid for LC-MS (>99%) were obtained from Fluka (Buchs, Switzerland). Acetonitrile for LC-MS was purchased from Riedel de Haen (Seelze, Germany). A MILLI-RO PLUS 90 apparatus (Millipore, Molsheim, France) was used to obtain the HPLC grade Milli-Q water used throughout the study. The cartridges employed for solid phase extraction were 3-mL disposable Oasis MCX (60 mg) from Waters Corp. (Milford, MA, USA). The chromatographic separation was performed using an Atlantis T3 column (2.1 x 150 mm, 3 µm) from Waters Corp. (Milford, MA, USA). All the reagents used for biomarker analyses were purchased from Sigma-Aldrich (Steinheim, Germany).

5.2.1 Experimental plan

Adult *Daphnia magna* specimens came from a single clone obtained from the Istituto Superiore di Sanità (Roma, Italy) and they were cultured (30 individuals/L) in a commercial mineral water (San Benedetto® - conductivity 415 µS cm^{-1} at 20 °C; pH 7.42; 301 mg/L HCO$_3$., 48.6 mg/L Ca^{2+}; 28.2 mg/L Mg^{2+}). Daphnids were cultured in 400 mL beakers (40 individuals/L) and fed *ad libitum* with a suspension of the unicellular green alga *Pseudokirchneriella subcapitata* (8 x 10^6 cells ind^{-1} day^{-1} until they were 8-day old, then 16 x 10^6 cells ind^{-1} day^{-1}) and the yeast *Saccharomyces cerevisiae* (15 x 10^6 cells mL^{-1}) three times a week. The culture medium was renewed every second day. Culture medium and exposure solutions were maintained at 20.0 ± 0.5 °C under a 16 h light: 8 h dark photoperiod, which are conditions ensuring continuous amictic parthenogenetic reproduction (Frey, 1982). Algae were cultured in ISO 8692:1989 medium in 2 L flask at 20.0 ± 2.0 °C under continuous light and shaken through aeration. Algae were harvested during their exponential growth and let for sedimentation in the dark at 4 °C for a week, supernatant was discharged and cell density was measured using a Burker chamber under a brightfield light microscope. For BE exposures we planned two experiments: a short-term BE exposure (48 h) and a 21 day-exposure (*D. magna* chronic test). Short-term exposure was performed using 8-day old individuals from the fourth reproduction cycle because they reached the minimum dimension

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allowing the video tracking of their swimming activity (personal observation). Exposures were performed in 200 mL beaker under semi-static conditions renewing BE solution (1 mg/L in ultrapure water) after 24 h from the beginning of the experiment. To confirm the effectiveness of the exposures, we collected a water sample from both control and exposure beakers 1 h after the spiking of BE and after 24-h of exposure. Twenty 8-day old individuals were transferred into each beaker and exposed for 48-h to two concentrations of BE (0.5 µg/L and 1.0 µg/L). Negative control beaker containing only culture water without chemical was included in all experimental replicate. Individuals were not fed during the experiments. We performed three independent experimental replicates per treatment to assess oxidative stress-related endpoints, and AChE activity. At the end of 48-h exposures individuals were transferred to a 1.5 mL Eppendorf tube, frozen in liquid nitrogen and stored at e 80 °C until the biochemical analyses.

5.2.2 Chemical analysis of BE in water

The chemical analysis of water samples to check BE concentrations was carried out by solid phase extraction (SPE) followed by high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) adapting a methodology previously published (Castiglioni et al., 2011). Aliquots of 5 mL for control samples, samples spiked at 0.5 µg/L and samples spiked at 1.0 µg/L were

extracted using mixed reverse-phase cation exchange cartridges (Oasis-MCX). Before extraction, the pH of each aliquot was adjusted to 2.0 with 37% HCl, and 2 ng of the labeled deuterated analog (benzoylecgonine-d3) was added to be used as internal standard. MCX cartridges were conditioned before use by washing with 5 mL methanol, 3 mL Milli-Q water, and 3 mL water acidified to pH 2. Samples were then passed through the cartridges at a flow rate of 5 mL/min. Cartridges were vacuum-dried for 10 min and eluted with 2 mL of methanol and 2 mL of a 2% ammonia solution in methanol. The eluates were pooled and dried under a gentle nitrogen stream. Dried samples were redissolved in 100 µL of Milli-Q water, centrifuged for 2 min at 2500 rpm, and transferred into glass vials for HPLC injection. HPLC-MS/MS determination was performed using a 1200 Series Binary Pump and Autosampler (Agilent Technologies, Santa Clara, CA, USA) coupled to a mass spectrometer with a triple quadrupole detector and a turbo ion spray source (API 5500, Applied Biosystemse-Sciex, Thornhill, Ontario, Canada). The chromatographic separation was performed by gradient elution using 0.1% acetic acid in Milli-Q water as solvent A and acetonitrile as solvent B at a flow rate of 200 µL/min. The analysis started with 99% of eluent A for 3 min, followed by a 20-min linear gradient to 60% of eluent B and a 1-min linear gradient to 100% of eluent B, which was maintained for 3 min. The initial conditions (99% of eluent A) were then achieved in 0.5 min and were maintained for 8 min to equilibrate

the column. The injection volume was 4 µL and the column was kept at room temperature. The MS analysis was done in the positive ion mode with a spray voltage of +5.5 kV and a source temperature of 400 °C. The Multiple Reaction Monitoring (MRM) mode was used for analysis, choosing the 2 most abundant fragmentation products of the protonated pseudo molecular ions of benzoylecgonine and its deuterated analog (benzoylecgonine-d3). Quantitation of BE was performed using the isotopic dilution method and calibration curves were made freshly before each analytical run. The method quantitation limit (MQL) was calculated as the concentration at which the signal-to-noise ratio was 10 and it was 0.8 ng/L.

5.2.3 Biomarker methods

The biomarker suite applied in the present study was performed on homogenates from a pool of all living *D. magna* individuals found in each beaker at the end of the exposure (0.5 µg/L and 1.0 µg /L BE, and negative control). Three independent experimental replicates (n = 15 individuals per each single replicate) were performed for each treatment. As it cannot be excluded that BE was removed from the outer carapax, *D. magna* individuals were washed trice before biochemical analyses with 0.5 mL of homogenization buffer to prevent potential bias caused by *in vitro* interactions. After the washes, individuals were homogenized using a motor pestle in a 100 mM potassium phosphate buffer (added with KCl 100 mM, EDTA 1 mM,

protease inhibitors 1:100 v/v and dithiothreitol 1 mM, pH 7.4). The homogenates were centrifuged at 15,000 x g for 15 min at 4 °C, then the supernatant was collected and immediately processed to determine protein content, SOD, CAT, GPx, GST and AChE activity through spectrophotometric methods. All the enzymatic activities were measure in triplicate per each pool. Briefly, SOD activity was assessed by measuring the inhibition of cytochrome c (10 µM) reduction by the superoxide anion generated by the xanthine oxidase (1.87 mU/mL)/hypoxanthine (50 µM) reaction for 1 min at $\Lambda = 550$ nm. Results were expressed as SOD units (1 SOD unit = 50% inhibition of the xanthine oxidase reaction). The CAT activity was assessed by measuring the consumption of H_2O_2 (50 mM) in potassium phosphate buffer (100 mM; pH 7) for 1 min at $\Lambda = 240$ nm. The GPx activity was assessed monitoring for 1 min the consumption of NADPH at $\Lambda = 340$ nm using H_2O_2 (0.2 mM) as substrate in potassium phosphate buffer (50 mM; pH 7) including glutathione (2 mM), sodium azide (1 mM), glutathione reductase (2 U/mL), and NADPH (120 µM). The GST activity was measured by adding reduced glutathione (1 mM) in phosphate buffer (100 mM; pH 7.4) and using CDNB (1 mM) as substrate. The reaction was monitored for method described by Jemec et al. (2007), with slight modifications. The reaction mixture (1.5 mL) was prepared in potassium phosphate buffer (100 mM, pH 7.4) containing acetylthiocholine chloride (1 mM) and 5,5' dithiobis-2-nitrobenzoic acid (0.5 mM), then 100 µL of supernatant was added.

The reaction was monitored for 15 min at $ʎ = 412$ nm and AChE activity was expressed as nmoles of acetylcholine chloride hydrolyzed min^{-1} mg $protein^{-1}$ ($ε = 13,600$ M^{-1} cm^{-1}). The activity of enzymes was normalized on protein concentration measured according to the Bradford method, using bovine serum albumin as a standard. ROS measurement was performed using dichlorofluorescein-diacetate (DCFH-DA), adapting the fluorescence method by Deng and co-workers (Deng et al., 2009). *D. magna* individuals (n = 15 per experimental replicate) were washed twice with homogenization buffer and homogenized in a 100 mM potassium phosphate buffer (see above). The homogenate was then centrifuged for 20 min at 15,000 x g at 4 °C. Twenty microliters of the homogenate was added to a 96-well plate and incubated for 5 min at room temperature. Then, 100 µL of PBS and 8.3 µL of DCFH-DA (10 mg/mL in DMSO) were added to each well and the plate was incubated at 37 °C for 30 min. The fluorescence intensity was measured by a microplate reader with excitation at $ʎ = 485$ and emission at $ʎ = 536$ nm, respectively. The ROS concentration was expressed in arbitrary units as AU DCF mg $protein^{-1}$. Lipid peroxidation was assessed by the thiobarbituric acid reactive substances (TBARS) method (Ohkawa et al., 1979), adapted to tissue homogenates of 8-day old individuals and expressed as nmol TBARS mg $protein^{-1}$.

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5.2.4 Video tracking and swimming activity analysis

We performed three independent replicates per treatment to assess changes in swimming activity induced by the exposure to the BE selected concentrations. Video tracking analyses were performed on all living individuals at the end of BE exposure into 24-well plates and tracked individually (each well contained 1 specimens and 3 mL of exposure medium). Video recordings were carried out by placing the 24-well plate (well dimension 25 mm x 25 mm x 10 mm) with 12-15 animals on a light panel, and the movement of each individual was tracked for 15 s for three times. We tracked the movement of at least 40 individuals per treatment. The three 1080p Full HD videos acquired for each specimens were analyzed using the software LoliTrack v.4 (Loligo Systems, Tjele, Denmark). This software was calibrated to measure the following endpoints: time of activity (%) and swimming velocity (mm/s). The tracking was based on differences in contrast between objects (animals) and background (water) without use of markers. When the object appeared against a contrasting background, the software assigned a coordinate pair (x, y) to the centroid of the contrasting object. Each well in the 24-well plates was defined as an arena, and each individual was considered as a single object. According to manufacturer manual, the lowest threshold for activity is defined as activity threshold (pixels; e.g. if the object moves a distance larger than this minimum distance between frames, the

object is scored as active). If the object is active, the speeds of movement(s) are calculated frame by frame.

5.2.5 Chronic toxicity test

Chronic toxicity evaluation at the two BE concentrations (0.5 µg/L and 1.0 µg/L) was performed in agreement with the standard 21 days chronic reproduction test (OECD, 2004). For each treatment group, 10 replicates of 1 specimens (<24 h old) were used. The exposures were conducted in glass beakers containing 50 mL of test medium. The exposure was performed at 20.5 ± 0.5 °C under a 16 h light: 8 h dark photoperiod. *D. magna* individuals were transferred every single day to clean glass beakers filled with freshly prepared medium and fed with *P. subcapitata* and *S. cerevisiae* (see above), to which was then added the exact amount on BE to reach the selected concentrations. Every day, the number of living, immobile or dead offspring were recorded, until the 21st day.

5.2.6 Statistical analysis

The effect of BE exposure on the amount of ROS, enzyme activity and swimming behaviour of 8-day old *D. magna* individuals was investigated by using linear mixed models (LMM) including the treatment as fixed effect factor and the exposure tank as random effect. When a significant effect of treatment was found, a Fisher LSD post-hoc test was applied to point out significant differences among

treatments. Significance was set at $p < 0.05$ (*) and $p < 0.01$ (**). Statistical analyses were performed using IBM SPSS Statistics 21.0 software package.

5.3 Results

5.3.1 Concentration of BE in water and immobilization/ mortality events

To check the reliability of the exposure we measured the concentration of BE in water from both control and exposure beakers. No residues of BE were found in control beakers, whereas no variation in BE concentration occurred neither in 0.5 µg/L nor in 1.0 µg/L treatment over the 24-h exposure. In detail, the mean (±standard deviation) BE concentration 1 h after the spike in water was 501.38 ± 12.86 ng/L (0.5 ± 0.01 µg/L) and 974.36 ± 123.59 ng/L (0.9 ± 0.1 µg/L), while after 24 h of exposure it was 501.14 ± 20.14 ng/L (0.5 ± 0.02 µg/L) and 944.47 ± 72.39 ng/L (0.9 ± 0.1 µg/L) for 0.5 µg/L and 1.0 µg/L treatment, respectively.

Immobilization/mortality at the end of the short-term toxicity test (48 h) was below 10% in all treatments (0.5 µg/L, 1.0 µg/L, and control) and for every replicates. Considering that 10% of immobilization/mortality is accepted in the control (OECD, 2004) for the standard *D. magna* acute toxicity test, we concluded that tested concentration were below acute toxicity range. Mortality (mean of all the independent replicates we performed in the present study; $n = 14$

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replicates per treatment for a total of 210 individuals per treatment) of *D. magna* individuals found at the end of the 48-h exposure was 4.3% in control, 6.7% and 3.9% in 0.5 and 1.0 µg/L beakers, respectively. No significant difference in *D. magna* individuals' mortality among treatments was found ($p > 0.05$).

5.3.2 Biomarker results

BE exposure induced a significant ($F = 6.030$; $p < 0.01$) increase of ROS in response to the highest tested concentration, with a 13% increase with respect to control (**Fig. 5.1a**). In spite of no significant effect of BE treatment on SOD ($F = 0.330$; $p > 0.05$) and CAT ($F = 0.877$; $p > 0.05$) activity (**Fig. 5.1b and d**), a significant increase of GPx ($F = 4.172$; $p < 0.05$) was noted at the end of the exposure to the highest BE concentration, showing a 68% increase compared to control (**Fig. 5.1c**). A significant ($F = 7.505$; $p < 0.01$) increase in GST activity was found at both the BE tested concentrations, with a 80% and 46% increase found at 0.5 µg/L and 1.0 µg/L with respect to control, respectively (**Fig. 5.1e**). A significant increase of lipid peroxidation ($F = 10.442$; $p < 0.01$) was found after the exposure to the highest BE concentration, with values 2-fold higher than the control (**Fig. 5.1f**). BE exposure had a significant effect ($F = 35.497$; $p < 0.01$) on AChE activity of *D. magna* individuals, showing a significant inhibition (-36%) at the end of the exposure to both the BE tested concentrations (**Fig. 5.2**).
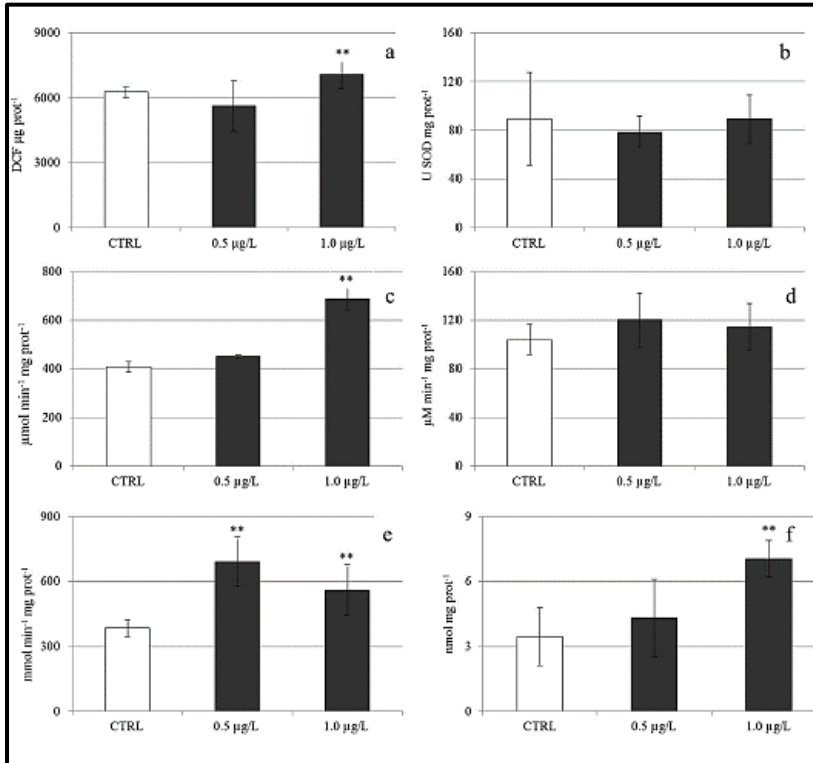
Figure 5.1 Mean (±SD) of the amount of reactive oxygen species (ROS; a)
SOD (b), GPx (c), CAT (d), GST (e) and lipid peroxidation (f) measured in
D. magna specimens after 48-h treatment with 0.5 µg/L and 1.0 µg/L of
BE. Asterisks above the histograms show significant differences between
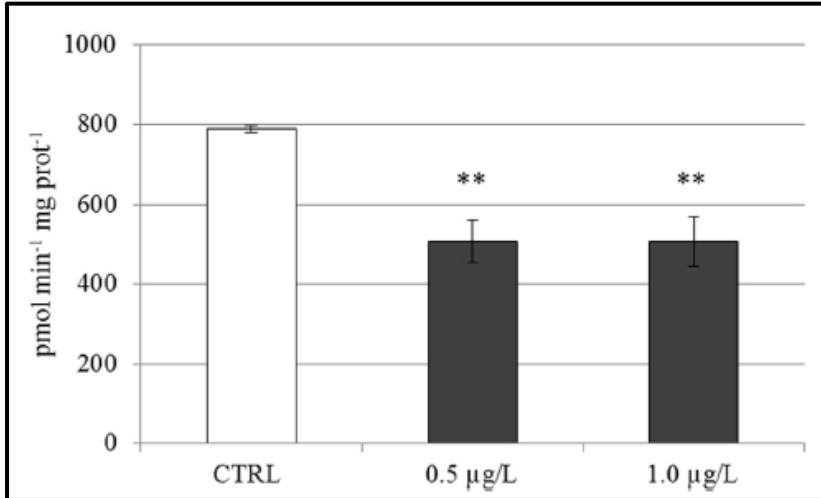treated and control specimens (*$p < 0.05$; **$p < 0.01$).

Figure 5.2 Mean (±SD) acetylcholinesterase (AChE) activity measured in *D. magna* specimens after 48-h treatment with 0.5 µg/L and 1.0 µg/L of BE. Asterisks above the histograms show significant differences between treated and control specimens (*$p < 0.05$; **$p < 0.01$).

5.3.3 Swimming activity results

BE exposure caused a significant ($F = 18.041$; $p < 0.01$) reduction in the activity of *D. magna* individuals, showing a 5% decrease in treated specimens at the highest tested concentration compared to the control (**Fig. 5.3a**). In contrast, the same treatment induced a significant increase of the swimming velocity ($F = 38.984$; $p < 0.01$) of treated specimens with respect to control (**Fig. 5.3b**).
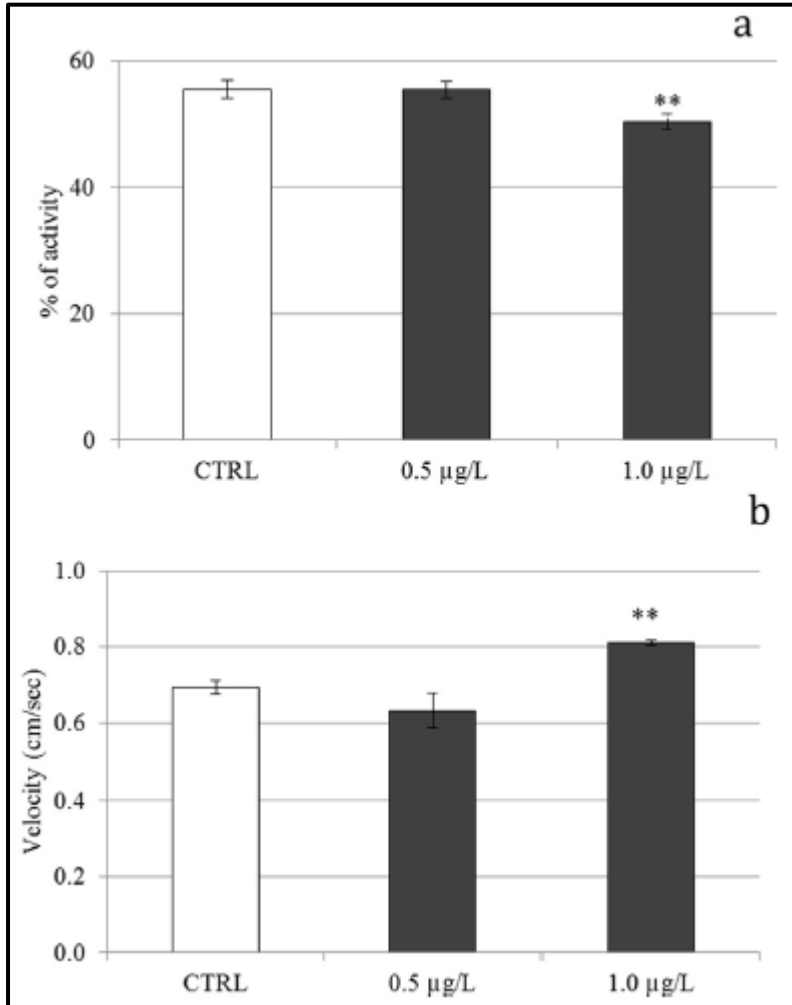
Fig. 5.3 Mean (±SD) swimming activity (a) and velocity (b) measured in *D. magna* specimens after 48-h treatment with 0.5 µg/L and 1.0 µg/L of BE. Asterisks above the histograms show significant differences between treated and control specimens (*p < 0.05; **p < 0.01).

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5.3.4 Chronic toxicity test results

A marginally significant effect of BE treatment (F = 3.635; p = 0.049) on the total number of offspring was noted, with a decrease of 43% and 39% caused by the exposure to 0.5 µg/L and 1.0 µg/L compared to control (**Fig. 5.4a**). Accordingly, a significant reduction of the number of parthenogenetic cycles (F = 4.533; p < 0.05) was found, showing that reproductive events of specimens treated with 0.5 µg/L and 1.0 µg/L of BE were 45% and 21% lower compared to the control (**Fig. 5.4b**). In contrast, no significant effects of BE treatments on the mean number of offspring per reproductive cycle (F = 0.211; p > 0.05) and on the mean number of days to the first reproduction event from the beginning of the experiment (F = 0.454; p > 0.05) was found (data not shown).
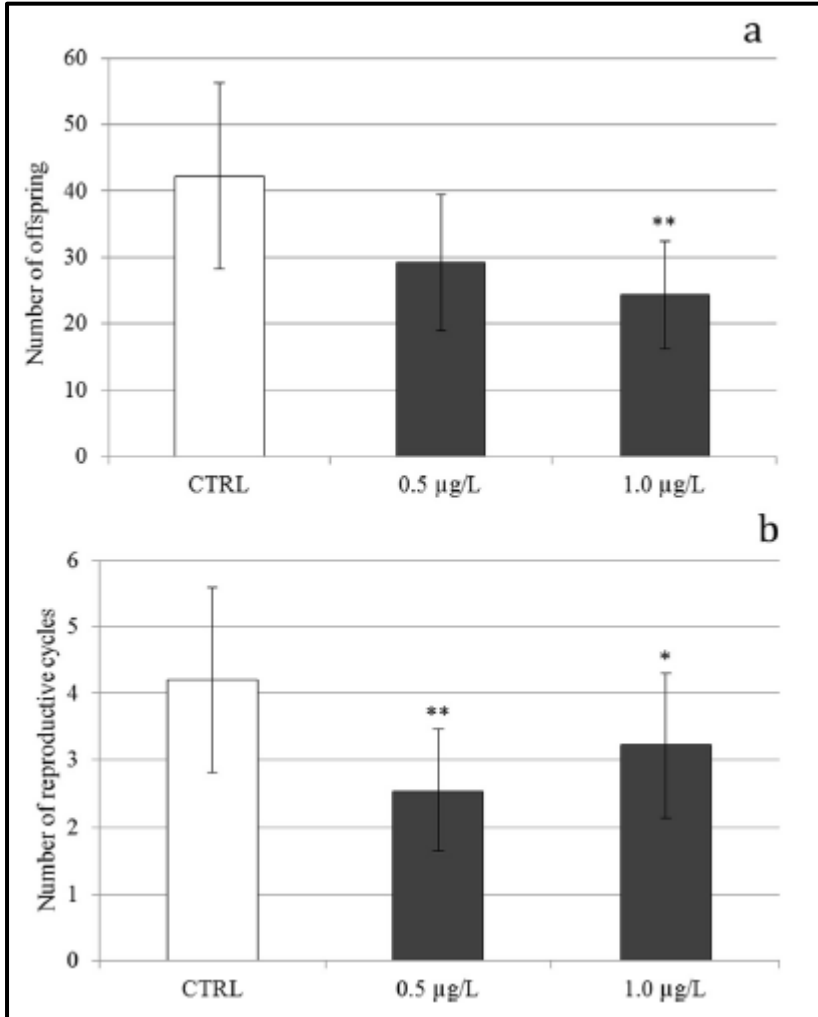
Figure 5.4 Mean number (±SD) of offspring (a) and parthenogenetic cycles
(b) of *D. magna* specimens after 21-day exposure to 0.5 µg/L and 1.0 µg/L
of BE. Asterisks above the histograms show significant differences between
treated and control specimens (*p < 0.05; **p < 0.01).

5.4 Discussion

A number of studies showed that cocaine causes damage on structure and function of diverse organs due to dissimilar mechanisms of actions. For instance, some detrimental effects are caused by the overstimulation of the adrenergic system, while the most of the direct toxic effects are promoted by oxidative stress and mitochondrial dysfunction occurring during metabolism (Riezzo et al., 2012). However, the toxicity of cocaine involves also its main metabolites, such as the benzoylecgonine. Some studies pointed out the bioactivity of BE, showing that this molecule can induce both physiological and behavioural effects on murine models (Morishima et al., 2001), but also on terrestrial and aquatic non-target organisms (Parolini et al., 2013, 2017; García-Cambero et al., 2015). Oxidative stress seems to be involved in the mechanism of action of BE, as demonstrated by the increased production of reactive oxygen (ROS) species in peritoneal macrophages isolated from treated mice, which can cause hepatic and cerebral toxicity (Vaz et al., 1994), as well as in the zebra mussel (Parolini et al., 2013; Pedriali et al., 2014) and in zebrafish embryos (Parolini et al., 2017). In addition, some studies showed that BE is neurotoxic for murine models (Nassogne et al., 1998; Bunney et al., 2001). Results from the present study showed that the exposure to two concentrations of BE similar to those found in the aquatic system worldwide altered the oxidative status and inhibited *Daphnia magna* AChE activity, affecting swimming behaviour and reproduction.

Although no increase of ROS was caused by the exposure to the lowest BE tested concentration, the highest one promoted a significant ROS overproduction (**Fig. 5.1a**), in accordance with a previous study performed on zebrafish embryos exposed up to 96 h post fertilization to similar BE concentrations (Parolini et al., 2017). Cocaine metabolites, including BE, may be involved in the activation of redox cycles, the depletion and/or the decrease of antioxidant enzymes, and the consequent overproduction of ROS, leading to oxidative stress situations, even if the exact mechanisms of toxicity are not fully understood (Neri et al., 2013). Accordingly to ROS measurements, no significant effects of BE on enzyme activity was found at the end of the exposure in 0.5 µg/L BE-treated individuals compared controls. However, the BE-induced increase of ROS levels due to 1.0 µg/L exposure modulated the antioxidant enzyme activity in treated *D. magna* specimens with respect to control. The lack of increase in SOD activity (**Fig. 5.1b**) suggests that both BE concentrations did not cause and overproduction of superoxide anion, and consequently of hydrogen peroxide, the final product of $O_2^{\bullet-}$ dismutation. However, the significant induction of GPx after 1.0 µg/L BE treatment (**Fig. 5.1d**) indicated that BE promoted the production of hydrogen peroxide, which could be produced by other cellular enzymes like those contained in peroxisomes (Khessiba and Roméo, 2005). In contrast, no significant effect of BE treatments on CAT activity was noted (**Fig. 5.1c**). Although GPx and CAT play a concomitant role to counteract

the toxicity of hydrogen peroxide, the discrepancy between their responses could be due to their competition for the same substrate (i.e. H_2O_2; Kappus, 1985) or, alternatively, to the activation of CAT exclusively when the concentration of H_2O_2 is extremely high (Pereira et al., 2013). These results are consistent with those found in our previous study performed on zebrafish embryos at 96 h post fertilization, in which concentrations of BE similar to those we tested here caused and induction of GPx but not of CAT (Parolini et al., 2017). In contrast, a 21-day exposure to the same BE treatments showed an opposed response in the zebra mussel *Dreissena polymorpha*, depending on the tested concentration. Whilst 0.5 µg/L of BE caused a significant increase of SOD, GPx and CAT activity, the exposure to 1.0 µg/L determined a significant inhibition of the three antioxidant enzymes (Parolini et al., 2013). The contrasting results occurring among biological models exposed to the same BE treatments may be related to the sensitivity to this cocaine metabolite at different developmental stage and/or to the duration of the exposure. In fact, some studies have demonstrated that early-life stages are more sensitive than adults and show an early response to the exposure to environmental pollutants. Other studies showed that the activity of antioxidant enzymes can increase when the organism is exposed to low concentrations of chemical or during short-term exposures, but it can decrease or be inhibited at high concentration or after prolonged exposure (Valavanidis et al., 2006;Wang et al., 2011).

Lastly, the induction of GST found after the exposure to both the BE concentrations suggests the involvement of phase II detoxification enzymes in the metabolism of BE in *D. magna*. Overall, the increase of ROS coupled with the impairment of antioxidant defences showed in individuals treated with 1.0 µg/L of BE may suggest an imbalance of the equilibrium between pro- and antioxidant molecules in favor to the former, leading to an oxidative stress situation that can negatively affect *D. magna* health status. In fact, when an organism undergoes oxidative stress, it can experience detrimental oxidative damage to cellular macromolecules, including lipids, proteins and DNA may occur, resulting in alteration of their structure and functionality, disruption of cellular activity and organ damage. The increase of lipid peroxidation in *D. magna* treated with the highest BE concentration (**Fig. 5.1f**) supported our hypothesis regarding the oxidative stress situation experienced by *D. magna* individuals because it suggested that ROS were not totally scavenged by the antioxidant enzymes and promoted oxidative damage to lipids. These results are in accordance with those found in a previous study where zebra mussel specimens were exposed for 14 days to the same BE concentration (Parolini et al., 2013). In addition, a number of studies have demonstrated that oxidative stress plays a crucial role in both the regulation and activity of AChE. For instance, an *in-vitro* study by Schallreuter et al. (2004) showed that low hydrogen peroxide activated human recombinant AChE, while high concentrations inhibited the enzyme activity.

Oxidative stress changed AChE activity in vivo during hypertension
(De Carvalho Corrêa et al., 2008), while ROS production due to
ethanol exposure alters the expression and the activity of AChE (Rico
et al., 2007). Hydrogen peroxide also inhibited AChE activity in
human erythrocyte membrane (Molochkina et al., 2005) and skin cells
(Schallreuter and Elwary, 2007). In addition, reduced expression and
activity of AChE were related to an increase of oxidative stress in
zebrafish embryos treated with the pro-oxidant molecule t-butyl
hydroperoxide (Rodríguez-Fuentes et al., 2015). A significant
inhibition of AChE was found in *D. magna* exposed to both BE
concentrations (**Fig. 5.2**). Since no evidence for direct action of BE on
AChE expression and/or activity was found in any species and the
amount of ROS was not increased by the exposure to 0.5 µg/L of BE,
the significant AChE inhibition at the lowest tested concentration was
unexpected and we do not have any reasonable explanation to this
result. In contrast, the ROS overproduction induced by the highest
treatment of BE may affect the activity of this crucial enzyme that
hydrolyzes the neurotransmitter acetylcholine in cholinergic synapses
allowing the effective control and modulation of the neural
transmission in both vertebrates and invertebrates (Oliveira et al.,
2012). By a functional point of view, a number of studies showed that
the decrease and/or the inhibition of AChE activity altered diverse
behavioural endpoints, which may subsequently affect fitness and
survival of the exposed organisms (Beauvais et al., 2000; Castro et al.,

2004; Cooper and Bidwell, 2006; Sismeiro-Vivas et al., 2007). For instance, the exposure to diverse contaminants can alter the swimming and filtering activity of *D. magna*; indeed, some studies showed that the swimming activity was reduced by the exposure to heavy metals or organic pollutants in diverse aquatic organisms (e.g. Little and Finger, 1990; Kavitha and Venkateswara Rao, 2007, 2008), including *D. magna* (e.g., Dodson et al., 1995; Baillieul and Blust, 1999; Untersteiner et al., 2003; Cerbin et al., 2010). Whilst, in accordance to biomarker results, no effects on the parameters chosen to assess effects of BE on locomotor activity were induced by the exposure to the lowest tested BE concentration, individuals treated with 1.0 µg/L of BE showed significant changes in both the considered endpoints (**Fig. 5.3**). In fact, in spite of a significant decrease of swimming activity caused by the highest BE treatment (**Fig. 5.3a**), an unexpected significant increase of swimming velocity (**Fig. 5.3b**) was noted. These findings are surprising because usually both the parameters are strictly correlated in *D. magna* (Untersteiner et al., 2003). In addition, many studies showed that swimming velocity is reduced in response to the exposure to some toxic chemicals (e.g., Baillieul and Blust, 1999; Untersteiner et al., 2003). This discrepancy may be related to the different mechanism of action occurring between diverse toxic molecules. Although the exposure to 1.0 µg/L BE decreased the total swimming activity of *D. magna* individuals, this molecule could promote high velocity jerky movements, which can results in an

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overall increase of individuals' velocity. This hypothesis was supported by previous studies on young rats, which showed seizures (Erickson et al., 1990), behavioural activation characterized by jumping, jerking and various degrees of tonic seizures after direct intraventricular injection of BE (Konkol et al., 1992a,b). Since the locomotion of *D. magna* depends on a continuative, high energy demanding, muscular activity, the decrease of swimming activity under BE exposure may be due to the high energy demand of the organism to support essential physiological processes to counteract the toxicity of the chemical. Thus, as swimming behaviour derives from the integration of physiological, sensorial, nervous and muscular systems (Charoy et al., 1995), our results should indicate a general impairment of the health status of BE-treated *D. magna*, which could lead to adverse effects on fitness and survival of the organism. The reduced performance in swimming we found could negatively affect the filtering activity and, consequently, the food uptake of treated individuals, which can lead to a drastic reduction in growth and reproduction (Baillieul, 1997), being food uptake one of the main driving forces of the latter (Enserink et al., 1993). According to this expectation, the exposure to the highest BE concentration reduced the number of parthenogenetic cycles and the total number of offspring (**Fig. 5.4a** and **b**, respectively). In addition, changes in locomotor activity may affect prey-predator relationship, causing a potential alteration to the trophic interactions occurring between phyto- and

zooplankton, as well as between zooplankton and fish (Uttieri et al., 2014). *D. magna* is one of the most important phytoplankton grazers in freshwater systems, thus changes in population dynamics of this cladoceran could result in serious consequences also on phytoplankton species. At the same time, *D. magna* represents one of the major dietary components of diverse fish species (Dodson et al., 1995) and its swimming behaviour is a pivotal component of prey selection and predator avoidance (Schmidt et al., 2005). Some studies showed that a reduced movement of the zooplankton could diminish the ability of the predator to locate its prey, decreasing the risk of predation (Zaret, 1980), while irregular movements may increase visibility to predators and predation risk (Strickler et al., 2005). For instance, Brewer and Coughlin (1996) showed that virtual *D. magna* with a higher hopping frequency were more vulnerable to attack by a predator such as the bluegill *Lepomis macrochirus*. Similar results were obtained by O'Keefe et al. (1998), which reported that faster swimming *D. magna* individuals were preferentially predated by the bluegill. Thus, the contrasting alteration of the considered swimming parameters suggests that exposure to BE may positively or negatively affect the predation risk of *D. magna* specimens by a fish predator. Due to the complexity of the obtained results and the ecological relevance of this issue, further study should be necessary to understand the role of BE in altering the ecological relationships between aquatic species.

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5.5 Conclusions

Our findings showed that the exposure to environmentally relevant concentrations of BE may induce notable adverse effects to *Daphnia magna* specimens at different level of biological organization. The exposure to $1.0 \, \mu g/L$ of BE induced an oxidative stress situation in *D. magna*, leading to behavioural and reproductive effects. The effects on *D. magna* reproduction may result particularly worrisome because they can negatively affect the population dynamic of this cladoceran species and, consequently, food web interactions. In addition, these results highlighted the linkage between biochemical, behavioural and reproductive endpoints, confirming the potential of biomarker techniques as early predictors of toxicant-induced alterations also at higher hierarchical level. Then, our findings confirmed the reliability of a suite of biomarkers to suppose the possible mechanisms of action of an emerging pollutant and the usefulness of behavioural and reproductive endpoints to clarify the eventual ecological hazard of a single focal chemical. Our results are particularly alarming because BE is the main illicit drug residue found in the aquatic system worldwide and the concentrations we tested in the present study are similar to those currently measured in aquatic environments. Moreover, considering the uninterrupted use of COC worldwide and the human metabolism, an incessant BE input in freshwater and its consequent increase in concentration is expected. This trend can confer to BE a sort of pseudo-persistence, representing a critical

aspect for the environmental risk assessment of this drug residue; indeed, aquatic organisms are exposed to BE, as well as to other drugs, for their whole life-span. This may result in possibly higher toxic effects than those we pointed out here. Considering these noteworthy issues, further investigations on the adverse effects of BE to aquatic organisms at different level of the ecological hierarchical scale should be a priority in order to shed light on its true ecological hazard for freshwater ecosystem.

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Chapter 6

Linking sub-individual and supra-individual effects in *Daphnia magna* exposed to sub-lethal concentrations of chlorpyrifos

Ferrario C.[1], Parolini M.[2], De Felice B.[2], Villa S.[1], Finizio A.[1]
Environmental Pollution (accepted)

Graphical abstract
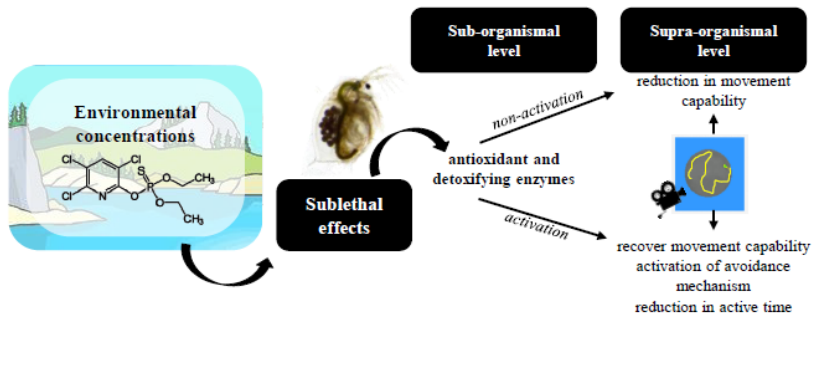
[1] Dept. of Earth and Environmental Sciences, University of Milano Bicocca, Piazza della Scienza 1, 20126 Milano.
[2] Dept. of Environmental Science and Policy, University of Milano, Milano.

Abstract

The main objective of the present study was to investigate possible links between sub-individual and supra-individual levels (i.e. population level) biomarkers in *D. magna* exposed to sublethal concentrations of the insecticide chlorpyrifos (CPF). To achieve the aim, 8 day old individuals were exposed for 96 hrs to two environmentally relevant concentrations of CPF (50 and 250 ng/L). Sub-individual level effects were investigated by measuring the activity of antioxidant (SOD, CAT, and GPx) and detoxifying (GST) enzymes, as well as by measuring the acetylcholinesterase (AChE) inhibition. In addition, the effects at supra-individual level were assessed by using a video-tracking system and analyzing changes in swimming capabilities (i.e. percentage of activity time, distance moved, and velocity). Our data have shown that daphnids exposed to both CPF concentrations were in a condition of stress which was highlighted by changes in both sub- and supra-individual biomarkers. Moreover, our results highlighted that the lowest tested CPF concentration did not modulate the antioxidant and detoxifying enzymes, whereas, an inhibition of AChE and a decrease of some parameters related to swimming behaviour (distance moved and velocity) were noted. On the contrary, significant changes in all the sub-individual biomarkers were measured at the highest tested concentration. In addition, organisms recovered the movement capability (distance moved) and also activate a mechanism of

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avoidance (increased swimming velocity). On the other hand, a reduction in the percent of active time was measured and this was attributed to the energy spent by organisms to activate antioxidant and detoxifying enzymes and the mechanism of avoidance. Based on these results, our study suggests the existence of a link between sub- and supra-individual levels, as the activation or non-activation in the antioxidant and detoxifying enzymes activities can led to different modifications of the swimming behaviour in *D. magna*.

Keywords: insecticide, oxidative stress, swimming behaviour, video tracking, *Daphnia magna*

6.1 Introduction

The ecotoxicological effects of chemical compounds are currently evaluated by means of standardised toxicity tests which are performed on organisms considered representative of the exposed ecosystems (Hood, 2005; Stadler, 2011). For the aquatic compartment, they include tests on algae, invertebrates and fish (the three levels of the trophic chain of the aquatic ecosystems) which are mainly focused on assessing acutely lethal concentrations (e.g., median lethal concentration, LC50) and chronic sub-lethal effects on developmental or reproductive endpoints. According to Amiard-Triquet (2012), in these tests a number of biochemical and physiological processes are completely overwhelmed as they do not allow organisms to cope with contaminants as they do in the field. However, at sub-lethal

concentrations (which are commonly measured in the aquatic environments) these mechanisms are functional, and many of them respond on the scale of days or weeks (Amiard-Triquet et al., 2011). The measurement of these sub-individual responses is the basis of the use of biomarkers in ecotoxicology as early warning indicators of potential risk (Forbes et al., 2006). All these mechanisms that are frequently involved in tolerance towards chemical stressors (adaptive mechanism) are energetically expensive, and thus may interfere with the allocation of energy, thereby governing the success of reproduction and growth of individuals and population and, in ultimate analysis, on the relative fitness (Sokolova et al., 2012). Thus the adaptive benefit of being tolerant may have negative counterparts in the long term period. In addition, the stress induced by chemical exposure can also have consequences at the higher hierarchical levels of the bio-ecological organization, from organism, population, up to the community levels (Parolini et al., 2017). For instance, at organism level, the presence of toxicants can lead to several behavioural changes (Boyd et al., 2002) such as the increase of the average speed (i.e., escape from contamination through the so called chemical avoidance), or the decreased swimming activity (protection reaction) (Wolf et al., 1998). Looking at the definition of biomarkers given by Depledge (Depledge and Fossi, 1994), behavioural changes can be included in this category (Forbes et al., 2006). In aquatic toxicology, behavioural responses of species have been used since the 80s as a

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method of monitoring and to measure potential environmental stress (Cairns and Gruber, 1980; Kramer et al., 1989; Diamond et al., 1990; Gerhardt et al., 1998; Van der Schalie et al., 2001). Nevertheless, only in recent years, with the improvement of video tracking technologies offering a better quantification of behavioural patterns, these studies are receiving the due attention (Asher, 2009; Little and Brewer, 2001; Amiard-Triquet, 2009; Sloman and McNeil, 2012).

At higher ecological hierarchy, impaired behaviour can have detrimental consequences at the population level through altered interactions with other members of the same species and at the community level through changes in competitive or predator/prey interactions. Ultimately, altered behaviour can affect ecosystem structure itself (Reichmuth et al., 2009; Duquesne and Küster, 2010). In a review of Faimali and coworkers (2017), it is reported that aquatic vertebrate and invertebrate behaviour such as predator-prey interactions, avoidance, and spatial movement have been impacted by toxicants at low concentrations and, for that, have a great potential as ecologically relevant end-points for contributing in ecological risk assessment mainly in the weight of evidence approach (Berninger et al., 2011; Boyd et al., 2002; Dodson and Hanazato, 1995; Gerhardt, 2007; Stanley et al., 2007; Valenti et al., 2009).

On these bases, it is evident that the investigation on how the responses to chemical stress are spread through the different levels of the ecological hierarchy is one of the challenges of modern

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ecotoxicology (Amiard-Triquet, 2009). In fact, the knowledge of the links between responses measured at a particular hierarchical level and those measured at the adjacent ones would be very effective in the risk assessment procedures, particularly for improving the use of biomarkers as early warning indicators of risk. Indeed, results obtained from studies at biochemical, molecular, cellular and even at organism level do not automatically allow predictions of stress responses at higher levels, such as population and community. For instance, it is difficult to determine whether the biomarker response indicates that an organism has been exposed to a chemical (and is dealing with it successfully) or whether it is being impaired by such exposure (Forbes, 2006). For these reasons, in the last two decades, the integration of several biomarkers at different levels of biological organization has been discussed as a tool to assess the extent of disturbances of a biological system and to quantify its actual state (Broeg et al., 2005; McCarthy and Munkittrick, 1996; Attrill and Depledge, 1997; Allen and Moore, 2004). For instance, Hagger and coworkers (2008) proposed a biomarker response index (BRI) to grade the level of biological impact of contaminants. However, more recently, the number of studies highlighting the link between sub-individual biomarkers responses and behavioural changes is constantly increasing (Ren et al., 2007; Baatrup, 2009; Ballesteros et al. 2009; Gravato and Guilhermino, 2009; Almeida et al., 2010, Mesquita et al, 2011; Oliveira et al., 2012; Silva et al. 2013; Van Praet

et al., 2014; Sabullah et al., 2015; Goodchild et al., 2016; Parolini et al., 2017). The present study is mainly aimed at highlighting the link of stress signals across two levels of bio-ecological hierarchy due to the exposure to chlorpyrifos (CPF). CPF is an organophosphorus insecticide widely used worldwide (George et al., 2014), with specific mode of action on aquatic invertebrates and vertebrates (Kavitha et al., 2008), which is frequently present in aquatic environments at concentration ranging from 0.01 to 1.95 µg/L (Palma et al., 2009).

Particularly, we focused the attention on the stress transition from the sub-individual to the supra-individual levels by measuring changes in molecular and behavioural biomarkers in *Daphnia magna* exposed to two sublethal concentrations of this organophosphorus compound. Regarding biomarkers (sub-individual level), we measured the activity of antioxidant (SOD, CAT, and GPx) and detoxifying (GST) enzymes, as well as the acetylcholinesterase (AChE) inhibition. At supra-individual level, we analyzed the changes in swimming behaviour of *D. magna* individuals due to CPF exposure by a video tracking approach, focusing on percentage active time, distance moved and active velocity.

6.2 Materials and methods

6.2.1 Test chemical and reagents

CPF (O,O-diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate; purity >99.7%) and the reagents used for biomarker analyses were

purchased from Sigma-Aldrich. All solvents (residue analysis grade; Merck Darmstadt, Germany) used for chemical analyses were checked by gas chromatography (GC) before use.

6.2.2 Test species

Daphnia magna Straus individuals were derived from a single clone obtained from the Istituto Superiore di Sanità (Roma, Italy). They were maintained (30 individuals/L) in commercial mineral water (San Benedetto® - conductivity 415 µS cm^{-1} at 20 °C; pH 7.42; 301 mg/L HCO^{3-}, 48.6 mg/L Ca^{2+}; 28.2 mg/L Mg^{2+}). The daphnids were cultured in 400 mL beakers (40 individuals/L of San Benedetto® water) and fed *ad libitum* three times a week with a suspension of the unicellular green algae *Raphidocelis subcapitata* (8×10^6 cells ind^{-1} day^{-1} until they were 8-day old, then 16×10^6 cells ind^{-1} day^{-1}) and the yeast *Saccharomyces cerevisiae* (15×10^6 cells mL^{-1}). The culture medium was renewed every two days. Culture medium, as well as the solutions used for the exposures, were maintained at 20.0 ± 0.5 °C under a 16h light: 8h dark photoperiod, which are conditions ensuring continuous parthenogenetic reproduction (Frey, 1982). Fourth generation were reared before the starting of the exposure experiments. Eight-day old *D. magna* individuals with dimensions allowing the video tracking of their swimming activity (personal observation) were utilized.

The algae were cultured in 2 L flask filled with ISO 8692/89 medium at 20.0 ± 2 °C under continuous light and shaken through aeration.

Algae were harvested during their exponential growth and let for sedimentation in the dark at 4 °C for a week. At the end of sedimentation, the density of algal suspension was determined through a Burker chamber under a brightfield light microscope.

6.2.3 Test conditions

All the experiments were performed in beakers of 400 mL under static conditions. Eight-day old *D. magna* individuals (in group of 20 specimens) were exposed for 96 hours to 50 and 250 ng/L of CPF (nominal concentrations). The stability of CPF in water was tested by spiking 740 ng/L of CPF, corresponding to the EC50 measured on *D. magna* by Palma et al. (2008). At the beginning of the stability test the CPF concentration was 740 ± 35 ng/L, while after 96 h it was 795 ± 25 ng/L, confirming that no degradation occurred in the water medium. Exposures were performed on 8-day old individuals because our preliminary analyses have shown that at this age they reached the minimum dimension allowing the video tracking of their swimming activity (see also Parolini et al., 2018, **Chapter 5**). Exposure concentrations were identified by considering both the EC_{50} (48-h) of CPF to *D. magna* (EC_{50mean} = 500 ng/L) (Pesticide Properties DataBase; Tomlin, 1994; Moore et al., 1998; Kikuchi et al., 2000; Printes and Callaghan, 2003; Palma et al., 2009) and the range of concentrations measured in surface waters (Palma et al., 2009). Individuals were not fed during the experiments. Stock solutions of

CPF (0.01 µg/mL and 0.1 µg/mL) were prepared in dimethylsulfoxide (DMSO) and the final concentrations of DMSO was under the level suggested by the OECD guidelines (OECD, 2004). Water solutions of CPF were prepared by spiking water with the stock solutions in DMSO in order to reach the two concentrations of exposure. Four independent experimental replicates were performed. Two negative control beakers (CTRL) containing each one 20 individuals were carried out during the period of exposure in all experimental replicate. Similarly, two control beakers containing 0.0005% of DMSO (DMSO) were also included to verify any carrier solvent effects.

6.2.4 Analysis of molecular biomarkers

The biomarker suite applied in the present study was performed on homogenates from a pool of all the alive *D. magna* individuals found in each jar at the end of the 96-h of static exposure (CTRL, DMSO, 50 ng/L and 250 ng/L). After video tracking (see the next paragraph), individuals were moved to a 1.5 mL Eppendorf tube, frozen in dry ice and stored at -80 °C until the biochemical analyses. As it cannot be excluded the complete removal of CPF from the outer carapax, individuals were washed trice with 0.5 mL of homogenization buffer to prevent potential bias caused by *in vitro* interactions. After washing, individuals (17-20 individuals per beaker) were homogenized using a pestle in 100 mM potassium phosphate buffer with the addition of 100 mM KCl, 1 mM EDTA, protease inhibitors 1:100 v/v and 1 mM

dithiothreitol (pH 7.4). The homogenates were centrifuged at 15.000 x g for 15 min at 4 °C, then the supernatant was collected and immediately processed to determine the activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and the inhibition of acetylcholinesterase (AChE) through spectrophotometric methods. All the enzymatic activities were measured in triplicate per each pool. SOD activity was assessed by measuring the inhibition of the reduction of cytochrome c (10 μM) caused by the superoxide anion produced by the xanthine oxidase (1.87 mU/mL)/hypoxanthine (50 μM) reaction for 1 min at $\lambda = 550$ nm (Mc Cord and Fridovich, 1969). We added 25 µL of supernatant to 1.5 mL of reaction mixture. Results were expressed as SOD units (1 SOD unit = 50% inhibition of the xanthine oxidase reaction). The CAT activity was assessed according to Aebi (1974) by measuring the consumption of H_2O_2 (50 mM) in potassium phosphate buffer (100 mM; pH 7) for 1 min at $\lambda = 240$ nm. We added 50 µL of supernatant to 3 mL of reaction mixture. The GPx activity was assessed according to Livingstone et al. (1992) monitoring for 1 min the consumption of NADPH at $\lambda = 340$ nm using H_2O_2 (0.2 mM) as substrate in potassium phosphate buffer (50 mM, pH 7) including glutathione (2 mM), sodium azide (1 mM), glutathione reductase (2 U/mL), and NADPH (120 μM). We added 50 µL of supernatant to 1 mL of reaction mixture. The GST activity was assessed monitoring the reaction of reduced glutathione (1 mM) in

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phosphate buffer (100 mM; pH 7.4) and CDNB (1 mM) for 1 min at $\lambda = 340$ nm (Habig et al., 1974). We added 20 µL of supernatant to 1 mL of reaction mixture. AChE activity was measured following the method described by Jemec et al. (2007), with slight modifications. The reaction mixture (1.5 mL) was prepared in potassium phosphate buffer (100 mM, pH 7.4) with the addition of acetylthiocholine chloride (1 mM) and 5,5' dithiobis-2-nitrobenzoic acid (0.5 mM). Then, 100 µL of supernatant was added to the mixture and the reaction was monitored for 15 min at $\lambda = 412$ nm. AChE activity was expressed as nmoles of acetylcholine chloride hydrolyzed min^{-1} mg protein^{-1} ($\varepsilon = 13,600$ M^{-1} cm^{-1}). The activity of all the enzymes was normalized on protein concentration determined with the Bradford method, using bovine serum albumin (BSA) as a standard.

6.2.5 Analysis of behavioral biomarkers

Video tracking analyses were performed on all the alive individuals at the end of the 96h exposures into 24-well plates. Each well contained 1 individual in 3 mL of culture medium, which was tracked individually. After a brief acclimation (3 minutes), video recordings were carried out by placing the 24-well plate with 17-20 animals on a light panel, and the movement of each individual was tracked three times for 15 seconds. The three 1080p Full HD videos acquired for each individual was analyzed using the software LoliTrack v.4 (Loligo Systems, Tjele, Denmark). This software was calibrated to measure

the following endpoints: swimming velocity (mm/s), distance moved (mm) and active/inactive time (%). Tracking was based on differences in contrast between objects (animals) and background (water) without use of markers. When the object appeared against a contrasting background, the software assigned a coordinate pair (x, y) to the centroid of the contrasting object. Each well in the 24-well plates was defined as an arena, and each individual was considered as a single object. Data were reported as the mean of the three replicates per each single individual.

6.2.6 Statistical analysis

The effects of CPF exposure on the activity of antioxidants, GST and AChE, as well on the swimming activity endpoints of 8-day old *D. magna* individuals were investigated by using a one-way Analysis of Variance (ANOVA), after controlling for normal distribution and homoscedasticity of data. Each single endpoint was considered as dependent variable, while the treatments as predictor. When a significant effect of treatment was found, a Fisher LSD *post-hoc* test was applied to point out significant differences between treatments. Significance was set at $p < 0.05$. Statistical analyses were performed by using STATISTICA 7.0 software package (StatSoft, Inc., 2004) and R 3.1.2 software (R core team, 2015).

6.3 Results and Discussion

At the end of the exposure period, no significant difference in mortality/immobilization was found among the treated and untreated samples ($p > 0.05$). In the following paragraphs the results obtained both for the molecular and behavioural biomarkers are presented and discussed.

6.3.1 Molecular biomarkers (sub-individual level)

In invertebrates, enzyme activities and other sub-cellular components are commonly used as biomarkers to identify causal mechanisms potentially responsible for effects at higher levels of bio-ecological organization. These include various defense enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST), and the acetylcholinesterase (AChE). SOD represent the first defense against free radicals, intervening by dismuting the most reactive and dangerous molecules, such as the superoxide anion, into ions that are less reactive (Shi et al., 2010), CAT and GPx decomposes the hydrogen peroxide into water and oxygen (Halliwell and Gutteridge, 2007). The GST catalyzes the conjugation of glutathione with diverse electrophilic molecules and contributes to the prevention of oxidative damage by conjugating glutathione to breakdown products of lipid peroxidation (Ketterer et al., 1983). In case the activities of these enzymes are not sufficiently adequate, the organism can be exposed to high levels of pro-oxidant

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molecules, which are produced during the metabolic pathways of contaminants (including pesticides) and this can lead to oxidative stress and consequent damage to lipids, proteins and DNA (Trypuć, 2017).

The measure of the acetylcholinesterase (AChE) activity is also frequently utilized as a useful biomarker to indicate that organisms have been exposed to a cholinesterase-inhibiting compound (such as organophosphate, carbamate insecticides, metals or detergents) at a sufficiently high level to elicit a significant effect (Lionetto et al., 2011). The inhibition of AChE suggests an over-accumulation of the acetylcholine, causing prolonged electrical activity at nerve endings and ultimately leading to death.

In the first part of our study, we measured changes in the activities of all the previously described biomarkers in 8-day old *D. magna* individuals after 96h of exposure to 50 ng/L and 250 ng/L of CPF (**Fig. 6.1**). This allowed us to get an overall picture about the effects at sub-individual level. Since the activity of CAT was significantly increased in DMSO treated specimens with respect to CTRL, we compared the effects of CPF both to CTRL and DMSO. A significant effect of the treatments was found for all the molecular biomarkers: SOD (F = 9.723 ; $p < 0.01$), CAT (F = 58.310; $p < 0.01$), GPx (F = 35.041; $p < 0.01$) and GST (F = 9.113; $p < 0.01$), AChE (F = 6.483; $p < 0.05$). Whilst the lowest tested concentration did not cause a significant modulation of antioxidant and detoxifying enzymes ($p > 0.05$ in all
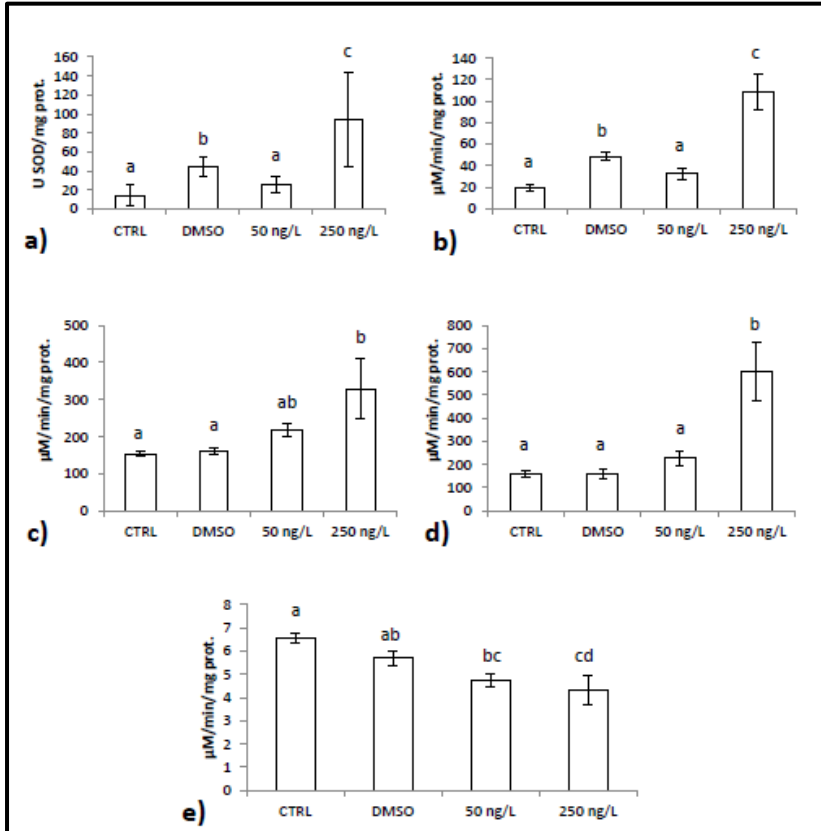
Figure 6.1 Mean activity (±SD) of SOD (a), CAT (b), GPx (c), GST (d) and AChE (e) measured in 8d old individuals after 96h of CPF exposure (50 ng/L and 250 ng/L). Different letters indicate significant difference among groups.

the cases, except of a significant reduction of CAT compared to DMSO), the exposure to 250 ng/L of CPF induced a 2- to 4-fold significant increase of SOD, CAT, GPx and GST. Similarly, CPF exposure had a significant effect on AChE activity of individuals,

which showed an inhibition accounting for the 22% compared to DMSO). However, no significant differences were found between the two tested concentrations.

- *SOD activity*

The enhancement in SOD activity of CPF-treated *D. magna* individuals suggested that this pesticide induced superoxide radicals ($O_2^{\cdot-}$) with the increase in concentrations. In an analogous experiment on *D. magna* exposed to CPF, Song and coworkers (2017), highlighted the dependency of the SOD activity in function of both the experiment duration and the exposure concentration. These authors, found that after 24h of exposure the SOD activity in 6-24 h old specimens of *D. magna* did not significantly change in all the experimental treatments (range 360-5,720 ng/L). However, after 48h of exposure, SOD activity showed an increasing trendfirst (reaching a peak at 720 ng/L of exposure) followed by a decrease according to the increase of the concentrations. This may be explained by the oxidation of SOD cysteine due to superoxide anions or their transformation to hydrogen peroxide (Dimitrova et al., 1994). Our observation on SOD activity somewhat confirmed and widened these findings; in fact, SOD activity increased with the increase of CPF concentrations. In addition, the higher time of exposure (96h) also increased the SOD activity. In fact, even if our highest tested concentration (250 ng/L) was less than 720 ng/L we observed an increase of SOD. In the study of Song et al. (2017), after 48h the peak of SOD was 59.33 U mg

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protein^{-1} whereas in our study we obtained a value of 94.5 U mg protein^{-1} after 96h.

- *CAT and GPx activity*

As previously stated, a clear induction of CAT and GPx activity was observed with the increase of CPF concentrations. CAT and GPx concur for the removal of H_2O_2, which is metabolized to O_2 and water. However, GPx is also considered an efficient enzyme in protection against lipid peroxidation (Winston and Di Giulio, 1991). CAT activity is directly regulated by the concentration of H_2O_2 (Fornazier et al., 2002). Our results showed that the trends of both CAT and GPx was consistent with the changes of SOD activity. This suggests that both enzymes are involved in the protective response by the Daphnia's antioxidant systems to counteract the adverse effects of hydrogen peroxide. Our results agree with the findings of Basopo and Ngabaza (2015) who measured an enhanced activity of CAT and GPx in the freshwater snail *Helisoma duryi* exposed to 25 ng/L of CPF.

- *GST activity*

GST is involved in the detoxification processes of different organic xenobiotics including CPF (Ecobichon, 1996). Exposure to CPF has been demonstrated to induce GST in chickens and rats (Vodela and Dalvi, 1995). In freshwater invertebrates, the experimental evidences on the role of GST are quite contradictory. McLoughlin and coworkers (2000) suggested low sensitivity of GST to organophosate

(OP) insecticides in annelids and crustaceans. In addition, Steevens and Benson (1999), found that GST was not affected by 48h CPF exposure but is inhibited after 96h in *Hyalella azteca*. On the contrary, other studies have shown the induction of GST in *Hydropsyche pellucidula* (Berra et al., 2006) and *Chironomus riparius* exposed to OPs (Callaghan et al., 2002; Choi et al., 2000). In mollusks, an induction of GST occurred in *Corbicula fluminea* after exposure to fenitrothion (Oneto et al., 2005). The same findings were highlighted in the recent study of Basopo and Ngabaza (2015). In fact, these authors found that the GST activity was significantly increased in snail *H. duryi*. Finally, Song and coworker (2017) in a study of *D. magna* showed that the GST was activated at low concentration and inhibited at high concentration of CPF reaching a maximum when the concentration was 360 ng/L and after 24h of exposure. The same authors reported an inhibition of GST activity after 48h exposure to increasing CPF concentrations (the lowest inhibition was obtained at the concentration of 360 ng/L). Our results partially confirmed these findings. In fact, we highlighted an increase of the GST activity following the increase of CPF concentrations even if at a higher time of exposure (96h) compared to previous studies. A possible explanation could be related to the tested concentrations. In our study, we were always below the concentration of 360 ng/L which was the peak of GST activity at 24h and the lowest level of inhibition at 48h. We hypothesize that the highest concentration of 250 ng/L tested in

our study was not sufficiently high to induce the inhibition of GST activity even with an exposure of 96h.

- *AChE activity*

Acetylcholinesterase activity is one of the most important biomarker in the evaluation of the exposure to OPs and carbamate pesticides, and several studies, in which AChE has been used as a biomarker for anticholinesterase insecticides, are present in literature (e.g. Fulton and Key, 2001; Printes and Callaghan, 2004, Xuereb et al., 2009). For crustaceans, several studies report a concentration-dependent inhibition of AChE with OPs pesticides (Domingues et al., 2009 and references therein). These observations are in accordance with the expectations based on the mechanism of action of OPs pesticides. In our study we measured significant difference of AChE activity in the individuals exposed to both the selected concentrations compared to CTRL and DMSO, without a reliance of the enzyme inhibition in function of the concentration of CPF. No significant difference was found between 50 ng/L and 250 ng/L (diff = 0.04, 95% CI: (-0.07) - (-0.15), p = 0.37). A possible explanation of our results could be related to the tested concentrations which were not sufficiently high to induce a drastic change in the AChE activity. Indeed, in a previous work, Barata et al. (2004) measured the response of AChE to single dose exposures of OPs and carbamates insecticides. These authors described the AChE inhibition by means of an allosteric decay model

with a period of no or low response at the low concentrations followed by an accelerated negative response as concentration increased.

6.3.2 Behavioral biomarkers (individual level) and potential link with molecular biomarkers (sub-individual level)

In this study, three swimming parameters (percent of active time, distance moved and swimming velocity) in *Daphnia magna* individuals were measured for each exposure condition. Behavioural responses were investigated in all the alive individuals at the end of the 96h exposure. Since the active velocity was significantly different in individuals treated with DMSO compared to the CTRL group (F = 29.25, $p < 0.0001$), the effects induced by CPF were compared to DMSO.

In **Figure 6.2** the increasing or decreasing effects on *D. magna* swimming behaviour induced by different concentrations of CPF are normalized to DMSO.
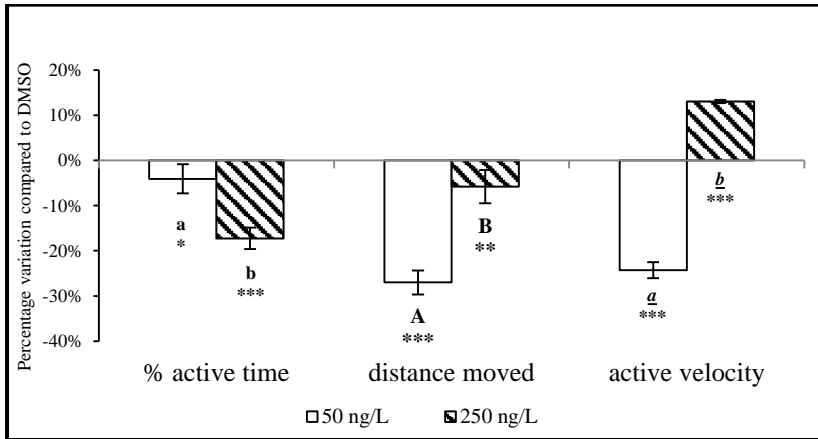
Figure 6.2 Histograms of increasing/decreasing effects (activity, distance moved and velocity) on swimming behaviour for *D. magna* exposed to different concentrations of CPF (50 ng/L and 250 ng/L). Data are normalized to DMSO. Different letters indicate significant difference between the tested concentrations ($P < 0.05$). Asterisks indicate significant difference with DMSO (Significance codes: $0 \leq$ '***', $0.001 \leq$ '**', $0.01 \leq$ '*' < 0.05).

An overall different profile is observed when the two concentrations of exposure are compared. In fact, at the lowest tested concentration (50 ng/L) a slight reduction in the % of active time of individuals can be observed (<5%), whereas a consistent decrease of both total distance moved and velocity (-24% and -25% respectively) is recorded. On the contrary, at CPF concentration of 250 ng/L the % of active time of individuals decreased notably (<17%), whereas a slight reduction in distance moved and an increase of velocity (-6% and 13% respectively) was noticed. The percent of activity time (i.e., how much of the time have the animal been in an active and inactive state) is calculated by considering, frame to frame, if the animal is moving a

distance longer than a minimum threshold value (in pixels). Based on our results, a concentration-dependent reduction of the percent active time for individuals is demonstrated, meaning that as higher is the concentration of exposure as higher will be the period of inactivity or immobility.

The inhibition of AChE has been historically related to the mode of action of OP insecticides such as CPF. Indeed, in previous studies several authors have tried to link the inhibition of AChE activity with adverse effects at the organism level, including growth, reproduction and mortality or immobilization (Depledge and Fossi, 1994; Jemec et al., 2010) with contrasting results. For instance, Ludke et al. (1974) suggested the 50% inhibition of AChE as a threshold limit of a life-threatening situation. This limit was somewhat confirmed by Barata et al. (2004) in a study on *D. magna*. On the other hand, Phillips et al. (2002) linked acute exposures to CPF at levels causing mortality, to enzyme inhibition of >71% and >90% in juvenile and larval walleye (*Stizostedion vitreum*) respectively. In addition, no immobility of *D. magna* exposed to 100 µM of the OP acephate was observed, although the 70% inhibition of the enzyme activity was reached (Printes and Callaghan, 2004). These authors also found that different cholinesterase-inhibiting pesticides had different inhibition level associated with immobilisation of the exposed daphnids. These studies indicated that although AChE activity has been associated with

mortality/immobilization, the association is species- and chemical-specific.

Given the key role of AChE in nervous system, it seems reasonable to relate swimming behaviour and the inhibition of this enzyme. Recently, Ren et al. (2017) investigated the role of AChE in swimming behaviour of *D. magna*. The authors concluded that 50% of AChE inhibition may cause changes in swimming behaviour in treated specimens. On the other hand, they also highlighted that there is no clear evidence for the role of AChE in the behaviour homeostasis. Similarly, in another study Xuereb and coworkers (2009) highlighted locomotor alterations in *Gammarus fossarum* exposed to CPF and the carbamate insecticide methomyl. The authors observed significant behaviour alterations for AChE inhibitions higher than 50% for both insecticides.

In our study we measured an inhibition of the AChE of about 22% (**Fig. 6.1**) without significant differences between the two tested concentrations, which is quite far from the threshold limits reported above. Therefore, we cannot establish a relationship between the AChE levels of inhibition and the percent of the reduced activity time. Chevalier and coworkers (2015) highlighted a variability in behavioural changes during time in D. magna exposed to different concentrations of several pollutants with different mechanism of action (including an AChE inhibitor). In our study, the temporal variability of metabolic changes and swimming behaviour was not

taken into consideration. Consequently, our results should be regarded as a snapshot after 96h of exposure to CPF and this could have limited a more appropriate evaluation of the link between AChE inhibition and behavioural changes.

The same concentration-dependent trend obtained for the time of activity cannot be observed for the other two considered parameters (distance moved and active velocity). Indeed, as previously described, the decreases in the distance moved is significantly higher at 50 ng/L than at 250 ng/L (diff= -0.11, 95% CI: (-0.15) – (-0.06), $p < 0.0001$). Moreover, when speed is considered, a contrasting result is obtained with a significant decrease at the lowest tested concentration and even an increase at the highest one (diff= -0.17, 95% CI: (-0.20) – (-0.15), $p < 0.0001$). Probably, the Stepwise Stress Model (SSM) (Gerhardt, 1999, 2001; Gerhardt et al., 2005) can be a useful starting point to explain our findings. According to SSM, a cascade of regulatory behavioural stress responses is performed by the organisms either by increasing the toxicant concentration or the exposure time.

We hypothesize that the concentration of 50 ng/L of CPF after 96h of exposure was too low to activate regulatory or compensatory mechanisms at sub-individual levels such as the activation of the detoxifying enzymes (**Fig. 6.1**) useful to maintain the homeostatic conditions. This situation has led to a significant reduction of both parameters indicating a condition of behavioural stress which can be associated to a mechanism of protective reaction due to a loss of

coordination (Ferrando and Andreu, 1993; Wolf et al., 1998). On the contrary, the concentration of 250 ng/L of CPF stimulated the activation of the detoxifying enzymes (**Fig. 6.1**). The activation of these regulatory mechanisms allowed the organisms to recover the movement capability (in terms of distance moved) and to activate another behavioural response that is the avoidance. In fact, the increased velocity of swimming can be associated to the attempt of the organism to "escape" from the polluted aquatic environment and this has been recognized as one of the first behaviour modulation in *Daphnia magna* (Ren et al., 2007). On the other hand, detoxification process and antioxidant protection as well as the avoidance behaviour require energy and this could help to explain also the reduction in the % of activity time in individuals exposed at the highest tested concentration.

6.4 Conclusions

This study was aimed at investigating potential links in the stress transition from the sub-individual to the supra-individual levels in aquatic organisms. Our goal was achieved by measuring changes in molecular and behavioural biomarkers in *Daphnia magna* exposed to sub-lethal concentrations of CPF.

The results have shown that daphnids were in a condition of stress in both conditions of exposure, however, with a contrasting pathway. In fact, at the lowest tested CPF concentration we measured a partial

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inhibition of the AChE and a significant decrease of some parameters related to swimming behaviour (distance moved and velocity), whereas the activity of antioxidant enzymes and GST (molecular biomarkers) were not different from the control. In addition, the percent of activity time (behavioural biomarkers) was slightly modulated in treated specimens in comparison with control. At the highest tested concentration, we did not measure further inhibition of AChE suggesting that this concentration was not sufficiently high to induce drastic changes in the activity of this enzyme. On the other hand, we measured significant changes in antioxidant activity and GST suggesting that at this concentration the organisms used a strategy of adaptation by synthesizing the antioxidant and detoxification enzymes. At supra-individual levels, organisms showed the tendency to recover the movement capability (distance moved) and also activated a mechanism of avoidance (increased swimming velocity). However, a reduction in the percent of active time was noticed, and this was attributed to the energy spent by organisms to activate the enzymes and the mechanism of avoidance. Overall, our results suggest the existence of a link from sub- and supra-individual levels as the activation or non-activation in the antioxidant and detoxifying enzymes activities can lead to different modifications of the swimming behaviour in *D. magna*. Finally, although sub-lethal concentrations of CPF elicited enzymatic and behavioural changes in *D. magna*, these cannot be directly related to effects on their fitness or

at higher ecological hierarchical level in a quantitative way. Therefore, they cannot be considered into an environmental risk assessment procedure at this time and more effort should be done in this direction.

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Chapter 7

Comparison of the behavioural effects induced by treated sewage effluents on *Diamesa cinerella gr* larvae (Diptera, Chironomidae) and *Daphnia magna* (Cladocera, Daphniidae)

Villa S.[1], Di Nica V.[1], Bellamoli F.[3], Pescatore T.[2], Ferrario C.[1], Finizio A.[1], Lencioni V.[3]

Paper in preparation

[1]Department of Earth and Environmental Sciences, University of Milano Bicocca, Piazza della Scienza 1, 20126 Milano, Italy
[2]Water Research Institute, National Research Council (IRSA-CNR), Via Salaria km 29.300, Monterotondo, 00015 Rome, Italy
[3]Section of Invertebrate Zoology and Hydrobiology, MUSE – Museo delle Scienze, Corso del Lavoro e della Scienza 3, 38122 Trento, Italy

7. Comparison of the behavioural effects induced by STPs on *D.cinerella gr* (Diptera, Chironomidae) and *D. magna* (Cladocera, Daphniidae)

Abstract

Even if identified as pristine, mountain freshwater ecosystems can be threatened by chemical pollutants through the discharge of effluents from Sewage Treatment Plants (STPs). Indeed, residues of pharmaceuticals and personal care products have been detected in different rivers downstream of STPs located in Alpine environments. Acute toxicity tests performed with *Daphnia magna* are among the most internationally used bioassays for monitoring the toxicity of effluents (whole effluent toxicity testing). More recently, the use of this organism has also been proposed in on line and real time biomonitoring programs as an early warning system for evaluating the STPs effluent quality. These systems are based on recording behavioural changes of the biomonitor organism, resulting from the stress produced by the effluents. Indeed, altered behavioural signals could be induced at sublethal concentrations which are significantly lower than the corresponding EC50. However, at this time, it is almost completely unknown if the sensitivity of *D. magna* can be representative of other aquatic organisms and particularly macroinvertebrates. Differences to stress responses could be due to different traits that organisms may have evolved in different environmental conditions. In this study, we analysed and compared the responses (mortality and behavioural changes) of *Daphnia magna* and *Diamesa cinerella gr.* larvae, a chironomid (Diptera Chironomidae) common in cold freshwaters in the Alps, and often

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associated to pristine environments. Both organisms were exposed for 24 and 48 hrs to different dilutions of effluents collected from a STP located nearby the Tonale Pass (1799 m a.s.l., Trentino Region, Italy). This study aimed to verify if *D. magna* can be employed in biomonitoring programs for STPs located in Alpine areas as surrogate of cold freshwater best adapted species. Mortality rate and behavioural responses (using video tracking systems) were compared. No significant mortality or change in behaviour was observed in the two organisms exposed to undiluted effluent. Exposure to serial dilutions of the treated effluent did not cause significant mortality events on both species, but altered notably their behaviour at both the exposure times (e.g., the time spent in activity in *D. magna*; the average speed of movement and the cumulative distance travelled in both), especially at a ten times diluted effluent. Overall, findings of this study emphasise that even if *D. magna* and *D. cinerella* use different behavioural strategies to cope adverse environmental conditions, their overall sensitivity to treated effluents is similar. Accordingly, the use of *D. magna* in Biological Early Warning Systems protocols seem to be sufficiently protective also for cold adapted local species, such as *Diamesa cinerella gr*, of Alpine freshwater ecosystems.

Keywords: Treated sewage effluents, D*aphnia magna, Diamesa cinerella gr*, behavioural changes, video tracking systems.

7.1 Introduction

Water contamination has become one of the major threat for the
survival of living organisms in aquatic systems (Reid et al., 2013).
Urban and industrial discharges are among the major drivers of the
degradation of these environments; indeed, a huge quantity of
wastewater-borne pollutants, such as suspended solids, nutrients,
microorganisms and toxic compounds is continuously discharged into
the water bodies (Boyd et al., 2003; Cristale et al., 2015; Fent, 1996;
Lindqvist et al., 2005; Lishman et al., 2006; Sun et al., 2015). In
addition, effluents from Sewage Treatment Plants (STPs) are
recognized as one of the main route of entry into aquatic environment
of emerging contaminants such as pharmaceuticals and personal care
products (EC, 2016). The European Union's (EU) environmental
regulations aim to reduce the pollution of surface water caused by
STPs (Council Directive 91/271/EEC 1991 as amended by the
Commission Directive 98/15/EEC of 27 February 1998; Directive
2000/60/EC). This requires the EU Member States to ensure that
discharge of urban wastewater and its effects are continuously
monitored (Farré et al., 2001). In this regard, the protection of the
receiving waters is the primary goal of the municipal STPs. Of
particular relevance is the control of the quality of the effluents
discharged from STPs which are located in Alpine environments. This
is not only because these ecosystems are intrinsically fragile (Kaul,
1993) but also in consideration of their relevance for water supply to

surrounding areas (Alpine Convention, 2009). Often identified as pristine, Alpine environments are threatened by chemical pollutants (Morselli et al., 2018, Ferrario et al., 2017 (**Chapter 3**), Chiogna et al., 2016, Villa et al., 2006) and discharges of STP effluents can further contribute to alpine freshwater pollution. For instance, Mandaric and coworkers (2017) measured the presence of pharmaceuticals and personal care products residues downstream of STPs in different Alpine rivers.

The measurement of several physical chemical parameters (pH, dissolved oxygen, BOD, COD, TOC, TDS, TSS) is generally used for evaluating the quality of STPs effluents. However, as it is often referred (Metcalf and Eddy, 2003; Movahedian et al., 2005; Teodorović et al., 2009) these parameters, taken alone, are not sufficient in obtaining reliable information about the toxicity of treated effluents. For this reason, toxicity tests are normally performed in combination with routine physical chemical analysis. Indeed, direct toxicity assessment of STPs effluents, can contribute to keep the good ecological status of water bodies. One of the most internationally used bioassays for monitoring the toxicity of effluents is the acute toxicity test performed with *Daphnia magna (*Koçbaş and Oral, 2015). The advantage of using this organism is related to its high sensitivity to toxic substances, its short generation time, easy acclimatization in laboratory condition, short term duration of the test (Tyagi et al.,

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2007). In addition, well standardized methods for the toxicity test are available for this organism (OECD, 2004, 2012).

More recently, the use of on-line biomonitoring for the surveillance of the STPs effluent quality has been proposed (Allan et al., 2006; Jeong et al., 2008; Jeong et al., 2014; Häder and Erzinger, 2017a, b). This approach allows determining the integrated toxicity of water and has the advantage that identification of the plethora of water pollutants which are present in wastewater effluents become not necessary (Chen et al., 2013). On-line biomonitors frequently use behaviour as an endpoint, which provides a visual and, thus, measurable response at the whole-organism level (Gerhardt, 1995). In addition, behavioural changes are recognized as more sensitive endpoints than the traditional ones (i.e. mortality or immobilization), as altered behavioural signals are induced at sublethal concentrations which are significantly lower than the corresponding L(E)C50 (Gerhardt, 2007; Hellou et al., 2008). *Daphnia magna* is again among the organisms which are frequently used in these biomonitoring activities. Indeed, when this organism faces a change in water quality or a toxic condition, its behaviour changes markedly. Consequently, the level of abnormality is determined from the recorded behavioural data.

As previously described, the wide use of *Daphnia magna* in toxicity and biomonitoring studies is mainly due to its easy acclimatization in laboratory as well as its high sensitivity to toxicants. For this reason, many regulations consider this organism as representative of the

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invertebrates in the aquatic ecosystems (EC, 2002, 2003, 2006). Nevertheless, this assumption will not always hold true (Wogram and Liess, 2001; Maltby et al., 2005; Wiberg-Larsen et al., 2016). Indeed, the sensitivity of macroinvertebrate species can be related to ecological traits (single trait or their combinations including respiration type, temperature preference, and current velocity preference, all related to skin thickness and permeability). For instance, according to biological traits-approaches, it has been demonstrated that Chironomidae are more sensitive to some categories of pollutants such as organochlorine and neonicotinoid pesticides than *D. magna* (Rico et al., 2015). It is completely unknown if chironomids are more sensitive than daphnids also towards others aquatic contaminants that could be found in STPs effluents such as pharmaceuticals and personal care products, or mixtures of them. Differences in sensitivity could be also exacerbated in the extreme conditions of cold ecosystems such as the Alpine environments, where organisms may have evolved different traits that could have great influences on the sensitivity of species towards toxicants.

In this context, the aim of this work was to analyse and compare the sensitivity of *Daphnia magna* and *Diamesa cinerella gr* larvae (an Alpine chironomid species) exposed to different dilutions of treated sewage effluents collected from a STP located at the Tonale Pass, in the Italian Alps (NE Italy). This area is characterized by a significant presence of tourists. Recently, downstream the selected STP, several

pharmaceuticals were measured in surface water at concentrations ranging from ng L^{-1} to µg L^{-1} (Mandaric et al., 2017). Particularly, during the study, differences in mortality and behavioural responses between the two species were investigated. For the behavioural responses, two different video tracking systems were utilized for each of the selected test organism. The comparison allowed us to verify whether or not *D. magna* could be utilized in toxicity and biomonitoring programs in STPs located in Alpine areas as surrogate of glacial habitats best adapted species. Indeed, *Diamesa* genus prevails in kryal habitats of high mountain and is often associated to pristine environments (Lencioni et al., 2012, Niedrist and Fureder, 2016).

7.2 Methods

7.2.1 Effluents sampling

The effluents were collected in a STP located at 1799 m a.s.l. along the Vermigliana river (a tributary of the Noce river) in the central part of the Italian Alps, within the Adamello National Park and nearby the Tonale Pass. The STP collects wastewater from an area of strong tourist vocation. It is designed to serve 10000 Equivalent Inhabitants (E I.), and is equipped with conventional treatments (oxidation, secondary sedimentation). The main technical information of the treatment processes can be downloaded by Agenzia per la Depurazione della Provincia di Trento webpage

(https://adep.provincia.tn.it/Agenzia-per-la-Depurazione-ADEP).

The sampling campaign was conducted on 6th March 2017, after tourist peak due to carnival holidays. All samples were collected in sterile graduated water sampling bottles in PP (from LP Italiana, Milan Italy); kept refrigerated during the transport and subsequently were stored at −20 °C until tests were performed.

7.2.2 Behavioural tests

7.2.2.1 Daphnia magna experimental plan

The tested organisms originated from a single clone of *Daphnia magna Straus* taken from the Istituto Superiore di Sanità (Roma, Italy). Organisms were kept (40 individuals L^{-1}) in 400 mL beakers with aerated commercial mineral water (San Benedetto®) having the following physical chemical characteristics: conductivity 415 µS cm^{-1} at 20 °C; pH 7.42; 301 mg L^{-1} HCO_3^{-}, 48.6 mg L^{-1} Ca^{2+}; 28.2 mg L^{-1} Mg^{2+}. Daphnids were fed three times a week by adding a suspension of the unicellular green alga *Raphidocelis subcapitata* (formerly *Pseudokirchneriella subcapitata*) and the yeast *Saccharomyces cerevisiae* to the culture medium. In particular, a suspension of 8×10^6 cells of the green algae per individual and per day was used to feed the 8-day old daphnids, and a suspension of 16×10^6 cells of the green algae per individual and per day was used to feed organisms older than 8 days. The suspension of the yeast employed was of 15×10^6 cells mL^{-1}. Culture medium was renewed every two days. An ISO

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8692:1989 medium were used to cultivate the algae in flask of 2 L. Algae were continuously kept at light and aerated through shaken at 20.0 ± 0.5 °C. During the exponential growth period, algae were collected and let to sediment for a week in a dark chamber at 4 °C. After discharge of the supernatant, the cell density was measured under a bright field light microscope with the use of a Burker chamber. Culture medium and test solutions were kept in a climate controlled chamber at 20.0 ± 0.5 °C with a 16:8 hrs light:dark cycle. 5-day old daphnids from the fourth reproduction cycle were used for the exposure tests. Five 5-day old individuals were exposed in 50 ml bottles for 48 hrs to four different dilutions (STP, STP/10, STP/100, STP/1000) of STP effluents. Test solutions were renewed after 24 hrs from the beginning of the experiment. Tests were performed in four replicates per treatment and two negative control (CTRL) containing each one five daphnids. Specimens were not fed during test. At the end of each exposure time, daphnids were transferred in the designed arena and the swimming activity of organisms was recorded as described below.

7.2.2.2 Daphnia magna video tracking analysis

Video tracking analyses were carried out on all the alive daphnids after 24 and 48 hours of exposure. At the end of each exposure time, daphnids from each bottle were transferred into the designed arena (80 mm x 75 mm x 5 mm) each containing 5 daphnids and 30 mL of the

test solution. Each arena was placed in front of a light panel and, after 30 minutes of acclimatization, video recordings were taken. The activity of five individuals was tracked contemporaneously for 10 minutes. The 1080p Full HD videos acquired were converted in .avi file and were partitioned into ten sections of 30s with FFMPEG, in order to have all videos from 900 frames (30 frames second^{-1}). Subsequently the mini-videos were analyzed using the software LoliTrack v.4 (Loligo Systems, Tjele, Denmark). The By adapting the RGB thresholds, the LoliTrack v.4 software identifies all pixels colored similarly in the image as objects in contrast respect to the background. In this way the software tracks the activities of daphnids (objects) in contrast to the water by assigning an X,Y-position to their centre. The software was optimized/calibrated to monitor and quantify three different swimming endpoints of *D. magna:* the time of activity (%), the average speed (AvgSpeed) of swimming (mm s^{-1}), and the total distance moved (mm).

7.2.2.3 Diamesa cinerella gr experimental plan

The IV instar larvae of *Diamesa cinerella gr* were collected with a 30 × 30 cm pond net (mesh size 100 μm) (Scubla SNC, Italy) from the Rio Presena stream at 2700 m a.s.l. in the Adamello National Park. The sampling campaign was conducted on 5th March 2017. Larvae were 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 hrs of sampling using a stereomicroscope (MZ 7.5; Leica Microsystems, Germany; 50×) according to Rossaro and Lencioni (2015). Animals were maintained in 1L glass aquaria with stream water in a thermostatic chamber (ISCO, model FTD250-plus; Teledyne Isco Inc., Lincoln, Nebraska) at 4 °C under aeration to maintain dissolved oxygen higher than 80% saturation. The incubation temperature (4 °C) approximated the water temperature measured in the stream with a multiparametric probe Hydrolab Quanta. To acclimate larvae to exposure conditions, 24 hrs prior to each experiment, IV-instar larvae randomly selected were removed from the rearing aquarium and transferred in 500 ml beaker (about 40 larvae per beaker), containing 200 ml of Hard Reconstituted Water (HRW) according to Lencioni et al. (2016): 4.36 mg L^{-1} NaHCO$_3$, 2.73 mg L^{-1} CaSO$_4$*2H$_2$O, 2.73 mg L^{-1} MgSO$_4$, 0.19 mg L^{-1} KCl (pH = 7.7). During acclimatization and exposure, larvae were kept at 4 ± 1 °C, without light and food but with aeration.

Larvae were exposed for 48 hours to four serial dilutions of STP effluent, as described above. Test solutions were renewed every 24 hrs. Video were taken after 24 hrs and 48 hrs of exposure.

After 24 hrs, control and treated larvae with their exposure solution were poured in a 50 mL- beaker on a worktable in a climate controlled room at 4 ± 0.1 °C where video were taken for 120 - 180s with a digital HD camera (Raspberry Pi 3 with Camera Module v2) at high-resolution (1920 x 1080 pixels), at about 10 cm from the bottom of the

beaker. During the video recording time, water temperature changed within a range of +0.1 to +0.3 °C measured with a portable thermometer (Koch 13211; ±1 °C).

7.2.2.4 Diamesa cinerella gr video tracking analysis

Videos were analysed using the software ImageJ (http://imagej.nih.gov/ij/), a public domain Java image processing program. 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). The software was optimized/calibrated to monitor three different behavioural endpoints of *D. cinerella gr* larvae (for details refer to Villa et al., 2018). Three endopoints were selected: the cumulative distance covered (mm), the AvgSpeed of movement (mm s^{-1}), and the frequency of the body bends (BBps = body bends per second).

Each original video was converted in .avi file and was partitioned into five sections of 30s with FFMPEG, an open source audio and video converter (http://www.ffmpeg.org), in order to have all videos from 900 frames (30 frames second^{-1}). Subsequently the mini-videos were processed with Image J and the data were converted from pixels to mm, and from pixels second-1 in mm s^{-1}. For do this, the length in mm of the backer was measured with a ruler from one edge to the other. In ImageJ, the same length was measured using the straight line tool: a

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line was drawn from one edge of the backer to the other and the length was measured in pixels (Brooks et al., 2016).

7.2.3 Statistical analysis

Behavioural results were analysed using Student's t test to detect statistically significant differences of each treatment group respect to the control at different exposure time (24 and 48 hrs) and between comparable endpoints (AvgSpeed and Distance) of the two tested organisms. The existence of outliers was investigated through the Grubbs test by using XLSTAT statistical software (Addinsoft 1995-2017). Statistical significance was indicated by asterisks: **** $P<0.0001$; ***$P<0.001$; **$P<0.01$; *$P<0.05$. Statistical analyses were performed using R-project software (R Core Team, 2017).

7.3 Results

7.3.1 Mortality

For both species, no mortality was observed in controls (CTRL) indicating the validity of the test (**Table SI-7.1** and **Table SI-7.2**). **Tables SI-7.1** and Supporting Information (**Chapter 9**) also report the numbers of dead organisms measured at different effluent dilutions after 24 and 48 hrs of exposure in *D. magna* e *D. cinerella gr* respectively.

In *D. magna* no mortality was detected in the undiluted samples of effluents (STP), whereas a progressively increment in mortality was

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observed with increasing dilutions and exposure time: 2 and 3 organisms after 24 hrs in 100 and 1000 times diluted effluent and 1, 3 and 4 after 48 hrs in 10, 100 and 100 times diluted solutions.

On the contrary, during the test no mortality was detected in *D. cinerella gr* (with a partial exception of 1 organism dead at the highest dilution after 24 hrs).

7.3.2 Behavioural test

In this study, a number of behavioural parameters of the two organisms were monitored:

- average speed is the velocity of movement calculated frame by frame as the length/time ratio (mm s^{-1}), where the length is sum of length of all movement vectors between frames given track;

- distance moved (mm) is the sum of the distance covered by animal from start to finish of the analysed video;

- activity is described as time of activity (%). The lowest threshold for activity is defined as activity threshold [pixels], e.g. if the object moves a distance larger than this minimum distance is scored as active;

- body bends per second (BBps) or the number of time that the organism assumes a curved shape in a second (frequency) calculated using angle, during all video frames.

The first two parameters (average speed and distance moved) are directly comparable for the two organisms, whereas the activity and

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BBps were specifically measured in *D. magna* and *D. cinerella gr* respectively. All measured data are detailed reported in Supporting Information (**Chapter 9**) section (**Table SI-7.1 and SI-7.2**). For both tested species, precision of measurements is within an acceptable range of variability, however, in *D. cinerella* the distance moved appeared to be more scattered as suggested by the high RDS values both in controls and in exposed specimens. The higher variability in *D. cinerella* is mainly due to the heterogeneity of individuals which were collected from natural populations, whereas *D. magna* specimens were obtained from a single clone cultured in laboratory. Anyway, we would like to point out that from an ecological point of view, a higher variability of responses could allow better adaptation and survival strategies for the species.

Fig. 7.1 (A, B, C, D) reports measured deviations from control of average speed and distance moved at different STP dilutions and time of exposure. Statistical significant differences with control are indicated by asterisks (** = $P < 0.01$; * = $P < 0.05$), whereas different letters indicate significant differences between the two organisms.

7. Comparison of the behavioural effects induced by STPs on *D.cinerella gr* and *D. magna*

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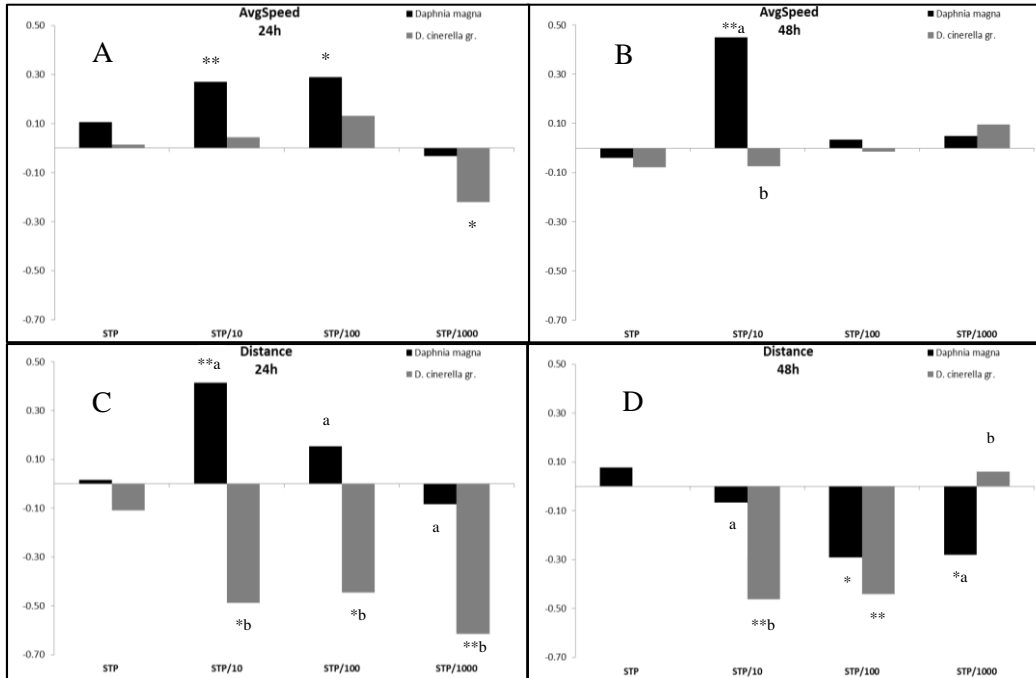
Figure 7.1 Histograms of effects on behavioural parameters (average speed and distance moved) in *D. magna* (black) and in *D. cinerella gr* (grey) exposed to different STP dilutions. Asterisks indicate significant difference with control (Significance codes: '**' ≤ 0.01; '*' < 0.05). Different letters indicate significant difference between the two tested organisms (P<0.05).

7. Comparison of the behavioural effects induced by STPs on *D.cinerella gr* (Diptera, Chironomidae) and *D. magna* (Cladocera, Daphniidae)

After 24 hrs and 48 hrs of exposure, both species showed the greatest behavioural deviations from the controls when exposed to diluted effluents.

In *D. magna* after 24 hrs of exposure, average speed and distance moved *(***Fig. 7.1 A** and **C***)*, were significantly increased at STP/10 dilution ($P < 0.01$), whereas at STP/100 dilution the organisms shown only a significant increase of the average speed ($P < 0.05$). Finally, no differences were observed for specimens exposed to STP and STP/1000 dilutions compared to controls.

After 48 hrs *(***Fig. 7.1 B** and **D**), the average speed was still significantly increased only at STP/10 dilution ($P < 0.01$). On the contrary, distance moved was significantly decreased at STP/100 and STP/1000 dilutions ($P < 0.05$), whereas at STP/10 dilution the organisms returned to the level of control.

D. cinerella gr gave quite different results; indeed, the average speed parameter seemed to be not particularly influenced during all the exposure period (**Fig. 7.1 A** and **B**) (with the partial exception of STP/1000 at 24 hrs where a reduction in the swimming speed was recorded; $P < 0.05$), whereas the distance moved (**Fig. 7.1 C** and **D**) was significantly reduced during all the exposure period and STP dilutions with a recovery at STP/1000 dilution after 48 hrs.

Figure 7.2 reports measured changes from control of active time and BBps parameters in *D. magna* and *D. cinerella gr* respectively.

7. Comparison of the behavioural effects induced by STPs on *D.cinerella gr* and *D. magna*
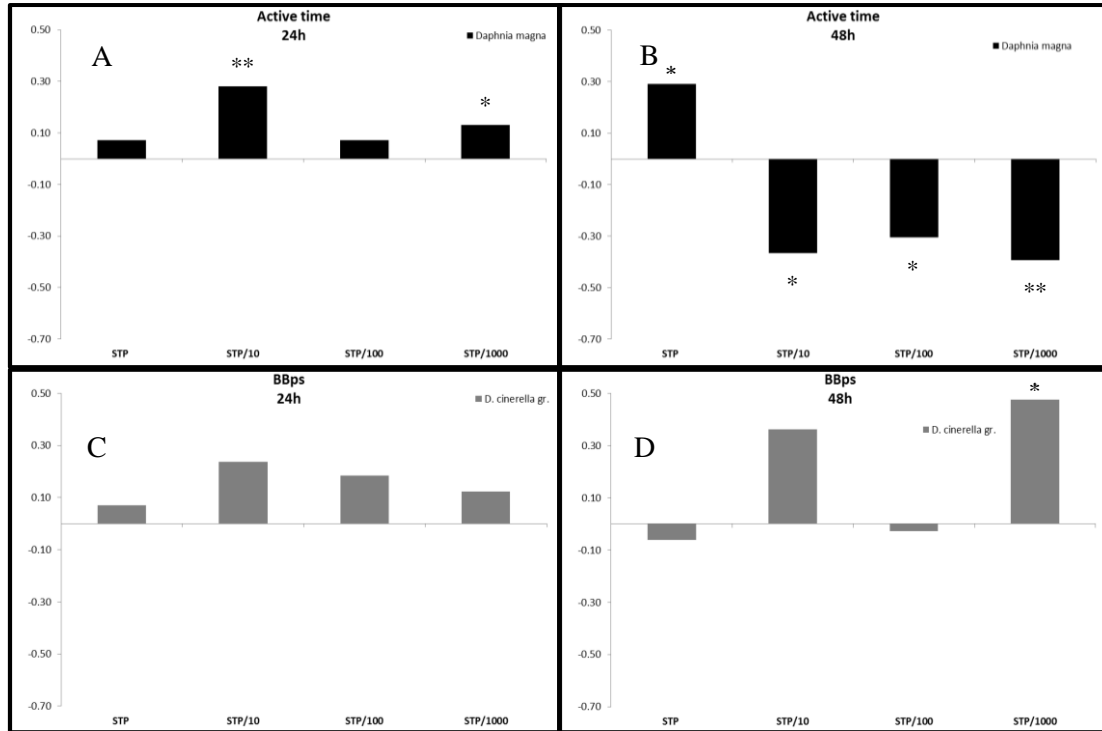
Figure 7.2 Histograms of effects on active time (in *D.magna,*) and BBps (in *D.cinerella gr,*)exposed to different STP dilutions. Asterisks indicate significant difference with control (Significance codes: '**'=$P \leq 0.01$; '*'=$P < 0.05$).

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Compared to control, after 24 hrs of exposure, *D. magna* shown an increased active time at STP/10 and STP/1000 dilutions (**Fig. 7.2 A**). Different results were gathered after 48h of exposure (**Fig. 7.2 B**). Indeed, in all the STP dilutions a significant reduction of this behavioural endpoint was observed, whereas an increase of active time was measured in organisms exposed to undiluted STP.

In *D. cinerella gr*, not significant changes of BBps were observed during all the exposure period, with the exception of the organisms exposed to the highest STP dilution (STP/1000) after 48 hrs of exposure. In these organisms an increase in the BBps number was noticed.

7.4 Discussion

Whole Effluent Toxicity (WET) testing were formalized for the first time in 1985 by the US EPA with the intent "to identify, characterize, and eliminate toxic effects of discharges on aquatic resources'' (Chapman, 2000; Chapman et al., 1995). In this context, scientists identified in the Biological Early Warning Systems (BEWS) a powerful tool to evaluate the WET. According to Jeong and coworkers (2014), BEWS have been diversified through the employment of various test organisms such as algae, fish and water fleas and are generally based on acute toxicity test (Baldwin et al., 1994; Benecke et al., 1982; Hendriks and Stouten, 1993). In recent years, the measure of behavioural changes in living organisms has been suggested as a

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powerful tool to be included in the BEWS. Indeed, impaired behaviour may have harmful effects at the population level (i.e., change in fitness), and even at higher levels of the ecological hierarchy, through changes in competitive or predator/prey interactions (Duquesne and Liess, 2010; Duquesne and Küster, 2010; Reichmuth et al., 2009). In BEWS protocols, *D. magna* is widely used because of its high sensitivity to pollutants and rapid behavioural responses when faces a change in water quality (Jeong et al., 2014). However, several studies clearly indicated that acute toxicity (single or in mixtures such as effluents) may change considerably among species (Maltby et al., 2005; Rubach et al., 2010; Wogram and Liess, 2001) depending on the toxicological mode of action of the chemicals (Escher and Hermens, 2002), and traits of species (Nyman et al., 2014).

To the best of our knowledge, there are a limited number of studies which compares sensitivity in terms of behavioural responses of different species. Consequently, there is a need to investigate in deeper details the range of species sensitivity to contaminants both in terms of toxicity and induced behavioural changes. This would be very important firstly for improving the knowledge of the potential harmful effects of STP effluents in aquatic environments. Secondly, to understand if using *D. magna* in BEWS protocols would be sufficiently protective to resident species, particularly when the concern is for pristine environments such as Alpine surface water ecosystems. Chironomids (order Diptera, family Chironomidae) have

been proposed as the best macroinvertebrate bioindicators of water quality at high altitudes because they typically dominate the fauna in terms of individual abundance and species number in these environments; species of the genus *Diamesa* in particular are associated with pristine conditions (Lencioni et al., 2012).

In the next paragraphs, we will discuss the obtained results in order to explore the variability of responses of the two species to STP dilutions and to verify if the use of *D. magna* as WET test species would be sufficiently protective for species such as *D. cinerella gr* which are present in Alpine environments.

Variability in D. magna

The results reported in **Table SI-7.1**, indicate an apparent inverse concentration-response relationship in *D. magna*. Indeed, the mortality increased with the increasing dilutions of STP effluents and exposure time. However, Dunnett's test and Tukey's test (p >0.05) indicate no significant differences among the control and undiluted and diluted STP effluents samples. According to US EPA (2000), this result may not indicate toxicity of the sampled effluent. Even if not statistical significant the slight mortality could be attributed to changes in abiotic conditions (e.g. hardness, pH). Moreover, changes in pH can influence the bioavailability and toxicity of chemical constituents, such as some metals (e.g., Cu, Zn).

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As previously reported in the results section (**Figs 7.1-2**), significantly changes in swimming capabilities (activity time, distance moved, and velocity) were measured in *D. magna* specimens exposed to different STP effluent dilutions, indicating an overall condition of stress for this organism. In general, the highest stress was measured at STP/10 dilution; indeed, all behavioural investigated parameters were significantly affected at this dilution. On the contrary, undiluted STP output seemed to not particularly influence swimming parameters (with the exception of active time after 48 hrs of exposure) whereas at STP/100 and STP/1000 measured changes were random.

At STP/10 dilution, the average speed significantly increased during all the exposure period, whereas at STP/100 a significant increase was measured only after 24 hrs of exposure with a recovery after 48 hrs. The increase of the average speed has been associated to the avoidance mechanism which is the escape from contamination or from undesirable abiotic conditions (Wolf et al., 1998). Contrarily to average speed, changes in the distance moved and active time have shown a different trend. Indeed, after 24 hrs of exposure both parameters were significantly increased at STP/10 (the active time also at STP/1000) whereas decreased after 48 hrs. The explanation of these opposite responses is not easy. Recently, Ferrario and coworkers (2017) highlighted a reduction in the active time and partially in the distance moved in *D. magna* specimens exposed to different concentrations of the insecticide chlorpyriphos after 48 hrs of

exposure; the authors suggested that the reduction in both parameters was due to the loss of energy spent by to organisms to activate detoxifying enzymes and the mechanism of avoidance (increased average speed). Analogously, in our study, we hypothesize that the organisms spent part of their available energy to activate these mechanisms of defense.

Variability in D. cinerella gr

The exposure of *D. cinerella gr* larvae to diluted STP effluents provoked some behavioural alterations in this organism. On the contrary, undiluted samples did not affect their behaviour. The most affected parameter seemed to be the distance covered, which has shown a significant reduction in all the diluted STP effluents samples both at 24 hrs and 48 hrs of exposure. Variations in average speed and BBps were randomly observed (STP/1000 after 24 hrs and 48 hrs for average speed and BBps respectively). To the best of our knowledge, there are no previous studies investigating the behavioural changes induced by contaminants in *D. cinerella*. Very recently, Villa and coworkers (2018) developed an experimental protocol to investigate behavioural changes on *D. cinerella* larvae exposed to several pharmaceuticals and plant protection products with different toxicological mode of action. The exposed organisms were sensitive to chemical stressors; indeed, behavioural alterations were measured at concentration below the LOECs (Lowest Observed Effect

Concentrations) of the investigated compounds. Also in this study, the most affected behavioural parameter was the moved distance which showed a general trend of reduction. Our study confirms these evidences and indicates this parameter as the most relevant to be included in behavioural studies for the genus *Diamesa*. As for *D. magna* a possible explanation of the reduction of the distance cumulatively travelled by *Diamesa* can be related to the attempt of organisms to contrast changed conditions (presence of contaminants, different abiotic conditions) and the loss of energy for muscle activity needed to overcome the friction of the aquatic medium during movement. Indeed, changes in external conditions, such as the burden imposed by toxic compounds or changes in the habitat conditions, 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) by activating antioxidant and detoxifying enzymes (Untersteiner et al., 2003; Wolf et al., 1988; Binelli et al., 2011; Colwill and Creton, 2011).

Comparison between D. magna and D. cinerella gr

A direct comparison of sensitivity of *D. magna* e *D. cinerella* to contaminants and/or effluents discharge is not a simple task. These organisms are present in different habitats: *D. magna* is a typical planktonic organism which inhabits most types of standing freshwater. On the contrary, chironomids are often a quantitatively

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important component of benthic invertebrate communities. In addition, *D. cinerella* is endemic of krion environments which comprise very cold brooks fed by water from melting glaciers. In these conditions it seems obvious that in their evolutionary path these organisms developed different traits and defence mechanisms to cope with changing conditions and stress. This become more evident in consideration that even within the same species there can be differences in sensitivity; for example, tests with the same effluents have found differences in response depending on which water flea species is tested (Chapman, 2000).

However, in consideration that in WET test *D. magna* is most utilized organism as a surrogate of resident species it seems relevant to verify even if sketchily if the use of *D. magna* in WET test would be sufficiently protective of Alpine environments where the presence of STPs is widespread. For instance, in our study the effluents were collected from a treatment facility located in an important touristic area in Northern Italy and where *D. cinerella* is present.

Based on the obtained results some consideration can be made:

- Both organisms were not particularly sensitive (in terms of mortality) to undiluted and diluted effluents. A slight higher mortality was observed in *D. magna* (not statically significant). This is in line with literature that indicates chironomids as least sensitive than daphnids in their responses to various toxicants (Cowgill and Williams, 1989). In addition, the undiluted STP

samples did not produced any variations in the behaviour of both species. In **Figs 7.1-2** no significant differences were found between the two species both after 24 hrs and 48 hrs of exposure.

- The obtained results highlighted that STPs diluted effluents have the potential to produce significant modifications in the behaviour of both organisms. Particularly, the two species have shown significantly behavioural changes when exposed to STP/10 dilution samples. In this *D. magna* seemed to be more sensitive than *D. cinerella* as all the three behavioural parameters were significantly affected at this dilution.

  On the other hand, the two organisms presented differences in terms of behavioural responses. In *D. magna* the most influenced swimming parameters was the average speed which increased significantly even after 48 hrs of exposure at STP/10 dilution. On the contrary, *D. cinerella* experienced a significant decrease in the distance moved. It is very hard to explain the bases of these differences. Probably, they derive from the different strategies of the two organisms to cope with unfavourable environments. *D. magna* is a pelagic organism and perhaps the easiest strategy to react to stress is the avoidance mechanism which results in swimming away from an unfavourable condition; as previously described avoidance has been associated to an increase of the average speed. On the contrary, in the benthonic organisms such as *D. cinerella* the strategy is to activate the adaption mechanisms

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that involve antioxidant and detoxifying enzymes. This led to an energy expenditure that reduces the moved distance of the organisms. The ecological meaning of a reduction in the distance moved cannot be easily understood as changes in the behaviour of a key-species such as *D. cinerella* can lead to unpredictable repercussions at higher levels of ecological organization. On the other hand, WET tests (and particularly behavioural tests) are performed to get signals of toxic effects (including behavioural changes) of the effluents. The lesson learned in this study is that both organisms seemed to react in a similar way to the tested STP effluent even if with different strategies. Consequently, the use of *D. magna* as WET test species could be sufficiently protective also for *C. cinerella*; indeed, behavioural changes in *D. magna* could be considered as a signal of changes in the chironomid. As mentioned above in the Introduction section, the use of *D. magna* would has the undoubtful advantage of the ease of handling and cost effective of culture conditions in the laboratory.

7.5 Conclusion remarks

In this study, we demonstrated that, even if the discharge of effluents from an alpine STP not provoke significant mortality events on *Daphnia magna* and *Diamesa cinerella gr,* it has the potential to affect the behaviour of both species. Particularly, both tested organisms have shown significant alteration of their behaviour. Surprisingly, the

undiluted effluent has not determinate any observable effects on the two tested species, while the greatest effect was recorded on both species exposed at a ten times diluted effluent. Moreover, it has been reported that *D. magna* and *D. cinerella* use different behavioural strategies to react to the presence of adverse environmental conditions; nevertheless, we have shown that their overall sensitivity is similar. On the basis of the reported results, it can be concluded that the use of *Daphnia magna* in Biological Early Warning Systems protocols seem to be sufficiently protective also for resident species of Alpine freshwater ecosystems.

Acknowledgements

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Chapter 8

General conclusions

The Ph.D. activities have been focused on two aspects which are recognized as current challenges in ecotoxicology with the purpose to improve knowledge and propose as well new approaches that would be useful in the risk assessment procedures.

In particular, the present work has studied the effectiveness of glaciers at mid-latitudes to act as cold condensers for contaminants undergone atmospheric transport with the aim of better understanding the environmental fate of these chemicals in temperate-zone mountain regions. On the other hand, the attention has been focused on sub-lethal effects on no-target organisms induced by the exposure to environmental concentrations of pollutants commonly detected in surface water systems. Especially, the efforts were devoted to understand the potential link of stress signals across different levels of bio-ecological hierarchy in order to unveil information able to predict the stress responses at levels of population and community.

8.1 Environmental fate of contaminants in temperate-zone mountain regions

In the present work, the contamination of high-altitude regions was investigated in order to improve the protection of these regions, often overlooked (Vighi et al. in Castro-Jiménez, 2007). In particular the attention was focused on the Italian Alps, which represent an

interesting area to investigate the presence of POPs and Emerging Contaminants (ECs). In fact, there are several studies showing the presence and the accumulation of POPs in these environments (Villa et al., 2003; 2006a, 2006b; Bogdal et al., 2009; Pavlova et al., 2016). In addition, these mountains lie close to industrialized and agricultural areas, which represent significant emission sources of ECs. However, it is largely unknown if these compounds, developed as a less persistent alternative of POPs and with atmospheric lifetimes too short to be transported to Polar Regions, can potentially reach remote cold areas because of MRAT (Medium Range Atmospheric Transport) process.

Within this work, a large number of airborne organic pollutants was found in meltwater samples collected in three Alpine glaciers. In particular, the obtained results underlined two contrasting processes leading to glaciers contamination. Indeed, although a declining trend of POPs (HCHs, HCB, DDTs, PCBs) was confirmed, the finding of Current Use Pesticides (CUPs) (chlorpyrifos and terbuthylazine) and musk fragrances (HHCB, AHTN) in meltwater samples has showed the potential accumulation processes of some ECs in the glaciers at mid-latitudes because of MRAT process. In particular, the constant level of musk fragrances recorded in melt water from Italian Alpine glaciers is associated to the high and continuous releases in the urbanized areas present in the Po River plain, which explains the widespread contamination of the Alps found for these compounds. At

the same time, the occurrence of CUPs has been recorded with a spatial and temporal variability, which is clearly related to the agricultural practices in the adjacent plain.

Based on the presence of ECs in alpine meltwater, a study of the potential degradation of chlorpyrifos (CPF) on glacier at mid-latitudes was carried out. Indeed, when deposited on glaciers, pollutants undergo partitioning among different environmental matrices (e.g. snow, ice, water, interstitial atmospheric gases and supraglacial sediments) and post-depositional alteration processes. Among those environmental matrices, cryoconite represents a potential sink for organic pollutants because of its high content of organic matter. However, its role in the accumulation of organic pollutants and its contribution to the removal them is excluded in models predicting the environmental fate of these compounds in cold areas. This is remarkable since the results of *in situ* microcosm experiment, carried out on Forni Glacier, highlighted that the degradation in the cryoconite hole ecosystem is a relevant process affecting CPF fate in the cryosphere. In particular, this study underlined that biodegradation contributes to the removal of this pesticide from the glacier surface more than photo- and chemical degradation and suggested that organic contaminants can represent a source of nutrient for microbial communities living on glaciers. This might suggest that microbial degradation can contrast the accumulation of pollutants transported on glaciers and the possible re-emission of contaminants back to the

atmosphere or to the freshwater systems. This is particularly true because cryoconite holes are present on glaciers during the same times of the year when most insecticides, such as CPF, are applied in lowland agricultural areas (late spring to late summer) and atmospheric conditions favour their transport to Alpine summits. These reasons and the relative extent of biodegradation documented in the present study implies that environmental fate models of pesticides in glacierized areas should account for biodegradative processes.

8.2 Sub-lethal effects induced by ECs environmental concentrations on no-target organisms

The consequences of exposure to xenobiotics in natural ecosystems are still largely unknown and this stands in stark contrast to the protection goals of Environmental Risk Assessment (ERA), which include the protection of populations and communities. This is particularly true for ECs, whose adverse effects towards non-target organisms have been only recently highlighted. Increasing laboratory evidences showed that the exposure to "environmentally relevant" concentrations of different ECs may induce several adverse effects to organism (Duquesne and Küster, 2010; Brodin et al., 2013; Bean et al., 2014), including changes in behaviour or physiology, which could affect fitness, and consequently the population dynamics (Brodin and Johansson, 2004; Smith et al., 2008). However, until now, there is a

dearth of information on the linkages among the different levels of bio-ecological organizations.

In this context, *Daphnia magna* individuals were exposed to environmentally relevant concentrations of two widespread ECs, namely CPF and benzoylecgonine (BE). The results of these studies showed that both ECs induce several adverse effects on the tested specie. In particular, BE concentrations similar to those found in aquatic ecosystems induced oxidative stress and inhibited AChE activity, thus affecting swimming behaviour and the reproduction of *D. magna* individuals. In the same way, daphnids exposure to environmental levels of CPF were in a condition of stress, which was highlighted by changes in both molecular and behavioural biomarkers. In addition, testing two different concentrations of CPF has allowed to notice that the activation - induced only by the higher considered concentration - or non-activation in some enzymes activities can lead to different modifications of the swimming behaviour in *D. magna* suggesting the existence of a link between sub- and supra-organismal levels.

These results showed that environmental concentrations of ECs can induce adverse effects on no-target organisms. This is particularly alarming because the continuous use of these chemicals can confer to them a sort of pseudo-persistence, and then their consequent increase in concentration can be expected.

Finally, although these studies demonstrated that sub-lethal concentrations of BE and CPF lead to enzymatic and behavioural

changes in *D. magna*, these effects cannot be directly related to those on organisms' fitness or at higher ecological hierarchical level in a quantitative way. Therefore, these endpoints cannot be considered into an environmental risk assessment procedure at this time and more effort should be done in this direction, especially for improving the use of biomarkers as early warning indicators of risk.

In addition, the results of the present work, suggested that a deeper knowledge of these endpoints could also contribute to the protection and safeguard of water quality of Alpine river streams. Indeed, comparing the behavioural responses, overall sensitivity to treated effluents from sewage treatment plants of *D. magna* is similar to that of *Diamesa cinerella* gr, which is considered a key-species in the Alpine aquatic ecosystems (Lencioni and Rossaro, 2010). Accordingly, the use of *D. magna* in behavioural ecotoxicological tests seem to be sufficiently protective also for the safeguard of cold adapted local species of Alpine freshwater ecosystems, which are threatened by chemical contaminants, such as organic pollutants undergone MRAT process.

8.3 The applicability of the results in ERA

The results of the present work provide evidence in support of the need to improve the ecological realism of exposure and the effect assessments in ERA, challenge that the European Commission is currently facing (EC, 2013).

About these challenges, the finding of CUPs in meltwater samples with a spatial and temporal variability, which is clearly related to the agricultural practices, confirmed the necessity of promoting the use of modelling that considers the properties and complexity of potentially exposed ecosystems, such as the spatiotemporal variability of environmental scenarios and pollutant emissions. The results of this project, mainly focused on cold regions, also suggested that environmental fate models organic contaminants in glacierized areas should account for biodegradation processes. Indeed the obtained outcomes showed that these pollutants could represent a source of nutrient for microbial communities living on glaciers, whose degradation activity can contrast significantly the accumulation of organic contaminants transported on glaciers and their possible re-emission back to the atmosphere or to the freshwater systems.

Regarding effect assessment, the results of the present work provide evidence in favour of a shift from ecotoxicology to the stress ecology approach in ERA in order to describe the consequences on ecosystems as changes in the combinations of functional traits. Currently the ecotoxicological effects of chemical compounds are evaluated by means of standardised toxicity tests, which are performed on target organisms considered representative of the exposed ecosystems (Hood, 2005; Stadler, 2011). In this context, the obtained results allowed to underline significant aspects. Firstly, these outcomes showed that "environmentally relevant" concentrations of different ECs can induce adverse effects on exposed organisms. In particular,

the recorded changes in molecular and behavioural biomarkers in *Daphnia magna* exposed to sub-lethal concentrations suggested that the PNECs estimated by the traditional ERA approach could be not sufficiently protective to avoid adverse effects on each element of the biological communities. On the other hand, the results highlighted the possible existence of a link between sub- and supra-organismal levels. This aspect is particularly important because the knowledge of the links between the responses measured at a particular hierarchical level and those measured at the adjacent levels would be very effective in risk assessment procedures, mostly for improving the use of biomarkers, such as behavioural changes, as early warning indicators of adverse effects of pollutants on populations and communities. However, until now, there is a dearth of information on the linkages among the different levels of bio-ecological organizations and more effort should be done in this direction. In addition, the present work investigated the use of Daphnia species as model organism in ecotoxicological tests to represent also the organisms adapted to extreme conditions. In this, the outcomes showed that *D. magna* and *D. cinerella*, a key-species in the Alpine aquatic ecosystems (Lencioni and Rossaro, 2010), use different behavioural strategies to react to the presence of adverse environmental conditions; nevertheless, their overall sensitivity is similar. Finally, on the basis of the reported results, it can be concluded that the use of *Daphnia magna* in behavioural ecotoxicological tests seems to be sufficiently protective for resident species of Alpine freshwater ecosystems and that a deeper

knowledge of behavioural endpoints could also contribute to the protection and safeguard of remote areas.

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Chapter 9

Supporting information

Legacy and emerging contaminants in meltwater of three Alpine glaciers

Section 3.1 Details on oven program and MS setting

Three oven programs were set.

- For ECs the oven program was the following: starting temperature of 50 °C, 15 °C/min to 160 °C, 4 °C/min to 200 °C, hold for 0.80 min, 1 °C/min to 205 °C, 30 °C/min to 280 °C, hold for 2.37 min.

- For PCBs the oven program was the following: starting temperature of 90 °C, 20 °C/min to 160 °C, 1.5 °C/min to 220 °C, hold for 6 min, 30 °C/min to 280 °C, hold for 3 min.

- For DDTs, HCB and HCHs the oven program was the following: starting temperature of 80 °C, 15 °C/min to 160 °C, 5 °C/min to 200 °C, hold for 1 min, 5 °C/min to 240 °C, 10 °C/min to 270 °C, 10 °C/min to 280 °C, hold for 3,33 min.

The MS was set in selected ion monitoring with SIM mode and retention time (min), identification and quantification ions of analytes are shown in the **Table SI-3.1.**

The detection limit of analyzed was from 2.1 to 6.3 pg/L for POPs considered, terbuthylazine, HHCB and AHTN, and from 5.2 to 15.6 pg/L for chlorpyrifos and pendimethalin.

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Table SI-3.1 Analyzed chemicals retention time and characteristic fragments

Chemical name	Retention time (min)	Characteristic fragments	Specific mass-to-charge ratio
α-HCH	12.04	181-219	181/219= 0.9
HCB	12.18	284-286	284/286=1.2
γ-HCH	13.10	181-219	181/291= 0.9
TBZ	15.87	214-229	214/229= 2.80
HHCB	17.38	243-258	243/258= 4.30
AHTN	17.38	243-258	243/258= 4.30
o,p' DDE	19.64	246-318	246/318= 2
CPF	20.32	314-197	314/197= 1.30
p,p' DDE	20.89	246-318	246/318= 0.8
o,p' DDD	21.12	235-237	235/237= 1.5
PEN	22.26	252-281	252/281= 7 .05
p,p' DDD	22.47	235-237	235/237= 1.5
o,p' DDT	22.57	235-237	235/237=1.5
p,p' DDT	23.80	235-237	235/237= 1.5

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Section 3.2

Section 3.2.1 The OECD Pov and LRTP screening tool model

This model is currently used as a screening tool for making comparative assessments of environmental hazard properties of non ionizing chemicals, using metrics of overall persistence (P_{OV}) and long-range transport potential (LRTP) (Öberg and Iqbal, 2012; Mostrag et al., 2010). It incorporates a steady-state fugacity-based model (Mackay, 2001) in which troposphere, soil surface layer and seawater surface layer are considered as the three main environmental compartments; furthermore, equilibrium partitioning is assumed between sub-compartments belonging to the same main compartment. Further details on the characteristics of the chemical fate model incorporated in the software can be found in Wegmann and coworkers (2009). As substance-specific inputs, the software requires the air-water partition coefficient (K_{AW}) and the octanol-water partition coefficient (K_{OW}), as well as the degradation half-lives (DT_{50}) in soil, water and air. For the compounds considered in this study, data are listed in **Table SI-3.2**.

From these inputs, P_{OV} and two LRTP indicator values (CTD: characteristic travel distance and TE%: transfer efficiency) are calculated. The values of these three indicators are dependent on the mode of emission (into air, water or soil); the software calculates their values for each of the three possible emission scenarios and selects the highest values found. The P_{OV} (days) gives a measure of degradation

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time of a chemical in the whole environment; it is calculated for each mode of emission according to Eq. (1) (Wegmann et al., 2009):

$$P_{OV,i} = \frac{M_{i,TOT}}{F_{DEG\,i,A} + F_{DEG\,i,W} + F_{DEG\,i,S}}$$

(1)

where $M_{i,TOT}$ (kg) is the total amount of contaminant at steady-state and $F_{DEG,i,A}$, $F_{DEG,i,W}$, and $F_{DEG,i,S}$ are the degradation mass fluxes in air (A), water (W) and soil (S) (kg/h), respectively.

The CTD (unit in km) is the distance at which the chemical's concentration has fallen to about 37% of its initial value (at the point of release), assuming that the chemical is transported by a constant flow of air (wind speed = 4 m/s) or water (0.02 m/s). It represents the potential of a chemical to be transported over long distances in air or water and is calculated using **Eq. (2)** (Wegmann et al., 2009):

$$CTD_i = \frac{M_{i,TOT}}{F_{i,E}} \times \frac{M_{i,i}}{M_{i,TOT}} \times v_i$$

(2)

The first term in the equation is the overall residence time in the multimedia environment (h), which is the ratio of the total mass at steady-state for the given mode of emission ($M_{i,TOT}$, kg) divided by the emission mass flux, $F_{i,E}$, that enters medium i. The second term in Eq. (2) is the dimensionless mass fraction in the mobile medium, which is the same as the medium that receives the emissions (Mi,i, kg) divided

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by the total mass at steady-state for the given mode of emission ($M_{i,TOT}$, kg). Finally, vi (km/h) is the assumed transport velocity in the mobile medium.

The TE (%) is an indicator of potential for atmospheric transport and deposition of the parent compound in a remote region and is calculated for emissions to air, water and soil according to **Eq. (3)** (Wegmann et al., 2009):

$$TE_i = \frac{F'_{i,D}}{F_{i,E}} \times 100$$

(3)

where $F_{i,D}$ (mol/h) is the atmospheric deposition mass flux in a target region and $F_{i,E}$ (mol/h) is the emission mass flux in a source region.

Table SI- 3.2 CUPs sales data in Northern Italy and some their relevant properties

Chemical Name	Sales data in North Italy[a] (tons of a.i.)	Molar Mass (g/mol)[b]	log K_{aw}[c]	log K_{ow}[b]	DT$_{50}$ air (h)	DT$_{50}$ water (h)	DT$_{50}$ soil[b] (h)
glyphosate	937	169.10	-10.07	-3.20	38.40[e]	1,656[e]	288
metam-sodium	746	129.19	-8.47	-2.91	2[d]	52.80[d]	168
fosetyl-aluminium	392	354.10	-12.89	-2.10	46[d]	103.2[d]	2.40
S-metolachlor	334	283.80	-6.05	3.05	5[e]	288[e]	360
terbuthylazine	292	229.71	-5.78	3.40	35[d]	4,704[f]	1,802
chlorpyrifos	231	350.89	-3.55	4.70	24[e]	720[e]	1,776
dithianon	191	296.32	-10.26	3.20	6.30[d]	12.12[d]	252
captan	164	300.61	-6.92	2.50	1.50[d]	4.90[d]	19.20
metam-potassium	94	145.28	-8.47	-2.91	2[d]	52.80[d]	168.
pendimethalin	83	281.31	-5.92	5.20	12[e]	504[e]	2,160
oxadiazon	44	345.20	-4.81	5.33	5.28[d]	3,048[d]	5,040
MCPA	43	200.62	-7.65	-0.81	18.72[e]	324[e]	576

[a] APPA, 2012, referred to Piemonte, Lombardy, Trentino Alto Adige, Friuli Venezia-Giulia,Veneto Regions
[b] PPBD Pesticide Properties Database; [c] Calculated from Henry's law constant (25°C) from PPDB
[d] EFSA Conclusion; [e] European Commission EU Pesticides Database; [f] Grenni, 2011

Section 3.2.2 Results of the application of the OECD Pov and LRTP screening tool model for the selection of CUPs to be included in the monitoring campaigns

Although important Italian cities (e.g. Milan, Turin, Bergamo, Verona) and industrial activities are located in the Po River plain, this area is still characterized by the presence of an intensive agriculture (almost the 30% of the total cultivated lands in Italy) (ISTAT, 2010). The agricultural activities are mainly focused in the production of arable crops (particularly maize), vineyards and fruit trees (**Fig. 3.1** in **Chapter 3**). These farming activities are intensive in the use of pesticides, consuming a total of about 14,800 tons of active ingredients in 2012 (APPA, 2012). During their spray application, a fraction of the applied dose can be lost in atmosphere. In addition, post-application emissions, involving volatilization from soil and plants and wind erosion of soil particles containing sorbed pesticides represent further significant pesticide input into the troposphere for several days or weeks after application (Bedos et al., 2002; Voutsas et al., 2005). The capability of pesticides to travel short or long distances depends on the amount of time it resides in the atmosphere, which is related to their chemical-physical properties and persistence. In addition, meteorological factors can influence the movement of polluted air masses (Addo et al., 1999).

In **Table SI-3.2**, the most widely used pesticides in North Italy are reported. From the available data, there are at least 12 CUPs that are used in quantities exceeding 10 tons of a.i. per year (many of them are

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used in quantities exceeding the 100 tons a.i. per year). This information can be useful as a preliminary step to identify those compounds that could have the potential of contaminating the investigated areas (the greater is the use, the higher the potential of contamination). However, as previously described, the capability of these compounds to be transported away from the area of emission is mainly depending by their properties. Based on this consideration, as a further screening, to identify among the 12 compounds those having the highest potential to reach the Alpine glaciers, "The OECD Tool" model was applied. In this way, a selection of CUPs to be included into the monitoring campaigns was made.

The results of "The OECD Tool" application are reported in **Fig. SI-3.1**. The thin black line in each plot defines the maximum LRTP that is possible for a given P_{OV}. In addition, in both plots vertical and horizontal reference lines are present. According to the classification scheme proposed by Klasmeier et al., 2006, the vertical line separates high and low persistent substances, whereas the horizontal line forms boundary between those chemicals with POP-like potential for long range transport from those substances that are expected to be less mobile in the environment. In both plots, the majority of the investigated CUPs is located in the bottom left quadrant.

Based on the calculated values of CTD and P_{OV}, pesticides are subdivided in two categories:

- in the first group are included glyphosate, oxadiazon, pendimethalin (PEN), chlorpyrifos (CPF) and terbuthylazine (TBZ) which have a P_{ov} higher than 100 days and a CTD above 150 km, value potentially sufficient to cover the distance from Po river plain to alpine cold remote sites;

- the second group comprises MCPA, captan, dithianon, fosetyl- aluminium, metam-sodium, metam -potassium and S-metolachlor and has a P_{ov} of about 1-26 days and a CTD shorter than 100 km. These substances can be classified not harmful for the alpine cold ecosystem due to their low persistence and low travel potential.

The second indicator used to describe LRTP is the estimation of how much contaminant can reach a certain distance. The majority of the selected CUPs exhibits a TE values below 0.1%, while only PEN, CPF and TBZ reached 0.2%, 0.5% and 0.8% respectively. Moreover, TBZ is the only substance falling in the bottom right quadrant.

Based on the considerations presented, PEN (P_{OV} of 129 days, CTD of 377 km), CPF (P_{OV} of 106 days, CTD of 457 km) and TBZ (P_{OV} of 282 days, CTD of 483 km) were selected for the analytical determinations in glacial melt water samples.

In literature, different CTDs, ranging from 62 to 430 km, are reported for CPF (Hoferkamp et al., 2010; Mackay et al., 2014; Muir et al., 2004). The variability of DT_{50} in air, from 3 to 24 hrs, determines the differences in CTD values. The long-range transport potential depends strongly by half-life in air which is influenced by •OH radical concentration. Mackay and coworkers (2014) reported that conservative value assumed lesser concentration of •OH and therefore higher DT_{50} in air, while minor levels of •OH are more appropriate for conditions in remote regions and at higher latitudes. The selection of the proper model input data is a crucial point in order to have reliable information on LRAT, but it has to be considered that all the three investigated alpine peaks are much closed to agricultural areas. In particular, apple and wine crops, where CPF is mostly used, are 70, 40 and 10 km far from Lys, Forni and Giogo Alto Glacier, respectively. In such situation, a shorter DT_{50} value is not relevant, as the distance is not sufficient to prevent CPF to reach the glaciers.
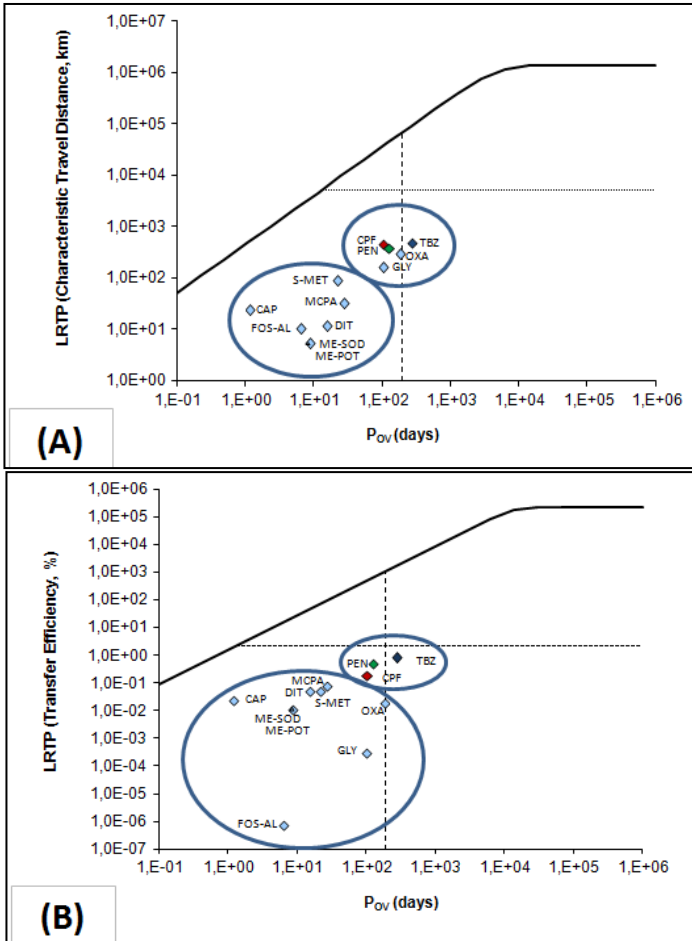
Figure SI- 3.1 The calculated P_{ov}, CTD and TE of the most sold CUPs in north of Italy . (A): P_{ov} vs CTD and (B) P_{ov} vs TE. CAP= captan, CPF=chlorpyrifos, DIT= dithianon, FOS-AL= fosetyl-aluminium, GLY= glyphosate, ME-POT=metam-potassium, ME-SOD = metam-sodium, OXA= oxadiazon, PEN= pendimethalin, S-MET= S-metolachlor, TBZ= terbuthylazine

Table SI-3.3 Chemicals concentrations (ng/L) in glacial melt water samples (nd= not detected)

	LYS July	LYS Oct	FORNI July	FORNI Sept	GIOGO July	GIOGO Sept
α-HCH	0.62	0.74	nd	nd	nd	nd
γ-HCH	0.34	0.44	nd	nd	nd	nd
∑HCHs	**0.96**	**1.18**	**nd**	**nd**	**nd**	**nd**
α/γ ratio	**1.8**	**1.67**	**-**	**-**	**-**	**-**
HCB	0.03	0.03	nd	0.04	nd	0.02
o,p' -DDE	nd	nd	nd	nd	nd	nd
p,p'- DDE	0.04	nd	nd	0.27	nd	0.10
o,p' -DDD	nd	nd	nd	nd	nd	nd
p,p' -DDD	nd	nd	nd	nd	nd	nd
o,p'-DDT	nd	nd	nd	nd	nd	nd
p,p'-DDT	0.23	0.33	0.46	0.23	nd	0.10
∑DDTs	**0.27**	**0.33**	**0.46**	**0.50**	**nd**	**0.20**
p,p'-DDE/p,p'-DDT ratio	**0.17**	**<1**	**<1**	**1.17**	**-**	**1**
PCB 28	0.45	nd	0.20	nd	nd	nd
PCB 52	0.30	0.24	0.14	0.28	0.43	0.58
PCB 101	0.01	nd	nd	nd	nd	0.01
PCB 153	nd	nd	nd	nd	nd	nd
PCB 180	nd	nd	nd	nd	nd	nd
∑PCBs	**0.76**	**0.24**	**0.35**	**0.28**	**0.43**	**0.60**
TBZ	1.98	1.23	0.29	0.13	nd	nd
CPF	0.43	0.21	1.06	0.50	1.02	0.37
PEN	nd	nd	nd	nd	nd	nd
∑CUPs	**2.41**	**1.44**	**1.35**	**0.63**	**1.02**	**0.37**

	LYS July	LYS Oct	FORNI July	FORNI Sept	GIOGO July	GIOGO Sept
AHTN	1.69	1.15	1.79	0.87	0.95	1.18
HHCB	1.15	1.28	1.57	1.06	0.99	1.46
∑Musks	**2.84**	**2.43**	**3.36**	**1.93**	**1.94**	**2.64**
HHCB /AHTN ratio	**0.68**	**1.11**	**0.88**	**1.22**	**1.04**	**0.97**

Bacteria contribute to pesticide degradation in cryoconite holes in an Alpine glacier

Table SI-4.1 Main features of reconstructed genomes.

Bin name	Completeness	Genome size	GC content	Relative coverage (%)
bin.001	99.10%	6566446	45.3	15.5
bin.002	57.00%	2078626	49.4	11.8
bin.003	22.40%	1130720	51.9	9.7
bin.004	0.90%	1619294	46	5.4
bin.005	86.00%	3406920	53	3.4
bin.006	91.60%	4866341	65.9	6.0
bin.007	76.60%	3407166	71.9	3.1
bin.008	84.10%	3557694	34.8	2.2
bin.009	28.00%	1587120	49.3	1.8
bin.010	89.70%	3244141	62.2	2.4
bin.011	87.90%	4248238	64	2.2
bin.012	71.00%	4372467	45.9	2.6
bin.013	91.60%	4965869	51.9	1.4
bin.014	61.70%	1340877	51.7	1.5
bin.015	38.30%	1212234	50.6	1.3
bin.016	96.30%	5462924	66.3	0.9

bin.017	96.30%	7193750	64.8	1.3
bin.018	5.60%	3017705	40.2	1.2
bin.019	44.90%	3505594	64.7	0.9
bin.020	94.40%	4107290	33.4	0.6
bin.021	66.40%	3801101	71.2	0.9
bin.022	42.10%	5174060	59.9	0.8
bin.023	96.30%	4446526	36.6	1.3
bin.024	95.30%	3211182	38.9	1.1
bin.025	68.20%	3345290	63.3	0.7
bin.026	81.30%	3094092	39.4	0.5
bin.027	63.60%	3552269	67.4	0.7
bin.028	41.10%	6613659	71	0.7
bin.029	88.80%	3409456	55.1	0.6
bin.030	17.80%	3376074	64	0.6
bin.031	53.30%	3903762	68.3	0.8
bin.032	92.50%	5449895	55	0.7
bin.033	93.50%	5200888	62.9	0.6
bin.034	77.60%	3495851	66.1	0.7
bin.035	72.00%	4178954	64.3	0.7
bin.036	32.70%	2724162	49.9	0.6

bin.037	78.50%	5229757	41	0.5
bin.038	24.30%	6136018	45.2	0.8
bin.039	90.70%	4033377	54.8	0.4
bin.040	93.50%	4792978	45	0.5
bin.041	62.60%	3881109	69.9	0.4
bin.042	15.90%	1784250	49.1	0.4
bin.043	92.50%	4235000	64.9	0.4
bin.044	72.00%	5419381	63.8	0.6
bin.045	81.30%	6458785	35.9	0.6
bin.046	66.40%	5122121	38.4	0.5
bin.047	82.20%	7324493	59.3	0.4
bin.048	30.80%	995751	54.9	0.4
bin.049	20.60%	5109457	61.9	0.5
bin.050	77.60%	5282656	57.2	0.5
bin.051	71.00%	6180564	62.1	0.4
bin.052	40.20%	3715025	67.1	0.3
bin.053	38.30%	2475458	54.7	0.3
bin.054	83.20%	4320545	54.7	0.4
bin.055	10.30%	892804	55.4	0.4
bin.056	41.10%	2770692	66.6	0.3

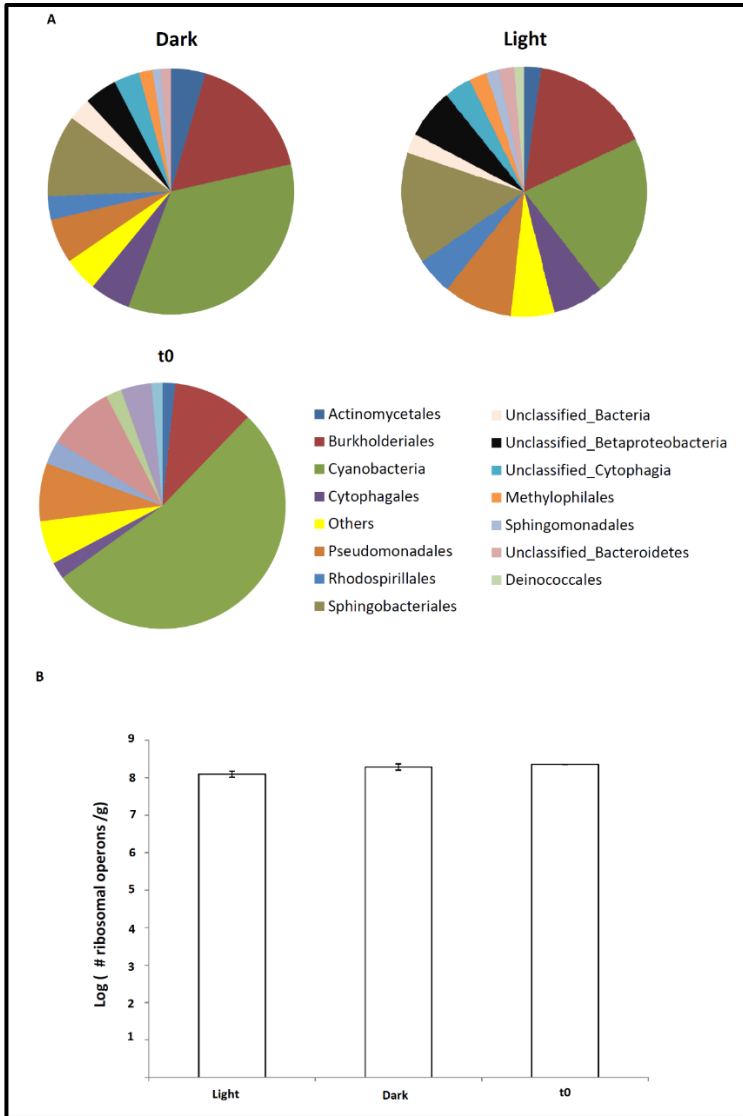| bin.057	42.10%	5235295	66.7	0.3
bin.058	72.00%	5867742	32.5	0.4
bin.059	17.80%	1817514	56.5	0.3
bin.060	17.80%	2647568	29.1	0.5
bin.061	53.30%	3250286	33.9	0.3
bin.062	87.90%	7532294	60.9	0.4
bin.063	0.00%	407315	49.4	0.8
bin.064	30.80%	2037137	42.6	0.3
bin.065	21.50%	1121985	47.9	0.3

Figure SI-4.1 Composition of bacterial communities in t_0 cryoconite and microcosms (A), and abundance of 16S rRNA gene copies (B).

Figure SI-4.2 One rack containing bottles used for microcosm experiments on the surface of Forni Glacier. Racks were covered with a plastic net and bound to a wooden stake drilled into the ice to avoid bottle overturning and dispersal.

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Comparison of the behavioural effects induced by treated sewage effluents on *Diamesa cinerella gr* larvae (Diptera, Chironomidae) and *Daphnia magna* (Cladocera, Daphniidae)

Table SI-7.1 Measured mean values (M), standard deviation (SD) and relative standard deviation (RSD) for the Active Time (%), the Average Speed (mm s^{-1}) and the total Distance Moved (mm) on <u>*Daphnia magna*</u> in CTRL, STP, STP/10, STP/100 and STP/1000 tests after 24 hrs and 48 hrs of exposure

Treatment	Dead	Active time			Avg. Speed			Distance moved		
	N	M	SD	RSD	M	SD	RSD	M	SD	RSD
24 hrs										
CTRL	0	24.3	3.04	0.12	2.03	0.57	0.28	12.9	3.25	0.25
STP	0	26.1	6.58	0.25	2.24	1.16	0.52	13.1	6.33	0.48
STP/10	0	31.1	7.69	0.24	2.57	0.62	0.24	18.2	5.40	0.3
STP/100	2	26.0	5.37	0.20	2.62	0.87	0.33	14.9	5.40	0.36
STP/1000	3	27.5	5.55	0.2	1.96	0.58	0.3	11.8	4.09	0.35
48 hrs										
CTRL	0	24.6	11.3	0.46	1.71	0.77	0.45	10.32	5.28	0.51
STP	0	31.7	11.4	0.36	1.64	0.61	0.37	11.10	4.49	0.40
STP/10	1	15.6	5.32	0.34	2.48	0.67	0.27	9.63	4.37	0.45
STP/100	3	17.10	5.18	0.3	1.77	0.46	0.26	7.32	2.44	0.33
STP/1000	4	14.92	6.75	0.45	1.79	0.69	0.39	7.43	4.33	0.58

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Table SI-2 Measured mean values for the Body Bend per second (n s^{-1}), the Average Speed (mm s^{-1}) and the total Distance Moved (mm) on _Diamesa cinerella-zerniy gr_ larvae in CTRL, STP, STP/10, STP/100 and STP/1000 tests after 24 hrs and 48 hrs of exposure.

Treatment	Dead	BBps			Avg. Speed			Distance moved		
	N	M	SD	RSD	M	SD	RSD	M	SD	RSD
24 hrs										
CTRL	0	0.68	0.29	0.43	0.74	0.29	0.36	6.67	5.38	0.81
STP	0	0.73	0.35	0.48	0.75	0.35	0.31	5.95	3.80	0.78
STP/10	0	0.84	0.38	0.45	0.77	0.38	0.58	3.41	1.94	0.79
STP/100	0	0.81	0.23	0.28	0.84	0.23	0.25	3.70	3.08	0.84
STP/1000	1	0.76	0.31	0.41	0.58	0.31	0.19	2.56	1.31	0.51
48hrs										
CTRL	0	0.67	0.36	0.54	0.85	0.21	0.25	8.70	5.36	0.62
STP	0	0.63	0.37	0.59	0.78	0.32	0.41	8.69	6.29	0.72
STP/10	0	0.91	0.53	0.58	0.79	0.39	0.49	4.67	2.76	0.59
STP/100	0	0.65	0.19	0.29	0.84	0.17	0.2	4.86	2.77	0.57
STP/1000	0	0.99	0.48	0.48	0.93	0.38	0.4	9.23	7.70	0.83

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