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ALPINE LAKES, INDICATORS OF GLOBAL CHANGE: ECOLOGICAL CHARACTERIZATION AND ENVIRONMENTAL PRESSURES IN TWO LAKES FROM ITALIAN ALPS

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ABSTRACT

Alpine lakes are among the most remote aquatic environments in Europe. Although remote, their small size and the high turnover of surface waters render Alpine catchments extremely receptive and vulnerable to anthropogenic impact on a local (i.e., water abstraction, tourism, introduction of alien species) and a global scale (long-range transport of pollutants, acid rain, global warming). Alpine lakes are indicators of global environmental change and “early warning systems” for the mountain environment. Many studies have considered single lakes or groups of lakes; however, because of the wide extent of the Italian Alpine area together with the difficulty of sampling, there have been few studies of the Alpine lakes as a whole. Since it was not possible to extend the research to all Italian Alpine lakes, two lakes (Balma Lake, Cottian Alps, 2100 m a.s.l. and Dimon Lake, Carnic Alps, 1872 m a.s.l.) were selected for this study according to the following criteria: a) the geographic location of the research groups involved in the project, Department of Life Sciences, University of Trieste and Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d’Aosta; b) site geomorphology and accessibility; 3) presence of fish after introduction for recreational fishing. The main aims of this PhD project were: a) conduct ecological characterization based on biotic components (macrobenthic invertebrates and fish); b) study the pressures and changes via analysis of biotic components; c) obtain background data for future research. The overarching objectives were: 1) obtain topographic and bathymetric maps of lakes using new technologies (drones); 2) characterize the hydrochemistry; 3) characterize macrobenthic invertebrates since typical biological components of Alpine lakes and widely used to assess ecological status of surface freshwater; 4) investigate changes in chironomids composition over time based on paleolimnological analysis. These biological components can yield information to different perturbations and, given a good time-control, it is possible to estimate phases and amplitudes of disturbance; 5) investigate environmental contamination through trace elements detection in macrobenthic invertebrates; 6) characterize fish communities, obtaining information about their biological and sanitary condition. Sampling was performed during the ice-free season (summer and autumn of 2017 and 2018) at both lakes. The physicochemical features of the lakes were in line with published literature but differed from each other due to the geo-lithological context of the two areas. The biodiversity of the littoral macrobenthic communities was comparable with other high-altitude environments, where Diptera Chironomidae and Oligochaeta generally predominate. Paleolimnological analysis of Balma Lake highlighted significant differences in subfossil chironomid communities before and after the introduction of fish and between modern and subfossil communities, with a significant reduction in diversity. Macrobenthic invertebrates from the two lakes were found to differ in trace element concentration, which was higher in Dimon Lake. Individuals of bullhead (*Cottus gobio*) and minnow (*Phoxinus phoxinus*) were captured from Dimon Lake, whereas only brook trout (*Salvelinus fontinalis*) were captured from Balma Lake, with individuals belonging to age classes 0+ to 4+. Health monitoring revealed hepatic steatosis in *C. gobio* from Dimon Lake, probably linked to an adaptation of the fish to the winter season. This PhD thesis has produced new data about the ecology and conservation status of two Alpine lakes, which may guide local administrations in their decisions to implement conservation and monitoring plans.

1. INTRODUCTION

Alpine lakes are remote and extreme ecosystems subject to harsh climatic conditions located in the alpine belt above the tree line (Boggero et al., 2005) where, due to the extreme winter temperatures, only alpine prairies or sparse vegetation can grow (Hinder et al., 1999). During most of the year (from October-November to June-July) the snow and ice cover the lakes, blocking sunlight from penetrating the underlying water column (Felip et al., 2002). Without the penetration of light, photosynthesis cannot take place and the lakes remain in darkness (Ventelä et al., 1998), becoming heterotrophic systems isolated from the surrounding area until the ice cover breaks. When the snow melts in early summer, the lakes quickly shift from extremely low to extremely high solar irradiance, with increasing levels of UV radiation directly correlated to altitude (Caldwell et al., 1980). The light can penetrate to great depth thanks to the low attenuation coefficient in clear water (Scully and Lean, 1994). The ice-free season lasts for a few months, generally from mid-June to late October. During this brief period of ideal conditions, some aquatic organisms can complete their life cycle before the snow covers the lakes again.

The harsh environmental conditions and the insular nature of alpine regions limit the biodiversity of these ecosystems. Moreover, Starkweather (1990) underlined a negative correlation between altitude and species richness, since high-altitude communities have scarce resource availability, lower habitat complexity, and exist under extreme physicochemical conditions.

1.1 Alpine lakes, climate change, and anthropogenic impact

Mountain regions are recognized as environments sensitive to climate change (Haeberli et al., 2007). Because the hydrochemistry of Alpine lakes is highly conditioned by the chemical composition of atmospheric deposition and by climate factors (Rogora et al., 2003), they are early response indicators of climate change (McGregor et al., 1995), atmospheric deposition, and air pollutants (Rogora et al., 2008).

Though mountain lakes are generally much less influenced by human activities than other habitats, global and local anthropogenic threats can alter their natural condition. The most alarming threats are water exploitation (CIPRA, 1992), alien species introduction (Eby et al., 2006; Pastorino et al., 2016, 2017a), climate change (Rogora et al., 2003, 2018), and medium-long range atmospheric transport of contaminates (Ørbæk et al., 2007; Camarero et al., 2009a).

Originally fishless because they are isolated ecosystems (Brancelj, 1999), Alpine lakes are sensitive to ecological damage caused by the invasion of alien species (Simberloff, 2001). The low taxon richness of Alpine freshwaters suggests that these habitats are not species saturated and are susceptible to invasive species or species that are expanding their ranges due to climate change (Rosset et al., 2008). The threat to biodiversity is greater due to the low diversity and structure of such communities (Ricciardi, 2001). Moreover, tourism and mountain farming are two other major sources of organic pollutants that can pose a local threat to aquatic biodiversity. Wastewater management and treatment in mountain regions is important for protecting natural ecosystem functioning (Tiberti et al., 2014a).

1.1.1 Climate change

Warming of the climate system in all its components (Earth's surface, oceans, and the atmosphere) is manifest: the sea level has risen, the ocean and atmosphere have warmed, the amount of snow and ice has diminished, and the number of extreme events has increased (IPCC, 2014). The period from 1983 to 2012 was likely the warmest 30-year period of the last 1400 years in the Northern Hemisphere (IPCC, 2014). The global average increase in surface temperature, considering land and ocean combined, from 1880 to 2012 was 0.85 °C (IPCC, 2014). The atmospheric concentration of carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) has increased since the pre-industrial era by anthropogenic greenhouse (GHG) emissions. Between 1750 and 2011, the cumulative anthropogenic CO_2 emissions to the atmosphere were 2040 ± 310 $GtCO_2$, about half of which occurred in the last 40 years (IPCC, 2014). About 60% of total anthropogenic emissions are removed from the atmosphere by the oceans and land (soils and plants), whereas the other 40% remain in the atmosphere.

The Fifth Assessment Report of the Intergovernmental Panel on Climate Change (IPCC, 2014) reported that it is extremely likely that more than half of the observed increased global surface temperature from 1951 to 2010 was caused by the anthropogenic increase in GHG concentrations and other anthropogenic forces. The effect of climate change on natural systems is strong. Climate warming is predicted to cause changes in the seasonality of precipitation, with more intense precipitation, increased intra-annual variability and more precipitation extremes (Gobiet et al., 2014). The change in precipitation and extreme events is happening on a regional scale (Rogora et al., 2018). Around our planet, changes in precipitation or melting snow and ice are altering regional hydrological systems and affecting water resources in quality and quantity. Many marine and freshwater species have shifted their geographical range, migration patterns, seasonal activities, species interactions and abundances in response to ongoing climate change (Walther et al., 2002; IPCC, 2014). The number of extreme events connected with climate change (such as heat waves, droughts, floods, cyclones, and wildfires) has increased in the last decades. The impact of climate-related extremes reveals the vulnerability and exposure of ecosystems and human systems (Easterling et al., 2000).

Anthropogenic GHG emissions are largely driven by population size, economic activity, lifestyle, energy use, land-use patterns, technology, and climate policy. There is growing evidence for a consistent and linear relationship between cumulative CO_2 emission and estimated global temperature change to 2100 (IPCC, 2014). In the future our climate will depend on the warming caused by past anthropogenic emissions, as well as future anthropogenic emissions and natural climate variability. For the period 2016-2035, the Intergovernmental Panel on Climate Change estimates an increase between 0.3-0.7 °C to 2.6-4.8 °C according to the models used for the projection (IPCC, 2014). Based on these projections, the risk of climate-related impacts on natural and human systems will be amplified and new risks created. Rising rates and magnitude of warming and other changes in the climate system, accompanied by ocean acidification, will increase the risk of severe, pervasive, and irreversible detrimental impacts in some cases. A large fraction of species faces increased risk of extinction due to climate change during and beyond the 21st century, especially as climate change interacts with other stressors. According to climate change projections, global warming will not be uniform but rather will vary considerably across regions: greater over land and at high-altitude (Auer et al., 2007; Gobiet et al., 2014). A reduction in snow cover over space and time, due to less precipitation and higher temperatures, means greater exposure of rocks and soils in watersheds, which enhances weathering processes.

Rogora et al. (2003) evaluated the possible effect of these processes on long-term changes in the chemistry of 35 Alpine lakes in the Ossola and Sesia Valleys (Central Alps) and reported an increase of solute contents in lakes lying in catchments with highly soluble rocks. The increase may be attributed to higher weathering rates due to climate warming. Recently, Rogora et al. (2018) presented a synthesis and review of the main results of ecological studies in mountain ecosystems in 20 sites in Italy, Switzerland, and Austria, with more than two decades of observations in most cases. The overall results highlight the rapid response of mountain ecosystems to climate change, with site-specific characteristics and rates. As temperatures have increased, the vegetation cover in alpine and subalpine summits has increased as well. Following years with a shorter duration of snow cover, there has been an increase in soil temperature and microbial biomass during the growing season. The effects on freshwater ecosystems were noted for increases in solutes, decreases in nitrates, and changes in plankton phenology and benthos communities.

1.1.2 Introduction of alien species in freshwater ecosystems

Alien species are species introduced by humans (either intentionally or accidentally) outside their natural past or present distribution. Widely used synonyms of “alien” are: exotic, allochthonous, introduced, not native species (NNS) or not indigenous. A species occurring in its original area is defined as autochthonous or native or indigenous. As regards freshwater fish, Zerunian (2003) reported the presence of 48 autochthonous freshwater fish species (with 22 endemisms) in Italy. Nocita and Zerunian (2007) reported 38 allochthonous species, 13 of which naturalized. The last check list drafted in 2019 by the Italian Association of Freshwaters Ichthyologists (AIIAD) reported the presence of 86 allochthonous fish species (60%) in Italian freshwater bodies (Lorenzoni et al., 2019).

The human-mediated transfer of a species from one place to another has a relevant impact on organisms around the globe (Dextrase and Mandrak, 2006; Olden et al., 2008). The introduction of alien species often creates severe threats to native species and can upset the ecological equilibrium. The introduction or the transfer of fish fauna is well documented in many habitats, and the ecological and biological damage that this can cause is often understood (Pastorino et al., 2017a). In Italy, the introduction of fish fauna in rivers and lakes is a common practice by local government administrations and fishery associations. The introduction of fish fauna from foreign countries, for both stocking and aquaculture, has continued for years. While fish fauna are an important food source and economic resource for the companies working in this sector, their introduction also can act as a vector for environmental contaminants and zoonotic disease (Aloo, 2000; Mantovani, 2001). Monitoring of the health status of freshwater fish fauna in public waters should be a fundamental tool for the preservation of the environment, species conservation, and for public health (Pastorino et al., 2017b). The reasons for NNS introduction in local ecosystems are related to aquaculture, recreational fisheries, stocking enhancement, biological control, and the aquarium fish industry (Pimentel et al., 2005; Gozlan, 2008; Vitule et al., 2009).

Not always but alien species can sometimes also be introduced involuntarily and enter a new habitat as a result of other human activities: a) live baits used by anglers; b) fish escaped from fish farms; c) accidentally, with other species during stocking plans. The most common negative effects of an alien species are: 1) predation; 2) competition with native species; 3) changes of the habitat; 4) hybridization with indigenous species; and 5) spread of new pathogenic agents (parasites, bacteria, virus, fungi) that may be more virulent in new hosts due to the lack of innate immunity in the autochthonous species (Sheath et al., 2015).

Problems of international trade are not just an Italian issue. Indeed, globalization and the paucity of rules and controls at international frontiers have led to the introduction of alien species in Member States and their spread throughout Europe. Biological invasions, with the threats they pose to aquatic biodiversity, pose a particularly difficult challenge to society in general and to decision makers in particular (Ricciardi et al., 2017). Across Europe, there is a wide range of policies, legislation, and management approaches (including citizen awareness initiatives) to deal with NNS and with invasive alien species (IAS) in particular. However, in some European countries, legislation and management for IAS are either entirely missing (e.g., Slovenia) or lack the regulatory structures (e.g., Croatia) needed to make them effective (Povž and Šumer, 2005; Piria et al., 2016). Also, there has been a general lack of coordination on how to approach IAS management both within and between Member States (Copp et al., 2005a; Ojaveer et al., 2014). These shortcomings impinge upon the increased risk of NNS introduction and consequent dispersal, which can eventually lead to adverse impacts on native biodiversity, local and national economies, ecosystem services, and human health (Scalera et al., 2012).

Regulation (EU) 1143/2014 (European Commission, 2014) on invasive alien species (the IAS Regulation) entered into force on 1 January 2015, fulfilling Action 16 of Target 5 of the EU 2020 Biodiversity Strategy, as well as Aichi Target 9 of the Strategic Plan for Biodiversity 2011-2020 under the Convention of Biological Diversity. The core of the IAS Regulation is the list of Invasive Alien Species of Union concern (the Union list). The IAS Regulation provides for a set of measures to be taken across the EU in relation to invasive alien species included on the Union list. Three distinct types of measures are envisaged that follow an internationally agreed hierarchical approach to combating IAS: 1)prevention: a number of robust measures aimed at preventing the intentional or unintentional introduction of IAS of Union concern into the EU; 2) early detection and rapid eradication: Member States must put in place a surveillance system to detect the presence of IAS of Union concern as early as possible and take rapid eradication measures to prevent them from establishing; 3) management: some IAS of Union concern are already established in certain Member States. Concerted management action is needed to prevent them from spreading any further and to minimize the harm they cause.

Currently, European regulations impose strict controls on aquaculture activities that breed fishes for stocking in public water, but the certification protocol applies only to viral diseases (Council Directive 2006/88/EC; Pastorino et al., 2017b). Unfortunately, the real health status of the introduced fish is not well investigated. The lack of appropriate health monitoring can lead to the introduction of viral, bacterial or parasitic diseases in public waters and can threaten native fish fauna, other aquaculture facilities located in the proximity of fish introduction, and public health.

1.1.2.1 Introduction of fish in fishless Alpine lakes

Alpine lakes are usually isolated from lower streams by physical barriers that have prevented natural colonization by fish (Pechlaner 1984; Knapp et al., 2001a; Miró and Ventura 2013).The introduction into these ecosystems of fish species mainly for recreational fishing is promoted by local administrations (Pister, 2001; Schindler and Parker, 2002). There is detailed historical evidence for the spread of salmonids in high-mountain areas of the western United States (Christenson, 1977; Bahls, 1992; Knapp, 1996; Wiley, 2003) and the Canadian Rockies (Schindler, 2000), where introductions are described chronologically from the end of the 19th and beginning of the 20th century initially by individual fishermen and a few decades later by governmental agencies (Christenson, 1977; Schindler, 2000). In contrast, information about stocking history in other mountain ranges is difficult to access. Historical information from the high-mountain ranges of Asia and South America (Vigliano and Alonso, 2007; Martín-Torrijos et al., 2016) are insufficient because of uncontrolled stocking (Vigliano and Alonso, 2007; Ortega et al., 2007). In contrast to the American continent, colonization in European high-mountain lakes has not been studied in detail (Gliwicz and Rowan, 1984; Pechlaner, 1984; Sostoa and Lobón-Cerviá, 1989). The first introductions in Alpine lakes are documented for the end of the 16th century (Pechlaner, 1984) and in the Tatra mountains at the end of the 19th century (Brancelj, 2000; Gliwicz and Rowan, 1984). In the Cantabric mountains (Iberian Peninsula) introductions also occurred at the end of the 19th century (Terrero, 1951) and more recently in the Sistema Central and Sistema Ibérico (Martinez-Solano et al., 2003; Toro et al., 2006). In the Pyrenees, the first introductions took place during the Middle Ages, for which the first written evidence of fish introductions dates from the 14th century (Miró, 2011).

In past decades, introduction was promoted by governmental agencies to meet the growing popularity of recreational fishing (Pister, 2001; Schindler and Parker, 2002). The first documented fish introductions in the Alps date from the Middle Ages to the late 16th century, probably during the reign of Kaiser Maximilian I (Pechlaner, 1984). However, as occurred in many mountain ranges across Europe, most fish introductions have taken place in recent decades to support recreational angling which became a popular activity in Italy starting in the 1960s (Cantonati et al., 2006). The presence of introduced species such as *Salmo trutta* L 1758 or species native to North America, such as *Oncorhynchus mykiss* (Walbaum 1792) and *Salvelinus fontinalis* (Mitchill 1814), is now common in Alpine lakes. Moreover, *Salvelinus umbla* (L 1758) was introduced in Italian Alpine lakes (Zerunian, 2003). Restocking with *S. umbla* is considered a conservation measure in some Alpine regions (Tiberti and Splendiani, 2019), though its status as an indigenous species in Italy is seriously doubted (Pechlaner, 1984; Machino, 1999; Piccinini et al., 2004) because this species does not require *ex situ* conservation actions. Also, the marble trout (*Salmo marmoratus* Cuvier, 1829), an alpine endemism, was introduced in Alpine lakes, as well as *Salvelinus namaycush* (Walbaum 1792) from North America. Finally, cyprinids, used as live baits by anglers, have been accidentally or voluntary released in many high-altitude lakes, and minnows (*Phoxinus* sp.) can establish reproductive populations.

A detailed history of fish introductions in Alpine lakes is lacking; information is often insufficient and scattered in archives and the grey literature. Also, the distribution of introduced fish has rarely been assessed for large regions. In their study conducted in the Eastern Alps, Jersabek et al. (2001) reported that fish fauna were present in 41% of high-altitude lakes (> 0.5 ha, above 1300 m a.s.l.). In Valle d'Aosta (Western Alps), where the first modern era documented fish introductions date to 1926 (Mammoliti Mochet, 1995), an accurate catalogue of ponds and lakes (Frezet, 2003) reports the presence of fish fauna in 43% of the lakes (>0.5 ha, above 1000 m a.s.l.), which rises to 50% except for lakes above 2900 m a.s.l., where fish survival is challenged by extreme temperatures and glacial influence. In Gran Paradiso National Park (Western Alps) 35% of the lakes (>0.5 ha) is occupied by fish (Tiberti et al., 2014b). These percentages are similar to those observed in other mountain ranges (Miró and Ventura, 2013; Bahls, 1992). The predatory action on native fauna by introduced fish brings ecological changes, since fish occupy a higher trophic level that was previously empty. Fish predation significantly reduces or eliminates autochthonous animal groups, such as amphibians and large macroinvertebrates in the littoral zone and crustaceans in the plankton. These profound effects raise concern for the conservation of the pristine ecological status of high-mountain lakes. There are multiple effects involved in the decline of the populations of aquatic vertebrates. And although all the taxa within these habitats are affected, amphibians are amongst the freshwater groups most threatened by alien fish. Predation by alien species is probably the most serious threat to amphibian populations (Gardner, 2001). Due to the absence of evolutionary adaptations to new predators, amphibians are particularly vulnerable to this threat (Kats and Ferrer, 2003). Introduced salmonids usually behave as opportunistic feeders in Alpine lakes, dramatically affecting the populations of more accessible prey, feeding mainly on eggs and larvae.

1.1.3 Contaminants input and Alpine lakes

Owing to their remote location, Alpine lakes are often pristine, unpolluted ecosystems. Since the 1980s, however, they been affected by global anthropogenic impacts and are now considered a receptor for medium atmospheric transported (MRAT) organic contaminants (Ferrario et al., 2017), as observed for the Arctic (Hung et al., 2016). In the European Alps, altitudinal transport can occur over relatively short distances from sources of pollution in the industrialized areas of Germany, Switzerland, Austria, and northern Italy (Poma et al., 2017).

Trace elements enter the aquatic environment from a variety of sources. Although most occur naturally through biogeochemical cycles, rapid industrialization has accelerated their flux into the environment through a variety of human activities, especially the combustion of fossil fuels (Förstner and Wittmann, 1981). Furthermore, trace elements attached to fine aerosols can be transported hundreds of kilometers away from the original source, are washed out into the aquatic ecosystems during precipitation events and enter the water column and the sediment (Pan and Wang, 2015). Trace element levels have increased in industrialized and remote areas as a result of atmospheric deposition, solubilization, and mobilization from sediments. These latter form the major sink for environmental contaminants (Karadede-Akin and Unlu, 2007). Mountain lakes are therefore excellent indicators of air pollution and its effects because they are not usually subject to other forms of disturbance (e.g., land-use). Moreover, due to climatic and geographical factors, high-mountain lakes may be more vulnerable to input than lakes in lowland areas. Likewise, fish inhabiting the lakes will be more vulnerable to pollutants because low temperatures and oligotrophic status cause low growth rates, which may result in a higher concentration of contaminants (Schindler et al., 1995). Despite the research in some European mountain areas for the analysis of organic pollutants in abiotic compartments (Vilanova et al., 1998), limited or no information is available concerning bioaccumulation of pollutants by organisms inhabiting mountain lakes. Far more is known about other ecosystems, especially contamination by trace elements in fish in freshwater (Elia et al., 2010; Squadrone et al., 2013a; Squadrone et al., 2014; Squadrone et al., 2015; Squadrone et al., 2016a) and marine (Giorgi et al., 2009; Squadrone et al., 2013b; Squadrone et al., 2016b) ecosystems. In contrast, the data are scarce for Alpine lakes (Köck et al., 1995; Yang et al., 2007). To date, no studies have investigated trace element accumulation in other biotic components such as macrobenthic invertebrates in high-mountain lakes.

1.1.3.1 Trace elements in aquatic ecosystems and associated risks to fish consumption

Trace elements are environmentally ubiquitous, quickly dissolved in and transported by water where they quickly taken up by aquatic organisms. Fish are at the top of the food-web and can accumulate large amounts of some metals from water. The accumulation patterns of contaminants in fish and other aquatic organisms are driven by both uptake and elimination rates (Guven et al., 1999). Trace elements and their compounds are taken up differentially by organs because of the affinity between them and are found at different concentrations in various organs of the body (Bervoets et al., 2001). In both freshwater and marine ecosystems, fish assimilate metals via several routes: ingestion of particulate material suspended in water, ingestion of food, ion-exchange of dissolved metals across lipophilic membranes (e.g., the gills, and adsorption via tissue and membrane surfaces). The distribution of metallic elements in tissues depends on the mode of exposure, essentially dietary or aqueous exposure or the sum of the two. The bioaccumulation of metals in fish is a valid index of the pollution status of water. Some trace elements have nutritional functions and are essential to life (e.g., copper, cobalt, selenium, molybdenum, manganese, and zinc) in humans and animals

(Vandermeersch et al., 2015). Other elements have no biological function and their intake can lead to adverse health effects in both animals and humans (e.g., mercury, cadmium, arsenic, lead, vanadium, tin, and aluminum). The World Health Organization (WHO) put arsenic, mercury, cadmium, and lead on its prioritized list of the ten chemicals of major public health concern (WHO, 2010). European Regulation 1881/2006 (European Commission, 2006) sets the maximum limits of Cd, Pb, and Hg in fish muscle for human consumption.

Cadmium is a non-essential, rare element distributed in the most abundant rocks of the earth's crust. A common source of Cd in industry is the production of nickel-cadmium rechargeable batteries, solar cells, and pigments. A portion of the Cd discharged into freshwater is absorbed into solids in suspension, but only the soluble forms are toxic to biota (Pereira et al., 2018).

Mercury is one of the most toxic heavy metals in our environment, including the lithosphere, hydrosphere, atmosphere, and biosphere. Exposure of humans to mercury is primarily through eating fish that contain some methyl mercury (CH_3Hg) in their tissues, the most toxic form of mercury. Since Hg is an intermediate volatility pollutant, it can be transported over long distances from its emission sources and contaminate aquatic habitats. It can be emitted by natural and anthropogenic sources (Pacyna et al., 2006) and is widespread in the atmosphere of the Northern Hemisphere (de March et al., 1998). Elemental gaseous mercury (Hg^0) is the predominant form in the atmosphere and can reach remote areas (e.g., Alpine lakes) by long-range atmospheric transport. Hg^0 can be oxidized to divalent mercury (Hg^{2+}) and deposited. In this way it contaminates reservoirs such as catchments and water. After it has been deposited, one fraction of Hg^{2+} can be methylated via biotic and abiotic pathways (Pongratz and Heumann, 1998; Celo et al., 2006), leading to an organo-metallic form: methylmercury (MeHg). This form is highly toxic, can accumulate in living organisms, and can be biomagnified through the food web. Intuitively, the greater the food web, the greater its biomagnification (Marusczak et al., 2011).

The principal sources of Hg in water are dry and wet deposition (Graydon et al., 2008). Studies investigating the bioaccumulation of Hg in fish from high-mountain lakes in Europe rarely report high values (Rognerud et al., 2002; Marusczak et al., 2011). Rognerud et al., (2002) gave several explanations for this observation: a) the trophic level is too low to allow significant accumulation of Hg in fish; b) the flow of mercury in sediment is low; c) in cold and clear waters (like those of Alpine lakes) the rate of methylation is low.

Lead is a non-essential element found in small amounts in the earth's crust. The main sources of Pb come from human activities. Lead is still used in a wide variety of products, including paint, gasoline, batteries, and cosmetics. In freshwater environments, lead primarily exists as the divalent cation (Pb^{2+}) under acidic conditions and forms lead carbonate ($PbCO_3$) and hydroxide ($Pb(OH)_2$) under alkaline conditions. Natural processes tend to remove lead from the water and deposit it in sediments, where it becomes available to aquatic organisms (Leach et al., 2010). Lead can move from one place to another in waterways as soluble complexes and ions. Its transport is largely controlled by the exchange of lead with sediments and the nature and size of the particles. In surface waters, the average residence time of biological particles containing lead has been estimated at 2-5 years (UNEP, 2010). The long-term route of lead is from rivers to estuaries to oceans.

1.2 Aims and objectives of the PhD project

The **aims** of the PhD project were:

1. perform ecological characterization of two Alpine lakes (Dimon Lake, northeast Italy, and Balma Lake, northwest Italy) using biotic components (macrobenthic invertebrates and fish);
2. study the pressures and the changes in the lakes based on analysis of biotic components;
3. obtain background data for future research since few studies are available to date.

The overall study **objectives** of the project were:

a) obtain topographic and bathymetric maps of the lakes using new technologies (drones), since no data about topographic and bathymetric profile of selected sites were available;
b) characterize the lake hydrochemistry to get information about nutrient loading, trophic status, stratification, and many other variables;
c) characterize macrobenthic invertebrates since typical biological components of Alpine lakes and widely used to assess ecological status of surface freshwater;
d) investigate changes in chironomids composition over time based on paleolimnological analysis. This biological component can yield information to different perturbations and, given a good time-control, it is possible to estimate phases and amplitudes of disturbance;
e) investigate environmental contamination through trace element detection in macrobenthic invertebrates;
f) characterize fish communities, obtaining information about their biological and sanitary condition.

It is important to point out that no previous investigations on hydrochemistry and biotic components were performed in the selected sites.

The following sections are mainly organized according to this scheme: preface, material and methods, results and discussion. Furthermore, at the end of the thesis a conclusion section has been reported.

2. STUDY AREAS

2.1 Dimon Lake

Dimon Lake (Fig. 1) is a high-mountain lake located in the Carnic Alps (municipality of Ligosullo, Udine Province, Friuli Venezia-Giulia Region, northeast Italy) at 1857 m a.s.l. The Carnic Alps are among the most remote areas of Italy. Anthropic impacts are very limited, except for pasturing. Dimon Lake is a typical glacial-origin lake, classified as a Site of Community Interest and Special Areas of Conservation (SCI/SAC-IT3320002 “Monti Dimon e Paularo”). Originally fishless, fish were introduced for recreational fishing (Ente Tutela Patrimonio Ittico, personal communication). The lake lies on sandstone and volcanic rock (Venturini, 2006).

The glacial site was created during Würm glaciation and the lake originated after glacial retreat. The site is assigned to the habitat type “oligotrophic to mesotrophic standing waters” according to the habitat description of Natura 2000 sites (Interpretation Manual of European Union Habitats, 2013).

The main habitats are hydrophilous tall herb fringe communities of the plains and the montane to alpine levels, species rich *Nardus grasslands*, on siliceous substrates in mountain areas (and sub-mountain areas in continental Europe), alpine and boreal heaths, siliceous alpine and boreal grasslands. Siliceous scree of the montane to snow levels (*Androsacetalia alpinae* and *Galeopsietalia ladani*) and siliceous rocky slopes with chasmophytic vegetation are present in the area. *Primula minima*, *Phyteuma hemisphaericum*, and *Rhododendron ferrugineum* are present in the meadows and pastures. Subalpine heath of *Loiseleuria procumbens* and scrubs with *Alnus viridis* can be found as well as beech and spruce woods. Ornithologically, the area is relevant for bird species typical of alpine zones, including *Aquila chrysaetos*, *Aegolius funereus*, and Tetraonids (i.e., *Tetrao urogallus*). As regards carnivores, *Ursus arctos* and *Lynx lynx* are reported as transient in the area. Regarding water environments within the SAC, valuable species such as *Austropotamobius pallipes* and *Cottus gobio* are present. Natura 2000 Standard Data Form (updated at March 2016) reports that the target species for the present project *Bombina variegata* and *Rana temporaria* are found in the area (FVG, 2012). Abandonment of pastoral systems and the lack of grazing have changed the landscape, with negative consequences for species that inhabit open spaces, such as *Crex crex* and *Lanius collurio*. In addition, habitat reduction, habitat fragmentation, and anthropogenic disturbance have had a negative impact on the settlement and reproduction of tetraonids in the area.

Figure 1. Dimon Lake (municipality of Ligosullo, Udine, Friuli Venezia-Giulia Region, Italy) (photo: Paolo Pastorino).

2.2 Balma Lake

Balma Lake (Fig. 2) lies at 2100 m above sea level (a.s.l.) in the municipality of Coazze, a small town about 40 km from Turin (Piedmont, northwest Italy). It is a typical glacial-origin lake in the Cottian Alps, within the Site of Community Importance (SCI)/Special Areas of Conservation (SAC) IT1110006 - Orsiera Rocciavrè. The lake is located above the tree line and is covered by ice from November to early June. The main pressures are grazing (cows in summer) and recreational fishing. Originally fishless, brook trout (*Salvelinus fontinalis*) was introduced for recreational fishing (Ente di Gestione delle Aree Protette delle Alpi Cozie, personal communication). The main core of the area is composed of ophiolite metamorphic bedrock.

Figure 2. Balma Lake (municipality of Coazze, Turin, Piedmont Region, Italy) (photo: Paolo Pastorino).

In the study area 14 habitats of community interest have been recognized and at least 850 plant species have been identified. Twenty of these species are present on the national red list. The flora are present with numerous species endemic to the Central Alps: *Campanula elatines*, *Campanula alpestris*, *Veronica allionii*, *Viola cenisia*; and several species rare in Piedmont (*Cortusa matthioli*, *Cerastium lineare*, *Aconitum anthora*, *Cardamine plumieri*). Two species are also listed in the Habitats Directive 92/43/CEE (Council of the European Commission, 1992): *Aquilegia alpina* and *Saxifraga valdensis*, endemic to the Cottian and Graie Alps (Debernardi, 1993). There is a rich diversity of birdlife: 73 nesting species are present. Typical alpine species include: *Accipiter gentilis*, *Carduelis spinus*, *Loxia curvirostra*, *Turdus torquatus*, *Nucifraga caryocatactes*, *Tichodroma muraria*, *Montifringilla nivalis*. There are 30 species of mammals, among which the most important are: *Rupicapra rupicapra*, *Capra ibex*, *Cervus elaphus*, and *Capreolus capreolus*. The wolf (*Canis lupus*) made its return in the 1990s, as its distribution expanded throughout the Alps. The study area is an important reproduction site for amphibians, including *Rana temporaria* and *Bufo bufo*. Six species of reptiles are present, *Coronella austriaca* included.

3. COMPLETE 3D RECONSTRUCTION OF THE EXTERNAL AND SUBMERGED DIGITAL TERRAIN MODEL OF THE LAKES

3.1 Preface

The digital terrain modeds (DTMs), also called digital surface models (DSMs) describe the 3D morphology of an area by considering, respectively, only the terrain, in the first case, and the soil plus all the structures and vegetation that cover it, in the second. These products are commonly used to represent digitally and in three dimensions a portion of the territory, to study typically tridimensional phenomena in cartographic and GIS (Geographic Information System) environment and as a basis for the production of orthophotos and true-orthophotos. The method commonly used to produce DTMs is the LIDAR (Kraus and Pfeifer, 2001; Liu, 2008; Chen et al., 2017) typically mounted on aircraft; it allows the acquisition of sparse 3D points on the ground even in the presence of vegetation. They are mainly used for small scale territorial representation of large areas, but sometimes and only in recent years, through the use of drones together with LIDAR instrumentation and an inertial measurement unit (IMU) of reduced size and weight can be used for restricted areas. These instrumentations, however, are always very expensive and do not assure very accurate results suitable for the desired big scale of representation. The most used alternative method is aerial photogrammetry. It is the method historically used for cartographic production. Nowadays, thanks to digital technology, the emergence of computer vision algorithms (Hartley and Mundy, 1993; Granshaw and Fraser, 2015) for automatic image analysis, the use of structure for motion techniques (SfM) (Spetsakis and Aloimonos, 1991; Boufama et al., 1993), and the use of UAVs (Niethammer et al., 2012), it is a method that is increasingly used by professionals and non-specialists for the survey of small areas and buildings. This type of approach is also called Low-Altitude Aerial Photogrammetry (LAAP) (Martínez-del-Pozo et al., 2013) or Close-range Aerial Photogrammetry (Murtiyoso and Grussenmeyer, 2017) or low-height aerial photogrammetry (Bitelli et al., 2003) because it allows the survey of portions of land from above (as in classical aerial photogrammetry) but with very high resolution because the data is acquired much closer to the object. With the photogrammetric method, it is possible to reconstruct automatically or semi-automatically DSM. DTMs can be obtained later by eliminating “non-terrain data” and interpolating the missing hidden parts. To reconstruct the depth profile of the lake many are the methods at disposal commercially (Menna et al., 2018). One the most simple, performant and cheap solution is to use sonars. The water transmits sound very well, so it is possible to propagate a sound underwater and read the returning signal back, allowing the identification of objects and the range measurements underwater. The survey work aims to build at large scale cartography of both lakes environment reconstructing accurately the complete digital terrain model of each area, which means considering both of the surrounding out of water terrain but also the submerged part, since no topographic and bathymetric data were available. The survey was supported by GeoStudio RC (Giaveno, Torino).

3.2 Material and Methods

Instrumentation

The photogrammetric technique was used for the reconstruction of the DTM around the lake. The used UAV was a DJI Phantom4 with the integrated digital camera FC330 (CMOS Sensor size: 1/2.3”, f=35mm/eq. with image max resolution 4000x3000 pixel) (Fig. 3a). The particular configuration of the ground, mostly rocky, bare of tall vegetation, and with sparse grass vegetation, allows this technique to be used for the direct extraction of the DTM. The used drone is a multi-rotor UAV ideal for the rapid acquisition of a small portion of land. The advantages of using a multirotor are: i) ease of use; ii) the ability to shoot at any altitude and to shoot even very close to the ground; ii) the massive payload, allowing, in fact, to carry sensors or groups of sensors even a few kilograms in weight; iii) the low cruising speed that allows you to take unmoved photographs with a significant overlap; iv) the ability to define in advance the route of the flight with the positions of the photographs.

For the underwater DTM, it was used a Sonar with Chirp Technology (Garmin Gpsmap 722XS) (Fig. 3b). The traditional sonar operates at a single frequency with consequent limitation in clarity and resolution of the UW image. CHIRP sonar sweeps a continuous range of frequencies, from low to high, in a single transmission. The returns from each frequency in that transmission are then interpreted to be drawn on the screen with a much more extensive range of information, creating a clear and high-resolution image.

Figure 3. a) DJI Phantom4, the unmanned aerial vehicle used for the reconstruction of the digital terrain model (DTM) around the lakes; b) sonar with Chirp Technology (Garmin Gpsmap 722XS) used for the underwater DTM (photo: Marco Rosa Clot).

The sonar was mounted on a small unmanned remote-controlled boat built by hand with a hull of about 1 meter in length and weighing about 10 kg (Fig. 4).

Figure 4. Equipped boat used for bathymetric survey. a) 360° Leica prism; b) Garmin Gpsmap 722XS (photo: Paolo Pastorino).

Work pipeline

The activity began with the study of the area planning the flight of the UAV. The main settings were: i) the flight height, ii) the fight speed, iii) the overlap between images and consequently iv) the shooting position for each image. Similar planning was also done for the boat navigation path identifying the waypoints and the frame rate of the depth data acquisition.

The second step was the survey on the field:

1. the UAV-photogrammetric acquisition;
2. the bathymetry survey using an equipped boat;
3. the topographic survey (total station survey and Global Navigation Satellite System-GNSS) to support and integrate together all the different acquisitions.

For the photogrammetric acquisition eight photogrammetric cross-targets were positioned on the ground around the lakes in order to cover all the areas (Fig. 5). The use of targets is mandatory in order to constrain the photogrammetric acquisition and avoid uncontrolled geometric deformation, and ii) to scale the DTM properly.

Figure 5. Positioning of photogrammetric cross-targets on the groud (photo: Paolo Pastorino).

The network was measured with a total station (LeicaTS15) in order to achieve an overall accuracy < 1 cm on the positioning determination of the points in a local reference system (Fig. 6).

Figure 6. Total station (LeicaTS15) used to measure the network (photo: Paolo Pastorino).

An additional GNSS acquisition was performed to georeference the area in the cartographic coordinate system (Gauss Boaga) using 3 points around the lakes in order to materialize a triangle containing all the other measurements inside (Fig. 7).

Figure 7. Location referencing with GNSS System Leica GS14 (photo: Paolo Pastorino).

The UAV flight was planned in order to achieve a mean GSD (Ground Sample Distance) of circa 2 cm and a mean overlap of 80 %. The flight was controlled manually by the operator.

A total-station acquisition also supported the bathymetric acquisition in order to measure the 3D position of the boat at each acquired depth measurement (Fig. 8). The total station was used in tracking-mode following a 360° Leica prism. Prism was mounted on the boat vertically respect to sonar head in a calibrated distance of 455 mm. For the water depth measurements, circa 300 points were collected materializing a virtual grid of acquired points of circa 5x5 meters.

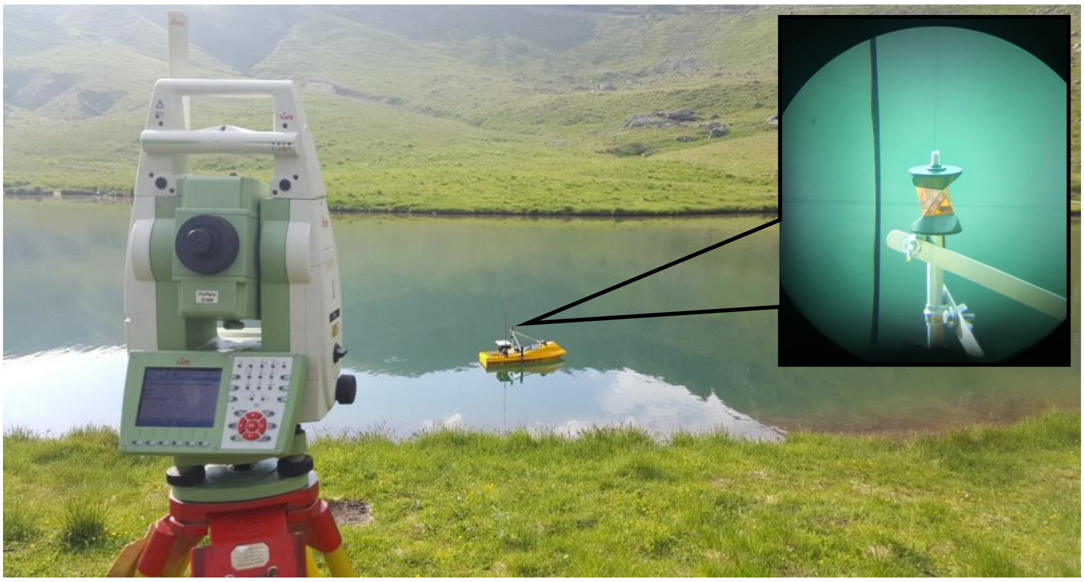

Figure 8. Total-station (LeicaTS15) acquisition to measure the 3D position of the boat at each acquired depth measurement (photo: Paolo Pastorino).

3.3 Results

The final results reported in Figure 9 for Balma Lake and in Figure 10 for Dimon Lake summarize the following information (Table 1):

1. a DTM of the outside area with the consequent calculation of contour lines, water free-surface altitude;
2. an orthophoto at a resolution of 2 cm/pix;
3. interpolation of the underwater depth with 1m resolution;
4. calculation of the water volume.

Table 1. Coordinates, water free-surface altitude, perimeter, surface, maximun depht and calculation of the water volume of study ares.

Lake	Coordinates	Water free-surface altitude (m a.s.l.)	Perimeter (m)	Surface (ha)	Maximum depht (m)	Water volume (m^3)
Balma	45° 02' 14" N 07° 10' 52" E	2100	414	1.21	6.42	41328
Dimon	46° 34 4.17" N 13° 03' 43.12" E	1857	368	0.58	4.27	15242

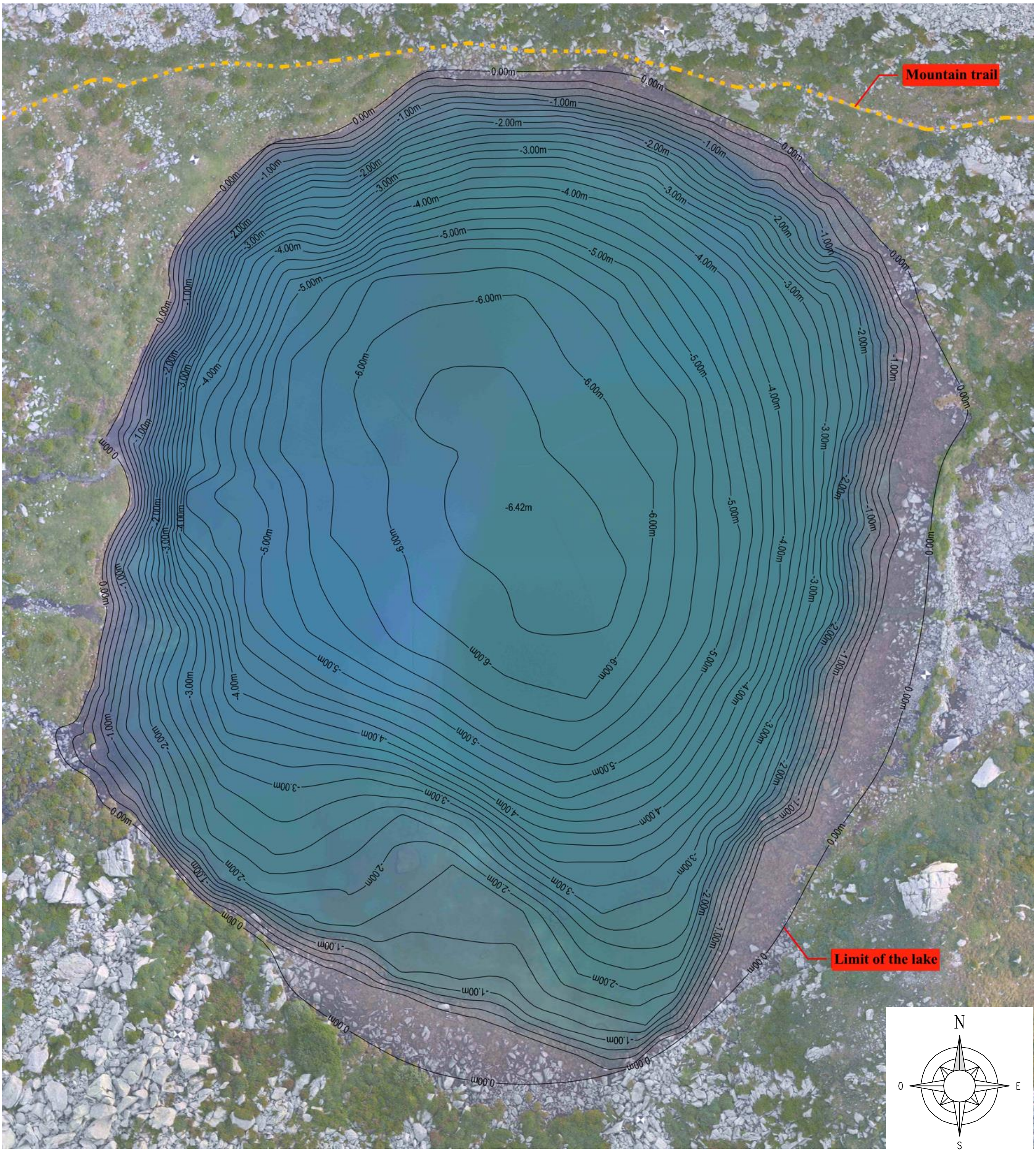

Figure 9. Orthophoto and interpolation of the underwater depth of Balma Lake.

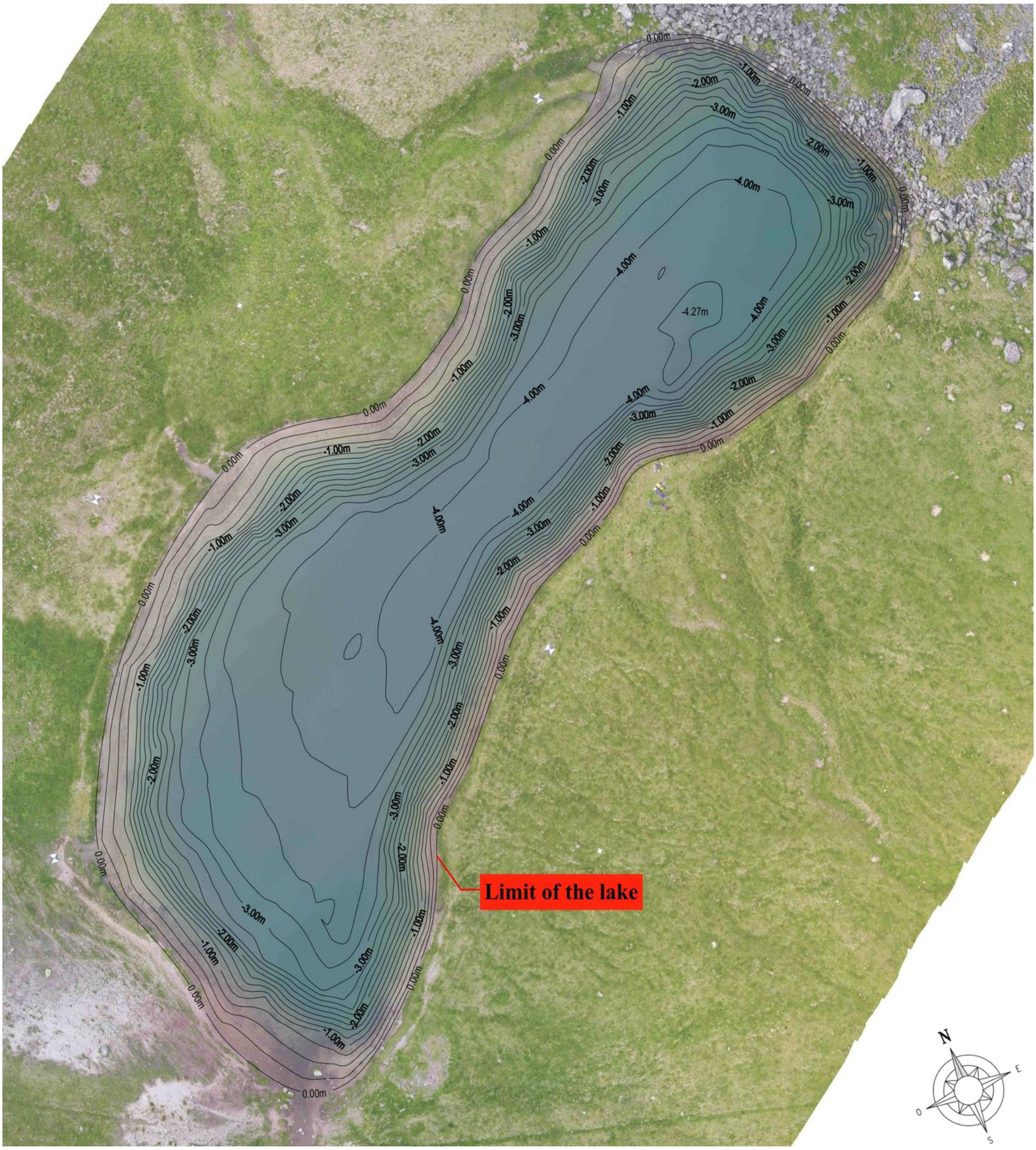

Figure 10. Orthophoto and interpolation of the underwater depth of Dimon Lake.

4. SELECTION OF SAMPLING SITES

In order to characterize hydrochemistry and biotic components, five littoral sites at both lakes (Table 2) were selected while walking the perimeter of each lake to identify the sampling sites along the shore (depth range 25-60 cm) with different habitats (grain size of the vegetation substrate) and tributaries or emissaries (Boggero et al., 2014; Dumnicka et al., 2015).

Three sites for deep sampling in each lake (Table 2) were chosen based on bathymetric investigations and substrate grain size (sand and mud). The sites at Lake Balma were located at 6.42 m, 5.0 m, and 4.30 m and the sites at Dimon Lake were located at a maximum depth (4.27 m), 3.50 m, and 2.70 m (Fig. 11a, 11b).

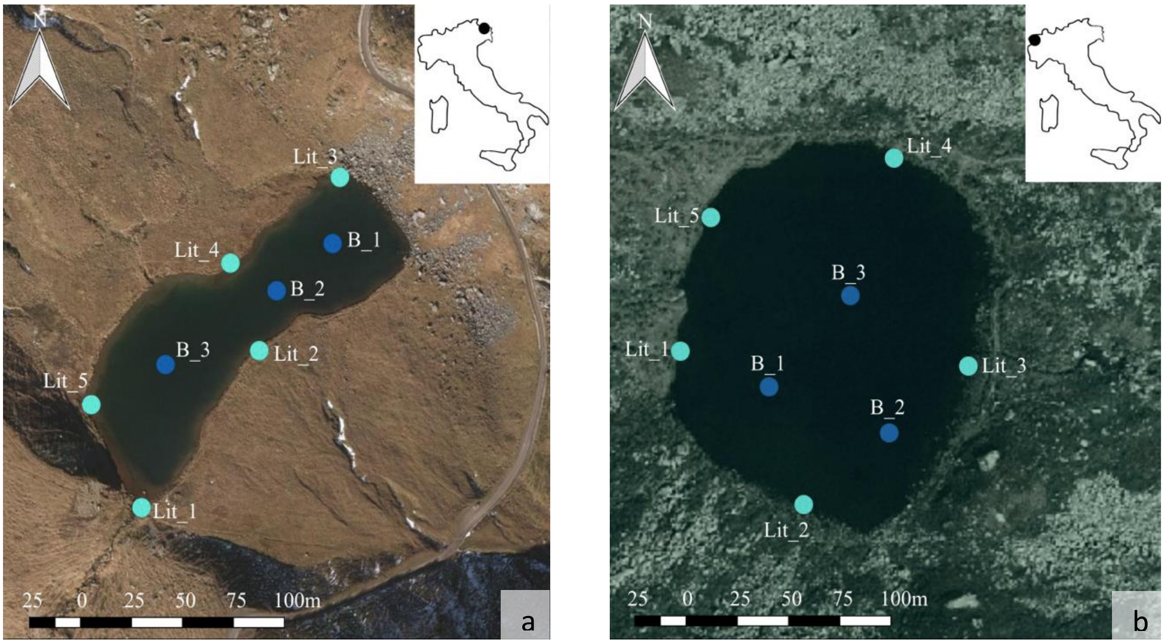

Figure 11. Location of sampling sites at Dimon Lake (a) and (b) Balma Lake. The littoral sites are indicated in blue; the deep sites in light green.

Table 2. Geographic coordinates of the sampling sites at Dimon Lake and Balma Lake.

	Site	Longitude	Latitude
Dimon	Lit_1	13°03'41.66"	46°34'01.93"
	Lit_2	13°03'43.62"	46°34'03.83"
	Lit_3	13°03'45.15"	46°34'05.85"
	Lit_4	13°03'43.11"	46°34'04.75"
	Lit_5	13°03'40.69"	46°34'02.88"
	B_1	13°03'44.89"	46°34'05.02"
	B_2	13°03'43.74"	46°34'04.42"
	B_3	13°03'42.20"	46°34'03.71"
Balma	Lit_1	07°10'49.22"	45°02'13.39"
	Lit_2	07°10'51.26"	45°02'11.83"
	Lit_3	07°10'53.78"	45°02'13.50"
	Lit_4	07°10'52.46"	45°02'15.76"
	Lit_5	07°10'49.63"	45°02'15.02"
	B_1	07°10'50.53"	45°02'13.24"
	B_2	07°10'52.82"	45°02'12.82"
	B_3	07°10'52.23"	45°02'14.28"

Dimon Lake

Site 1 was situated near the outlet (Fig. 12) at the southwest end of the lake, where the substrate is made up of pebbles and gravel and the accumulation of organic substance partially covers the substrate, especially in summer. The sampling depth in both seasons was 25 cm. Site 2 was situated along the eastern shore (Fig. 13) in an area where the lake bed descends suddenly towards the center of the lake; the substratum is composed of gravel and the sampling depth was 40 cm in summer and autumn. *Carex* sp. was present at the site. Site 3 was situated next to a landslide on the northeastern shore (Fig. 14), where large boulders lie on a fine-grained substrate (sand). Sampling was performed at a depth of 25-60 cm. The boulders were covered in algae, especially in summer. Site 4 was situated on the northwestern shore (Fig. 15) in a bed consisting mainly of gravel. Finally, site 5 was located on the south-western shore of the lake (Fig. 16). The substratum changes from pebbles to sandy, with a reduced portion of gravel. Sampling was performed at a depth of 25-30 cm.

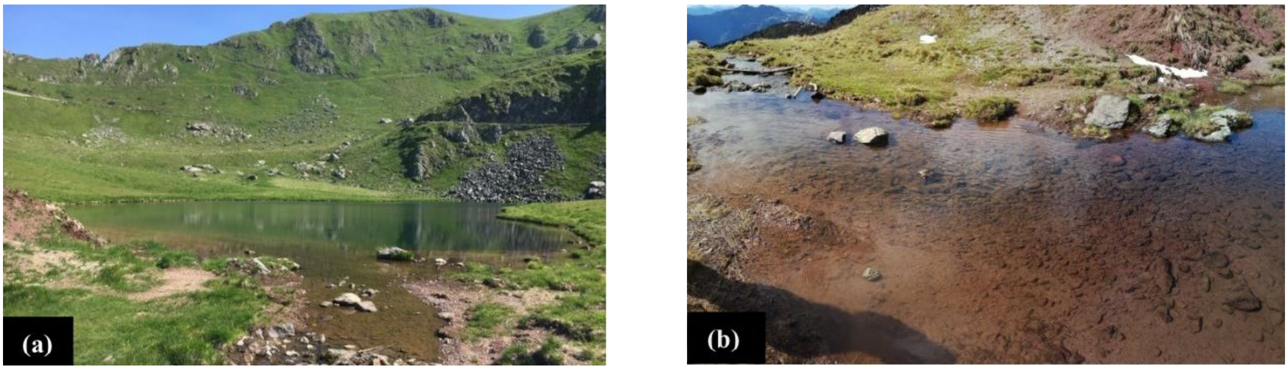

Figure 12. Site 1 (Dimon Lake): summer sampling (a) (photo: Pastorino, 2017) and autumn sampling (b) (photo: Perilli, 2017).

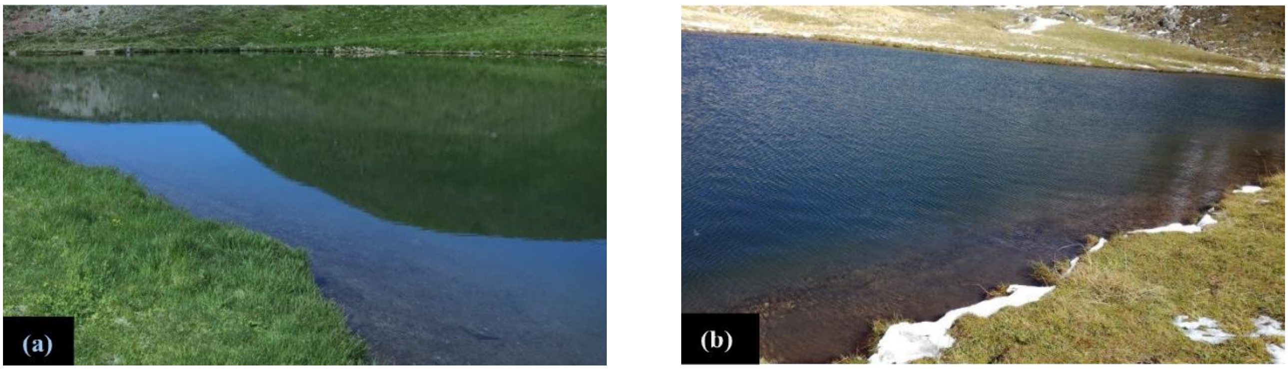

Figure 13. Site 2 (Dimon Lake): summer sampling (a) (photo: Pastorino, 2017) and autumn sampling (b) (photo: Perilli, 2017).

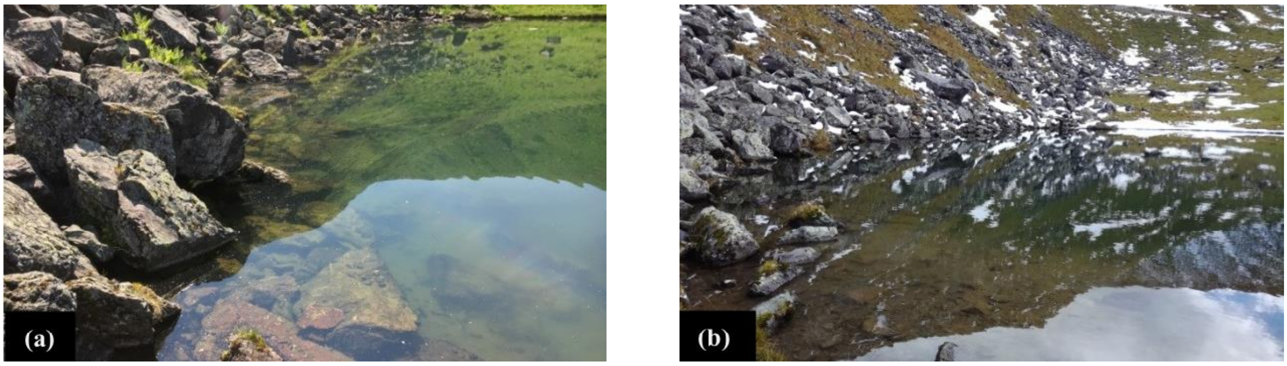

Figure 14. Site 3 (Dimon Lake): summer sampling (a) (photo: Pastorino, 2017) and autumn sampling (b) (photo: Perilli, 2017).

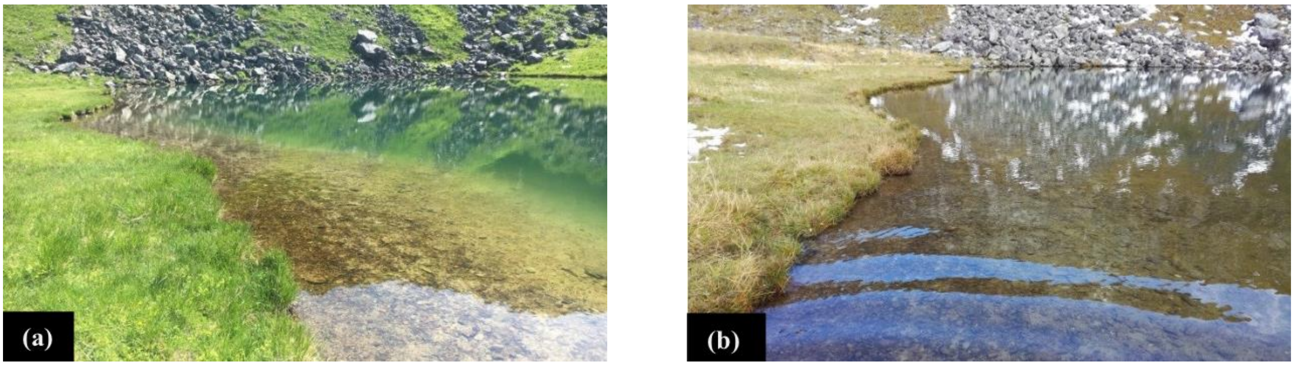

Figure 15. Site 4 (Dimon Lake): summer sampling (a) (photo: Pastorino, 2017) and autumn sampling (b) (photo: Perilli, 2017).

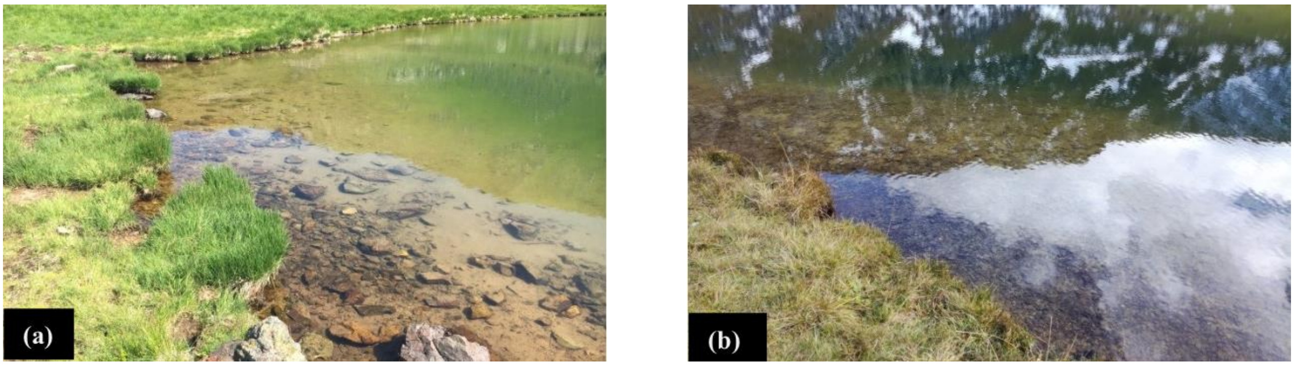

Figure 16. Site 5 (Dimon Lake): summer sampling (a) (photo: Pastorino, 2017) and autumn sampling (b) (photo: Perilli, 2017).

Balma Lake

Site 1 was located on the western shore where a weak current was present near the inlet area (Fig. 17). The substrate was composed of gravel and pebbles. Sampling was performed at a depth of 25 cm. Site 2 (Fig. 18) was located on the southern shore, where large submerged boulders (the predominant substrate) and medium-sized pebbles are present. Sampling of macrobenthic invertebrates was taken at a depth of 40 cm in both summer and autumn. Site 3 (Fig. 19) was located on the eastern lakeshore. Although a real surface emissary is not visible, Balma Creek originates at this point by water filtering through the sediments that delimit the lake. The substrate consists mainly of large boulders. The sampling depth was 40 and 60 cm. Site 4 (Fig. 20) was located on the northern shore where there the bed is composed of medium and large pebbles. The samples were collected at a depth of 30 cm. Finally, site 5 (Fig. 21) was located on the northwestern shore near a bed with a pebble and gravel substrate and accumulation of organic material. Sampling of macroinvertebrates was taken at a depth of 30 cm.

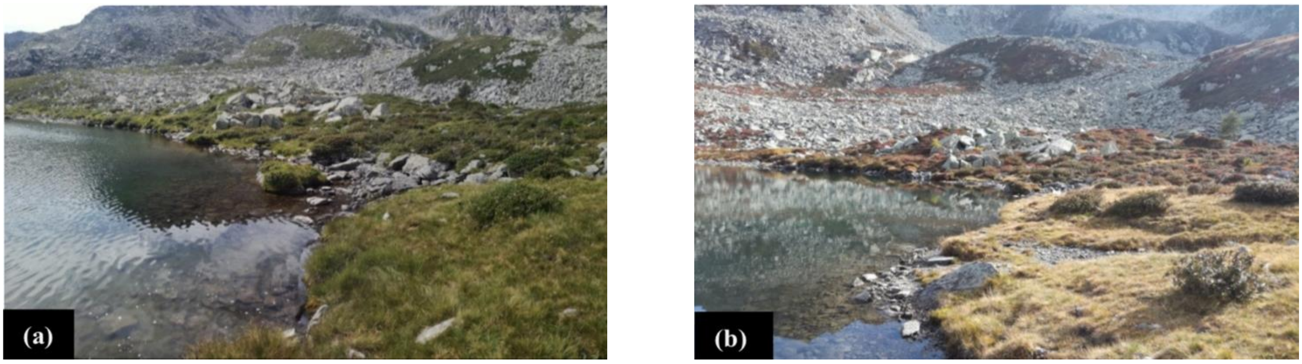

Figure 17. Site 1 (Balma Lake): summer (a) (photo: Pastorino, 2017) and autumn sampling (b) (photo: Perilli, 2017).

Figure 18. Site 2 (Balma Lake): summer (a) (photo: Pastorino, 2017) and autumn sampling (b) (photo: Bertoli, 2017).

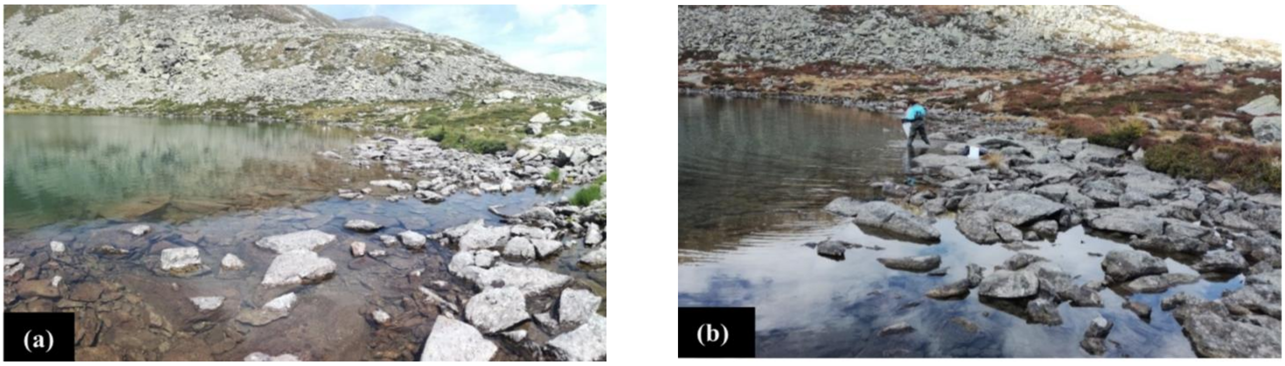

Figure 19. Site 3 (Balma Lake): summer (a) (photo: Pastorino, 2017) and autumn sampling (b) (photo: Bertoli, 2017).

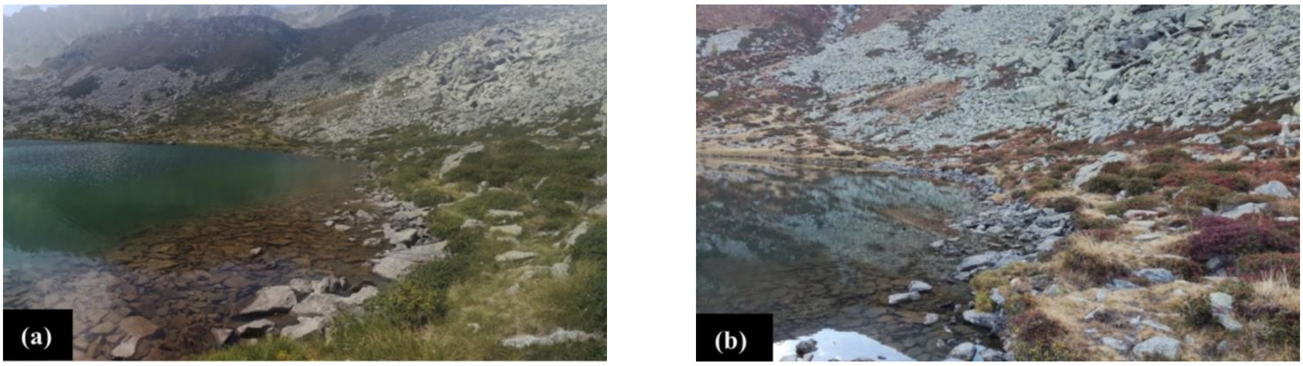

Figure 20. Site 4 (Balma Lake): summer (a) (photo: Pastorino, 2017) and autumn sampling (b) (photo: Bertoli, 2017).

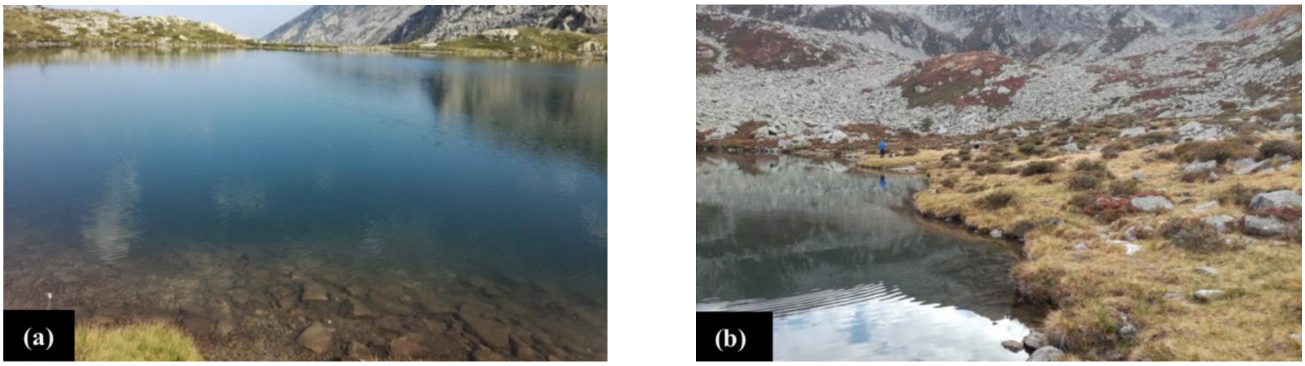

Figure 21. Site 5 (Balma Lake): summer (a) (photo: Pastorino, 2017) and autumn sampling (b) (photo: Bertoli, 2017).

5. HYDROCHEMISTRY, SEDIMENT CORE CHEMISTRY AND DATING

5.1 Preface

Water chemistry is an important indicator of a lake's condition. Numerous materials are dissolved in lake water or suspended in the water column, and many more insoluble forms are associated with the lake sediment (Baccini, 2013). Many are present in more than one form and can be transformed through chemical or biological processes into different forms (Baccini, 2013). Concentrations of various elements provide information about biological processes, nutrient loading, contaminant input, trophic status, stratification, and many other variables. The chemical composition of Alpine lakes depends on several variables that are unique to each catchment and lake, such as local climatic features including amount and duration of ice and snow cover (Rogora et al., 2003; Camarero et al., 2009b; Tiberti et al., 2010), geological composition and land cover in the catchment (Marchetto et al., 1994, 1995; Kamenik et al., 2001) and human stressors, e.g., organic pollution (Tiberti et al., 2014a) and introduced fish (Tiberti et al., 2014b). The most common measured physicochemical parameters are temperature, conductivity, pH, dissolved oxygen, nitrogen, and phosphorus. In particular, the latter three are the most important for biological processes. Other chemical variables also affect lake status and biological processes, but they will not be considered here. Other important parameters for the environmental status estimation of aquatic ecosystems are the total organic carbon (TOC) and total nitrogen (TN) content in sediments (Avramidis et al., 2015). The sediments TOC and TN are mainly derived by decomposition of the plants and animals or plankton or anthropogenic sources such as chemical contaminant or organic rich waste (Avramidis et al., 2015). Regardless of the source the portion of TOC and TN affect, especially in aquatic systems, the faunal communities (Schaanning, 1994; Carroll et al., 2003), the primary production in an ecosystem as well as the eutrophication status (Nixon, 1995). Moreover, organic matter and nitrogen content ratios have been used widely as biomarkers for the reconstruction of sedimentological depositional environments and the environmental changes of the past (Avramidis et al., 2013, 2014).

As already mentioned in the introduction (section 1), Alpine lakes are particularly sensitive to anthropogenic impacts that have caused several changes on both abiotic and biotic compartments. In particular, sediments are expected to contain a range of physical, chemical and biological records of the response of the ecosystem to these changes (Appleby, 2000). In order to investigate the relationship between impacts (i.e. fish introduction) and sediment records, it was essential to determine a reliable chronology for sediments on both investigated sites.

In this first part of the study, the aims were: a) obtain information about the main physicochemical and nutrients parameters of water; b) measure the concentration of TOC and TN in the littoral sites and in the sediment core samples; c) perform a chronology of the core sediment for each study area using radiocarbon and non-radiocarbon data.

5.2 Material and Methods

Physicochemical parameters of water, Total Organic Carbon and Total Nitrogen analysis

During summer (July) and autumn (October) 2017 the main physicochemical and nutrients features were monitored at each sampling site (littoral and deep). Water temperature (°C), dissolved oxygen (mg L^{-1}), oxygen saturation (% saturation), conductivity (µS cm^{-1}), and pH were recorded using field meters (HI 9033 conductivity meter, HI 9125 pH/ORP meter, HI 9147 oximeter; Hanna Instruments Inc. Woonsocket, RI, USA). At the deep sites, depth and transparency were also measured using a graduated rope and Secchi disk. Water samples were collected at each site in sterile containers, taking care not to include sediment particles, and then brought to the laboratory for analysis. Concentrations of NH_4^+ (mg L^{-1}), NO_3^- (mg L^{-1}), and PO_4^{3-} (mg L^{-1}) were measured on a multi-parameter benchtop photometer (HI 83200-02 (Hanna Instruments Inc.). Concentration of NO_3^- (mg L^{-1}) was obtained by measuring the absorbance at 525 nm via adaptation of the cadmium reduction method (APHA et al., 1998); concentration of NH_4^+ (mg L^{-1}) was obtained by measuring the absorbance at 420 nm (ASTM, 2015) via adaptation of the Nessler method; PO_4^{3-} (mg L^{-1}) concentration was obtained by measuring absorbance at 610 nm via adaptation of the ascorbic acid method (APHA et al., 1998). Analyses were carried out on a multi-parameter spectrophotometer (HI83200-02, Hanna Instruments Inc.) and three replicates for each parameter.

Surface sediment (0-2 cm) was collected at each littoral and deep site in both lakes to determine the total organic carbon (TOC, %) and total nitrogen (TN, %). For analysis, 15 mg bulk samples were obtained for each site manually powdered with an agatha mill, and ovendried at 105 °C for 24 h. The samples were then acidified in HCl, increasing the concentration up to 18%, and then processed by frontal chromatography on a CHN Analyzer (model ECS 4010 CHNSO, Costech Analytical Technologies Inc., Valencia, CA, U.S.A) (Hedges and Stern, 1984).

Sediment core sampling and TOC and TN analysis

A coring campaign was conducted in October 2017 in both lakes, using a 50 mm gravity Kajak-type sediment corer (Kajak et al., 1965; Renberg, 1991; Brooks et al., 2007). Two long core sediment samples (30 cm for Balma Lake; 36 cm for Dimon Lake) were retrieved from the deepest sampling site (B3-Balma Lake; B1-Dimon Lake) in both lakes. The core samples were sealed in a sampling tube, brought to the laboratory and stored at 4 °C until further analyses. Subsamples were obtained by cutting each core in transverse sections of 2 cm. The choice of section thickness was dictated by the need to obtain a sample suitable for analysis with a minimum volume equal to 30 cm^3 of wet sediment, as indicated for mountain lakes due to their high sedimentation rates (Heiri and Lotter, 2003; Heiri et al., 2003; Brooks et al., 2007). Total organic carbon (TOC, %) and total nitrogen (TN) were also measured in each core section for both lakes.

Sediment core samples dating

Two bulk sediment samples (8-10 cm and 20-22 cm core depth for Balma Lake; 2-4 cm and 21-23 cm core depth for Dimon Lake) were used for AMS ^{14}C carbon analyses, which were performed at the Poznan Radiocarbon Laboratory (Poznan, Poland). Analysis of bulk sediment sample for dating was performed because no vegetal remains were found in the core samples. In addition to radiocarbon dating, sediment dating was investigated using non-radiocarbon data. In particular, the total lead (208Pb) concentration in core sections was determined by Inductively Coupled Plasma - Mass Spectrometry (ICP-MS). Following an adaptation from EPA Method 3052, freeze dried samples were manually powdered using an agatha mill. Then, 0.300 g aliquots were solubilized with a mixture of 5 mL of nitric acid (69%) and 1 mL of hydrogen peroxide (30%) and subjected to microwave digestion for 55 minutes using a Multiwave PRO Anton Paar reaction system (Anton Paar, Graz, Austria). Samples were then centrifuged to remove residue, transferred to flasks, and filled to the mark with MilliQ water (25 mL final volume). Samples were diluted to 1:20 and analyzed by ICP-MS NEXION 350X (manufactured by Perkin Elmer, Waltham, Massachusetts, U.S.A.). Lead concentrations were determined using the calibration curve method obtained from the analysis of five standard solutions (range 0 - 100 ppb).

Beginning of lead emissions could be dated back to 6000-8000 years ago due to human utilization (Needleman, 1999) and the first indication regarding the atmospheric deposition of lead pollution are dated between 2000 and 1500 BC (Renberg et al., 2000). However, atmospheric lead pollution dramatically increased during the past century reaching main peak in the 1970s with increased fossil fuel combustion and the extensive use of leaded petrol (Nizich et al., 2000; Renberg et al., 2000; Brännvall et al., 2001; Siver and Wonziak, 2001). Due to the deposition of atmospheric pollution, lead concentration increased over time also in soils, where concentration trends followed the history of atmospheric ones (Renberg et al., 2000). During the peak in the early 1970s, the deposition rate was about 15 mg m^{-2} $year^{-1}$ (approximately 1000-fold the background rate) and it declined to about 1 m^{-2} $year^{-1}$ (Rühling et al., 1996; Renberg et al., 2001) after implementation of stricter emission standards and the introduction of lead-free petrol (Brännvall et al., 2001; Renberg et al., 2001; Siver and Wonziak, 2001). Since the 1970s, lead emissions decreased by 60-70% (Pacyna et al., 2007), showing a strong declining trend in Italy as well (ISPRA, 2009). In this context, indirect dating could be hypothesized for the top core sections through comparisons with other studies carried out in European lakes. As the highest peak in total Pb concentrations within core sediments is reported during the 1970s (Brännvall et al., 2001; Renberg et al., 2001; Arnaud et al., 2003, 2004; Spadini et al., 2003; Camarero et al., 2009a; Nedjai et al., 2011), the detection of this peak in the top sections of the Balma core could allow the identification of the core section corresponding to the 1970s period, when fish was introduced in the Piedmont Alpine lakes (Forneris et al., 1984).

Statistical analysis

The Wilcoxon test was used to assess significant differences in physicochemical parameters, water nutrients, TOC, and TN between two lakes. Results were considered statistically significant at p values < 0.05. The calibration of the radiocarbon dates was based on the IntCal13 dataset (Reimer et al., 2013). Balma Lake age depth model was obtained using RStudio Package Clam 2.3.2 (Blaauw, 2010) through smooth interpolation function, using 14C AMS dates and total Pb peak.

5.3 Results

Hydrochemistry, TOC and TN concentration

The lake water temperature was lower in autumn, consistent with the season. The lowest temperatures were recorded for Lake Dimon during autumn (range: 3.20-3.85 °C). No thermal stratification was found at either lake. Conductivity differed significantly between the two lakes (Wilcoxon test, n=32; $p < 0.01$), with higher values measured for Dimon Lake (range: 122-143 $\mu S\ cm^{-1}$). As expected for mountain lakes, water oxygenation was high, with levels of oxygen saturation (>100%), except for the levels measured at the littoral sites in Balma Lake during summer (range: 77-92%). The pH was significantly higher at Dimon Lake (Wilcoxon test, n=32; $p < 0.001$). Table 3 presents the physicochemical parameters. There were no significant differences in NH_4^+ and NO_3^- concentrations between the two lakes, whereas the PO_4^{3-} concentration was significantly higher in Dimon Lake than in Balma Lake (Wilcoxon test, n=20; $p < 0.01$) (Table 4). The results of TOC and TN content analysis in surface littoral and deep samples from Balma Lake and Dimon Lake are reported in Table 5.

Table 3. Physicochemical parameters for Dimon Lake and Balma Lake in summer and autumn (Lit_1-5 = littoral sites; B_1-3 = deep sites).

Lake	Season	Site	T (°C)	O_2 (mg L^{-1})	O_2 saturation (%)	pH	Conductivity (uS cm^{-1})	Secchi disk (m)
Dimon	Summer	Lit_1	16.70	08.70	109	8.90	124	-
		Lit_2	15.60	08.40	106	8.57	125	-
		Lit_3	16.80	09.00	115	8.81	132	-
		Lit_4	16.00	08.60	110	8.79	122	-
		Lit_5	15.80	08.70	108	8.77	122	-
		B_1	16.00	08.40	109	8.65	134	4.00
		B_2	16.00	08.50	108	8.64	135	3.43
		B_3	16.00	08.45	109	8.65	135	3.72
	Autumn	Lit_1	03.50	10.30	101	9.43	141	-
		Lit_2	03.30	10.50	101	9.38	143	-
		Lit_3	03.20	10.20	98	9.50	141	-
		Lit_4	03.25	10.80	105	9.48	141	-
		Lit_5	03.85	10.10	99	9.39	134	-
		B_1	03.62	10.20	100	9.43	141	4.00
		B_2	03.55	10.30	101	9.21	140	3.00
		B_3	03.51	10.10	99	9.39	138	3.20
Balma	Summer	Lit_1	15.60	06.10	77	6.53	18	-
		Lit_2	15.80	07.16	91	6.50	18	-
		Lit_3	15.90	06.88	86	6.66	18	-
		Lit_4	16.10	07.02	92	7.31	20	-
		Lit_5	17.00	06.95	92	6.52	19	-
		B_1	14.60	08.80	103	6.50	17	3.25
		B_2	15.10	07.93	100	6.55	19	3.25
		B_3	14.85	08.37	101	6.53	18	3.25
	Autumn	Lit_1	07.20	05.96	102	7.53	21	-
		Lit_2	08.80	06.20	104	7.69	18	-
		Lit_3	08.40	05.55	106	7.55	18	-
		Lit_4	08.70	05.85	104	7.73	18	-
		Lit_5	08.70	06.05	101	7.70	18	-
		B_1	08.20	09.00	106	7.65	18	2.80
		B_2	07.90	10.12	108	7.90	19	2.80
		B_3	08.05	09.56	107	7.78	19	2.80

Table 4. Concentration (mg L^{-1}) of water nutrients (NH_4^+, NO_3^- and PO_4^{3-}) in Dimon Lake and Balma Lake in summer and autumn (Lit_1-5 = littoral sites).

Lake	Season	Site	NH_4^+ (mg L^{-1})	NO_3^- (mg L^{-1})	PO_4^{3-} (mg L^{-1})
Dimon	Summer	Lit_1	0.09	13.70	0.02
		Lit_2	0.70	12.50	0.00
		Lit_3	0.36	27.60	0.02
		Lit_4	0.06	27.60	0.08
		Lit_5	0.13	13.90	0.09
	Autumn	Lit_1	0.06	1.73	0.29
		Lit_2	0.15	1.33	0.05
		Lit_3	0.07	1.30	0.20
		Lit_4	0.06	1.60	0.08
		Lit_5	0.07	1.40	0.04
Balma	Summer	Lit_1	0.13	8.87	0.01
		Lit_2	0.04	9.30	0.01
		Lit_3	0.05	4.40	0.02
		Lit_4	0.20	12.00	0.01
		Lit_5	0.05	8.20	0.01
	Autumn	Lit_1	0.05	7.50	0.01
		Lit_2	0.06	9.20	0.01
		Lit_3	0.14	4.00	0.00
		Lit_4	0.13	8.15	0.02
		Lit_5	0.05	5.25	0.01

Table 5. Concentration of total organic carbon (TOC; %), total nitrogen (TN; %), and C/N ratio in Dimon Lake and Balma Lake (Lit_1-5 = littoral sites; B_1-3 = deep sites).

Lake	Site	TOC (%)	TN (%)	C/N
Dimon	Lit_1	5.72	0.49	14.04
	Lit_2	5.11	0.47	8.18
	Lit_3	0.32	0.08	4.65
	Lit_4	0.30	0.07	4.98
	Lit_5	0.20	0.05	6.13
	B_1	3.18	0.31	11.18
	B_2	4.02	0.40	11.71
	B_3	3.34	0.34	11.56
Balma	Lit_1	14.74	1.27	13.56
	Lit_2	11.52	1.29	10.43
	Lit_3	3.20	0.35	10.88
	Lit_4	12.99	1.00	15.12
	Lit_5	19.21	1.33	16.86
	B_1	5.98	0.47	13.95
	B_2	3.28	0.29	13.18
	B_3	2.65	0.24	12.88

Generally, levels of TOC (%) were significantly higher at the littoral sites than the deep sites in Dimon Lake (Wilcoxon test, n=16; $p < 0.01$), whereas both TN (%) and TOC (%) were significantly higher at the littoral sites than the deep sites in Balma Lake (Wilcoxon test, n=16; $p < 0.01$). As regard core sediment samples, in Balma Lake TOC and TN values increase with the rising depth in the core sediment, ranging from a minimum value of 2.48% to a maximum of 4.89% and 0.16% to 0.37% respectively (Table 6a). Both TOC and TN reach higher values at 20-22 cm. The corresponding C/N ratios gradually increased downcore from 9.71 to 13.6. On the contrary, in Dimon Lake TOC and TN showed high fluctuation values along the core sediment sections likewise the corresponding C/N ratios (Table 6b).

Table 6. Total organic carbon (TOC; %), total nitrogen (TN; %) in core sediment samples from Balma Lake (a) and Dimon Lake (b).

(a) Balma Lake			
Depth (cm)	**TOC (%)**	**TN (%)**	**C/N**
0-2	3.97	0.33	9.71
2-4	3.57	0.32	9.59
4-6	3.06	0.24	10.94
6-8	2.92	0.24	10.21
8-10	2.54	0.19	10.57
10-12	2.48	0.19	11.14
12-14	2.78	0.24	11.22
14-16	3,53	0.30	11.26
16-18	3.85	0.27	11.46
18-20	3.56	0.23	12.47
20-22	4.89	0.37	11.81
22-24	4.18	0.31	12.12
24-26	3.63	0.24	12.39
26-28	3.42	0.20	13.52
28-30	2.51	0.16	13.66

(b) Dimon Lake			
Depth (cm)	**TOC (%)**	**TN (%)**	**C/N**
0-2	4.31	0.45	11.12
2-4	5.68	0.61	11.33
4-6	5.20	0.51	11.99
6-8	4.77	0.43	13.17
8-10	4.99	0.45	12.81
10-12	4.76	0.43	12.61
12-14	4.48	0.37	13.55
14-16	4.25	0.35	14.85
16-18	3.84	0.32	15.39
18-20	2.97	0.23	17.52
20-22	2.50	0.21	17.30
22-24	2.26	0.18	17.37
24-26	1.94	0.14	18.33
26-28	1.40	0.11	19.83
28-30	1.92	0.16	18.52
30-32	1.62	0.13	19.28
32-34	1.68	0.14	18.86
34-36	1.61	0.13	17.22

The results of total lead (208Pb) determination for both lakes are reported in Figure 22. The Pb profile in Dimon Lake showed values with high fluctuations down the core sample with higher value recorded in the upper part of the core (109.54 µg g^{-1}) and at 30 cm (109.44 µg g^{-1}). Instead, in Balma Lake, Pb concentrations showed lower values at the bottom of the core, between 18-20 and 28-30 cm, ranging from 6.54 µg g^{-1} to 8.56 µg g^{-1}. Values started to increase from a depth of 16-18 cm and remained fairly constant until a depth of 8-10 cm (16.27-19.04 µg g^{-1}); at 4-6 cm the increase become more evident (30.95 µg g^{-1}). Finally, Pb concentration reached a peak in the 2-4 cm section (73.07 µg g^{-1}) and then decreased at the surface samples (40.75 µg g^{-1}). The measured Pb data have been compared with others different Pb analyses recorded on cores collected in nearby lake basins (Arnaud et al., 2003, 2004; Camarero et al., 2009a; Nedjai et al., 2011). Also considering data published by ISPRA (2009), which showed a decrease in lead emissions in Italy after 1990, it was possible to estimate that the observed peak could be likely related to the time period 1970-1980.

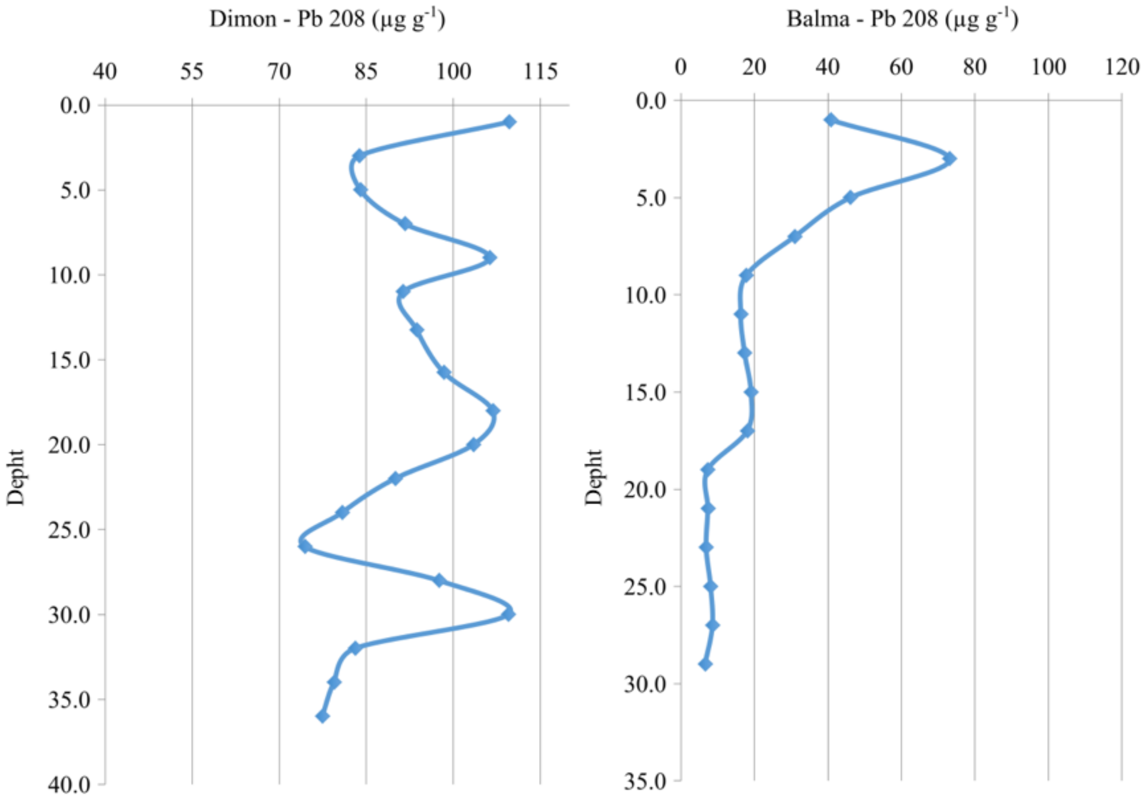

Figure 22. Values of total lead Pb (μg g^{-1}) in each section of sediment core samples from both Dimon Lake and Balma Lake.

Finally, the results of radiocarbon analyses allowed to date the 8-10 cm and 20-22 cm samples (Table 7) in both lakes.

Table 7. Radiocarbon ages for sediment core in Balma Lake and Dimon Lake.

Lake	Depth of sample	Lab code	14C Age (Year BP)
Balma	8-10	Poz-111888	800 ± 30
	20-22	Poz-111887	1515 ± 30
Dimon	8-10	Poz-117166	475 ± 30
	20-22	Poz-117167	2010 ± 30

The data and the information obtained from Pb determination allowed us to date core sections only in Balma Lake, since in Dimon Lake a high fluctuation values of TOC, TN and Pb supported by dubious values from radiocarbon analysis lead us to assume a sediment mixing due to several landslides and detrital input (Venturini, 2003, 2006).

Ages obtained from the smooth interpolation model for Balma Lake are reported in Table 8, while the graphical representation of the used age-depth model is reported in Figure 23.

Table 8. Ages obtained from the smooth interpolation model for Balma Lake.

Balma Lake	
Depth (cm)	**Age depth model (Years BP-2σ calibrated)**
0-2	0
43557	47
43620	153
43683	310
43746	493
43809	680
41974	851
14-16	1007
16-18	1152
18-20	1288
20-22	1421
22-24	1553
24-26	1685
26-28	1817
28-30	1949

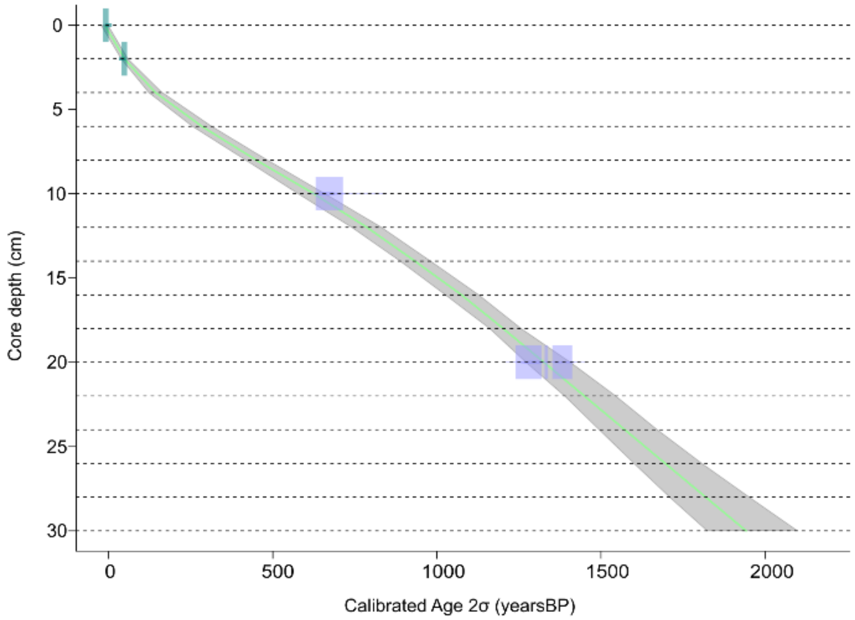

Figure 23. Age-depth model based on smooth interpolation of best point calibration estimates from ^{14}C dates and non-carbon dates (Pb concentrations in core sections), obtained using he Clam 2.3.2 Package for RStudio. Grey area shows the 95% confidence intervals based on 1000 interactions; green line represents the best fitted age. Accumulation rate for the Balma Lake was equal to 0.018 cm y^{-1}.

5.4 Discussion

Lake water temperature differed due to season and the absence of thermal stratification because of the shallow depth of the two lakes (maximum depth 6.42 m Balma Lake; 4.27 m Dimon Lake) and the high transparency of the water column, which can contribute to attenuation of stratification (Tiberti et al., 2010). As expected for high-mountain lakes, the oxygenation values were generally high, with frequent supersaturation conditions, as previously observed by Tiberti et al. (2010) in 12 alpine lakes in Gran Paradiso National Park (Western Alps, Italy). The pH values were significantly higher at Dimon Lake than at Balma Lake (pH range 8.64-9.50 and 6.50-7.78, respectively) due to the geo-lithological context of the study areas: lower values are noted for lakes lying over a granite bed than those over limestone or sandstone (Camarero et al., 2009b), which usually have a pH > 8 (Füreder et al. 2006). In fact, Dimon Lake lies over sandstone and volcanic rocks (Venturini, 2003, 2006). The observed pH values were generally consistent with the data for high-altitude environments (Kownacki et al., 2000; Boggero et al., 2006; Füreder et al. 2006; Fjellheim et al., 2009; Hamerlík et al., 2017). The conductivity at Dimon Lake (range 122-143 $\mu S\ cm^{-1}$) was in line with data reported by Boggero et al. (2006) and Fjellheim et al. (2009) for high-altitude lakes located in the Julian Alps (Northeast Italy and Slovenia), whereas the values recorded at Balma Lake were significantly lower (range 17-21 µS cm^{-1}) but still generally in line with published data (Kownacki et al., 2000; Boggero et al., 2006; Fjellheim et al., 2009; Hamerlík et al., 2017). The differences in conductivity between Dimon Lake and Balma Lake can be explained by their geo-lithology: siliceous basins tend to have a conductivity < 50 µS cm^{-1}, whereas basins with a higher percentage of carbonate component have higher values (250 µS cm^{-1}) (Boggero et al., 2006; Füreder et al., 2006). Regarding water nutrients, phosphate concentrations were significantly higher at Dimon Lake than at Balma Lake, suggesting a higher trophic condition probably linked to human activities. Animals are grazed around both Dimon Lake and Balma Lake (sheep and cattle, respectively). The magnitude of the impact of nutrient enrichment due to these activities was not exactly quantifiable, however. Furthermore, the phosphate concentration measured for Dimon Lake was consistent with data reported by Boggero et al. (2006) (0.01-0.08 mg L^{-1}) and Fjellheim et al. (2009) (0.01-0.08 mg L^{-1}) for lakes in the Eastern Alps (Julian Alps), suggesting other causes for the observed differences. A possible explanation could be the greater rainfall in Friuli Venezia-Giulia (2500 mm per year; source: ARPA FVG, 2017) compared to Piedmont (1296 mm per year; source: ARPA Piemonte, 2017). Because Alpine lakes are typically oligotrophic environments, they are sensitive to even small changes in nutrients transported by the atmosphere (Reche et al., 2009; Brahney et al., 2015). No difference in the concentration of NO_3^- and NH_4^+ between two lakes was found, though the average values tended to be slightly higher in Dimon Lake. Further investigations with more frequent samplings are necessary to support this observation. In Balma Lake, levels of TN and TOC decreased from the surface down to about 10 cm probably due to the mineralization of organic matter by bottom organisms, while the increasingly evident oscillation in strata between 14 and 20 cm was probably due to an inefficient recycling of organic matter. The observed increase in C/N ratios with depth is expected because of a bacterial preference for degrading nitrogen-rich compounds (Fenchel et al., 1998). However, values of C/N ratios around 10, as observed in the top sections, could indicate an origin of non-vascular aquatic plant whereas values of about 20 or higher are related to a higher contribute of terrestrial organic matter (OM) (Klug et al., 2009). In the deeper sediments, C/N ratios are found to be quite uniform in value thus suggesting that degradation processes are most intense because higher proportion of terrestrial OM. Similar results was reached in oligotrophic Slovenian mountain lake sediments (Muri and Simčič, 2004) which found that respiratory activity was most intense in the upper 3-5 cm of the sediments where autochthonous material and perhaps some labile terrestrial components were degraded than the deeper sediments where terrestrial organic matter recorded increasing values. As mentioned above, for what concert Dimon Lake the high fluctuation of TOC, TN and Pb along the core sediment lead us to assume a sediment mixing due to several landslides and detrital input, not allowing the core dating.

The trend observed in the Balma core showed the highest Pb peak within the 2-4 core section, which was significantly higher than the other observed values. No evidence of sediment movement after sedimentation emerged during the sample analysis. This peak observed for one of the top core sections could be related to the 1970s period, in agreement to other studies carried out in Europe (Brännvall et al., 2001; Renberg et al., 2001; Arnaud et al., 2003, 2004; Spadini et al., 2003; Camarero et al., 2009a; Nedjai et al., 2011). Despite the low number of points and despite the impossibility to conduct further analyses, the age depth-model obtained for the Balma Lake core is comparable to those reported in studies carried out in other European lakes (Table 9). In addition, the accumulation rate value (0.018 cm y^{-1}) observed for the Balma Lake is also in agreement with those reported in literature. However, accumulation rates observed in the Alpine lakes are generally very low (Appleby, 2000; Camarero et al., 2009a). Moreover, accumulation rates could vary between different lakes (Bennet and Buck, 2016) showing wide ranges due to several factors such as catchment-lake relationship, erosion dynamics and meteorological conditions (Arnaud et al., 2016).

Table 9. Comparison between age depth-model obtained for Balma Lake and other European lakes.

Lake	Geographic area	Altitude (m a.s.l.)	Dating method	Core depth (cm)	Age obtained from age-depth model 30 cm depth		Mean sedimentation rate (cm y^{-1})	Reference
					cal BP 2σ	BC/AD		
Balma	Cottian Alps, Italy	2100	AMS ^{14}C	30	1949		0.018	Present work
Mutterbergersee	Tyrol region, Austria	2483	^{210}Pb-CRS and AMS ^{14}C	33		1360 AD	0.046	Ilyashuk et al., 2019
Marboré	Central Pyrenees, Spain	2612	AMS ^{14}C	700	400		0.061	Leunda et al., 2017
Schwarzsee ob Sölden	Central Eastern Alps, Austria	2796	AMS ^{14}C	159	2200		-	Ilyashuk et al., 2011
Lucern	Central Switzerland	434	^{137}Cs peak	163		1750 AD	0.070	Thevenon et al., 2011
Meidsee	Southern Switzerland	2661		121	1800	200 AD	0.020	Thevenon et al., 2011, 2012
Suorijaure 1	Sweden	962	Radiocarbon	400	600		0.470	Rubensdotter and Rosqvist, 2009
Suorijaure 2		962		110	2000		0.100	
Nero di Cornisello	Central Alps, Italy	2233	AMS ^{14}C	175	2500		0.011	Filippi et al., 2005
Hinterburgsee	Central Switzerland	1515	AMS ^{14}C	900	1600		-	Heiri et al., 2003

6. MACROBENTHIC INVERTEBRATES

6.1 Characterization of macrobenthic invertebrates' communities

6.1.1 Preface

The ecology of macrobenthic invertebrates in Alpine lakes is influenced by two orders of factors: local (nature of the substratum, vegetation cover, presence of tributaries or emissaries) and regional (biogeographic history of the lake, climate, and transport of long-range air pollution) (Fjellheim et al., 2009). Macrobenthic communities in Alpine lakes can be characterized according to the bathymetric profile: communities established in the littoral and sublittoral zones and communities established in the deep zone. More than 70% of the macrobenthic community in these lakes can be made up of Diptera Chironomidae and Oligochaetes (Laville, 1971; Juget and Giani, 1974; Stoichev, 2000; Boggero et al., 2006; Kownacki et al., 2006; Dumnicka and Boggero, 2007), achieve greater density (Kownacki et al., 2006; Oertli et al., 2008), and species diversification (Kownacki et al., 2000). Diptera of the Chironomidae family are found in the littoral and the deep zone, in sediments and on submerged, woody or rocky hard surfaces (Lencioni and Lazzara, 2004). Around 15000 species are known in the world (Armitage, 1995), at least 500 of which are present in Italy. Chironomids are excellent bioindicators (Wiederholm, 1984) for investigating the trophic state of lakes over time (Sæther, 1979; Hofmann, 1988). Chironomid survival in an environment depends on diverse environmental factors, including water temperature, pH, and dissolved oxygen (Oliver, 1983; Lencioni and Lazzara, 2004). The ecological establishment of Chironomids in a wide range of trophic conditions is a consequence of their physiological adaptability, which allows them to live in extreme conditions. They are able to colonize many types of environment and substrates (Berg and Hellenthal, 1992; Armitage, 1995). Stenotherm species are usually found in Alpine lakes, where they constitute typical communities (Kownacki et al., 2000; Füreder et al., 2006; Čiamporova-Zat'ovičová et al., 2010). Chironomids are generally the predominant taxon in these kinds of environments, with organisms of the subfamily Orthocladiinae, which constitutes patterns of species typical of high-altitude environments (Bretschko, 1974; Kownacki et al., 2000; Füreder et al., 2006). Also frequent is the subfamily Diamesinae (Brittain and Milner, 2001).

Among other taxa, the subclasses Oligochaetes can be found in the littoral, sublittoral, and deep zones, where they generally prefer fine substrates (Dumnicka, 1994, Verdonschot, 2001). They are classified in the functional groups of collectors and predators (Sansoni, 1988). Chironomid communities are also studied to reconstruct natural or anthropogenic changes caused by eutrophication (Walker and Mathewes, 1987; Hofmann, 1988; Walker, 2001; Porinchu and MacDonald, 2003), poor oxygenation

(Brodersen and Quinlan, 2006), water acidification (Brooks et al., 2007) or climate change (Walker et al., 2002; Levesque et al., 1993). Moreover, recent studies have identified fish as one of the major factors that influence changes in Chironomid community composition (Milardi et al. 2016; Perrine, 2017; Raposeiro et al., 2017). The presence of fish can threaten the free-living Chironomid taxa and/or larger individuals, since they are more easily subject to predation (Uutala, 1990; Johnson et al., 1990; Armitage, 1995; Heiri and Lotter, 2003; Porinchu and MacDonald, 2003). Furthermore, Chironomid communities can also change with the disappearance of a fish community (Perrine, 2017). The aims of the present study were to characterize the macrobenthic invertebrate community in Balma Lake and Dimon Lake in summer and autumn 2017 in relation to environmental parameters.

6.1.2 Material and Methods

Macrobenthic invertebrate sampling

Macrobenthic invertebrate sampling was carried out during two campaigns in 2017: one in summer (August) and one at the beginning of autumn (October). The choice of these periods was dictated by the need to exploit the time in which the lake is accessible (generally from June to early November), with the intent to study the macrobenthic community in seasons when the abundance and biomass of these organisms are greatest (Fjellheim et al., 2000, 2009). A Surber net (mesh 250 µm; 0.1 m^2 subtended area) was used for sampling the macrobenthic communities at 5 sites of the littoral zone (depth 25 to 60 cm), and 3 subsamples were collected at each site with a different microhabitat and presence of tributaries or emissaries (Boggero et al., 2014; Dumnicka et al., 2015) (see section 4). Single samples were collected from the deep sites using a Van Veen grab (2 liters, 260 cm^2 sampling surface) and sieved through a net with a mesh size of 250 µm (Boggero et al., 2014).

To standardize the sampling procedure, all substrates were disturbed for at least 2 min (Fjellheim et al., 2009; Füreder et al., 2006). After collection, the littoral and deep samples were stored *in situ* with 70% ethanol solution and brought to the laboratory. The organisms were counted and determined where possible to the species level with the help of dichotomous keys (Minelli, 1977; Ferrarese and Rossaro 1981; Rossaro, 1982; Belfiore, 1983; Carchini, 1983, Ferrarese, 1983; Moretti, 1983; Oliver, 1983; Pinder and Reiss 1983; Sæther, 1983; Rivosecchi, 1984; Nocentini, 1985; Campaioli et al., 1994, 1999; Sansoni 1988; Zwick 2004; Lencioni et al., 2007; Moller Pillot 2009a, 2009b, 2009c; Timm, 2009; Andersen et al., 2013; Sambugar and Giacomazzi, 2013).

Microscopic preparations of the Diptera Chironomids were set up such that the head of each larva was separated from the body by positioning the head ventrally and the body dorsally; the sample was dissected at the stereomicroscope to avoid damaging the specimens and with the aid of dissecting needles (Lencioni et al., 2007). Slides were prepared using a semi-permanent preservation technique involving the use of Faure liquid. The slides were analyzed by transmission optical microscopy at magnifications from 60x to 100x. Microscopic preparations of Oligochaeta were set up by mounting the specimens on temporary slides and applying a drop of distilled water to identify them under an optical microscope.

Statistical analyses

The average number of genera for each sampling site was determined to characterize the communities, and the Shannon-Wiener index and the Equitability index (Evenness) were calculated. The indices were calculated based on the number of organisms identified at the genus level. The presence of significant differences between values for the two lakes was tested using the non-parametric Wilcoxon test.

The density data for the macrobenthic invertebrate community were used to investigate the differences in the structure of the communities from the two lakes. For this purpose, one-way PERMANOVA was used. Furthermore, the contribution of the taxa to the observed variability was investigated using the SIMPER test. Finally, canonical correspondence analysis (CCA) was performed to correlate the environmental parameters to the taxa density at the littoral sites (Bazzanti et al., 2010). To avoid/reduce multicollinearity, a non-redundant subset of variables was chosen through stepwise selection. Variables with Variance Inflation Factors (VIF) higher than 4 were dropped from the model (Hair et al., 2010). Taxa recorded only in one replicate per site or present at very low densities (one individual) were not included in the analysis (Bazzanti et al., 2010).

Data were transformed (log [x + 1]) before analyses. Statistical analysis was performed using RStudio software, version 1.1.383. A *p*-level of 0.05 was set to interpret the significance of the test results.

6.1.3 Results

Macrobenthic communities

A total of 10657 specimens of macrobenthic invertebrates were collected and identified, 6242 of which from Dimon Lake and 4415 from Balma Lake. The number of observed genera (Fig. 24) did not differ significantly between the two lakes nor did the diversity (Shannon-Wiener index) or the equitability (Evennes) (Wilcoxon test: n=60, $p > 0.66$ for all comparisons) (Fig. 25).

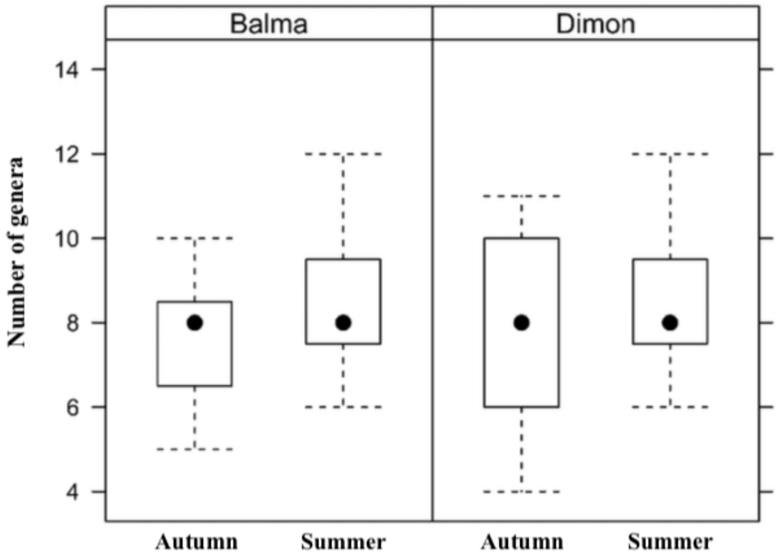

Figure 24. Box plots showing the total number of genera observed for Dimon Lake and Balma Lake in summer and autumn.

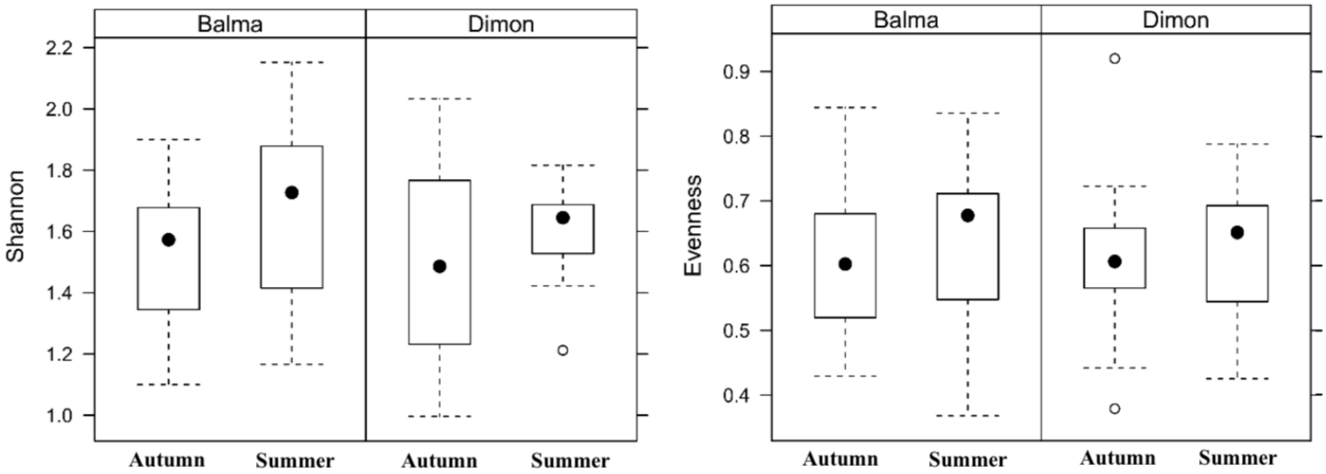

Figure 25. Box plots of the Shannon index and equality (Evenness) observed at the littoral sites in Dimon Lake and Balma Lake in summer and autumn.

Balma Lake

A total of 4415 specimens from the macrobenthos of Balma Lake were collected and identified. In the littoral zone sampling sites, the number of genera ranged from 6 to 12 in summer and from 5 to 10 in autumn, for a total of 1765 and 2556 individuals, respectively.

During the summer, the community (Fig. 26) was composed chiefly of Hexapoda belonging to Diptera Chironomids (density 556.7 ind m^{-2} to 786.7 ind m^{-2}), with five subfamilies (Fig. 27): Prodiamesinae, Chironominae, Orthocladiinae, Diamesinae, and Tanypodinae. The Orthocladiinae were represented by *Psectrocladius* gr. *psilopterus* (density 76.7-350.0 ind m^{-2}), *Hydrobaenus* (density 16.7-280.0 ind m^{-2}), and species of the genera *Cricotopus*/*Orthocladius* (density 26.7 ind m^{-2}). The Tanypodinae family was represented by the genera *Zavrelimyia* (density 150.0-510.0 ind m^{-2}) and *Macropelopia* (density 3.3-153.3 ind m^{-2}). The genera *Paratanytarsus* (density 106.7-286.7 ind m^{-2}) and *Micropsectra* (density 33.3 ind m^{-2} only at station 1) of the Tanytarsini tribe were also recorded.

The second most abundant taxon was the Oligochaeta (Fig. 28). A higher density of the subfamily Tubificinae was recorded for the species *Spirosperma ferox* (density 3.3-1066.7 ind m^{-2}) and for the genus *Potamothrix* (density 13.3- 40.0 ind m^{-2}) and a lower density for the species *Tubifex tubifex* (density 3.3-56.7 ind m^{-2}). The Lumbriculidae family was present with only one species *Lumbriculus variegatus* (density 33-130 ind m^{-2}), and the Enchytreidae family with the genera *Cognettia* (density 23.3-146.7 ind m^{-2}) and *Henlea* (density 10.0-13.3 ind m^{-2}). Bivalvia of the genus *Pisidium* (density 6.7-66.7 ind m^{-2}) were present, as were the families Ephemeroptera (genus *Ecdyonurus*), Plecoptera (*Nemoura cinerea*), and Trichoptera (genus *Limnephilus*).

During the autumn sampling, the community was still mainly composed of the order Hexapoda (Fig. 26), almost entirely Diptera Chironomidae (density 650.0-1773.3 ind m^{-2}). The most abundant were the Orthocladiinae and the Tanypodinae families, followed by the Tanytarsini tribe (Fig. 27). Orthocladiinae was represented by *Psectrocladius* gr. *psilopterus* (density 300.0-956.7 ind m^{-2}), *Hydrobaenus* (density 16.7 ind m^{-2} only at site 1), and species of the genera *Cricotopus*/*Orthocladius* (density 66.7-186.7 ind m^{-2}). The Tanypodinae family consisted of the genera *Zavrelymia* (density 173.3-413.3 ind m^{-2}) and *Macropelopia* (density 13.3-66.7 ind m^{-2}). The genera *Paratanytarsus* (density 73.3-573.3 ind m^{-2}) and *Micropsectra* (density 123.3 ind m^{-2} only at station 1) of the Tanytarsini tribe were recorded. The second most abundant taxon was Oligochaeta (Fig. 28), mainly the species *Spirosperma ferox* (density 76.7-390.0 ind m^{-2}) and the genus *Potamothrix* (density 16.7-220.0 ind m^{-2}). *Lumbriculus variegatus* (density 26.7-180.0 ind m^{-2}) was also present.

Bivalves of the genus *Pisidium* (density 10.0-110.0 ind m^{-2}) were recorded in autumn. Only at site 1 were recorded specimens of the genus *Ecdyonurus* (Ephemoptera), the species *Nemoura cinerea* (Plecoptera) (density 13.3 ind m^{-2} and 3.3 ind m^{-2}, respectively), the genus *Limnephilus* and the species *Plectrocnemia conspersa* (Trichoptera) (density 13.3 ind m^{-2} and 3.3 ind m^{-2}, respectively).

Samples collected from the deep sites revealed the presence of Oligocheta in both seasons, with only two species of Tubificinae: *Tubifex tubifex* and *Spirosperma ferox* (Fig. 29).

The average density (ind m^{-2}) and functional feeding guilds (FFG) of taxa recorded in littoral sites of Balma Lake are reported in Appendix 1.

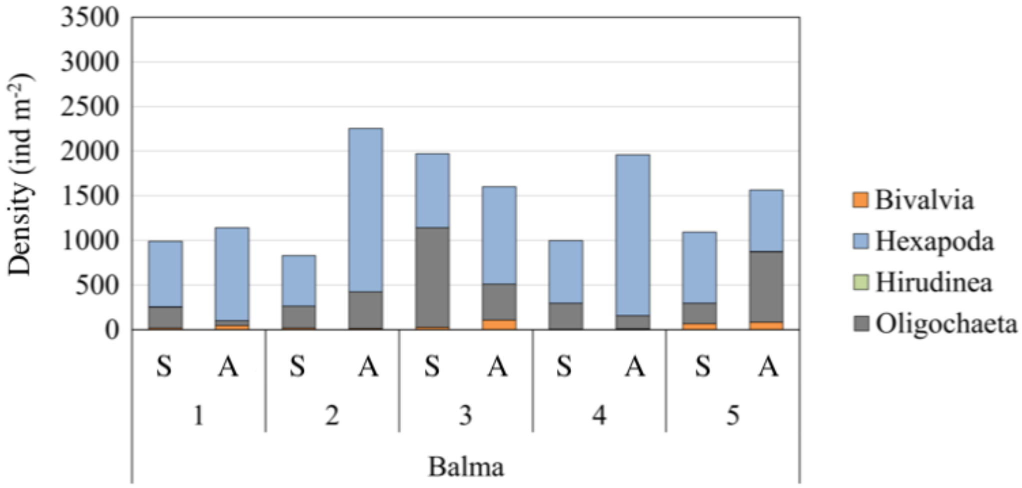

Figure 26. Average density (ind m^{-2}) of the main classes of macrobenthic invertebrates observed at Balma Lake in both seasons (S = summer; A = autumn).

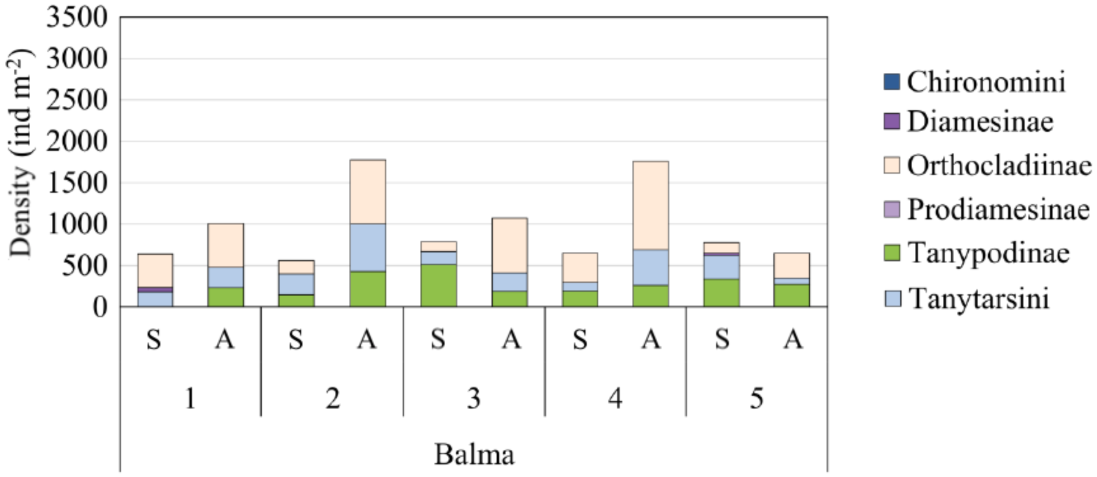

Figure 27. Average density (ind m^{-2}) of the main subfamilies and tribe of Dipera Chironomidae observed at Balma Lake in both seasons (S = summer; A = autumn).

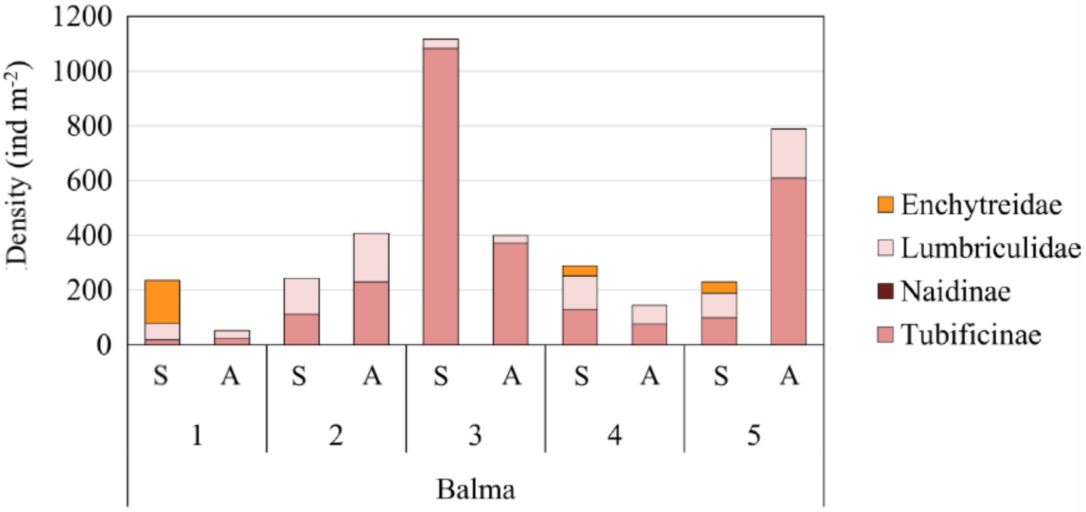

Figure 28. Average density (ind m^{-2}) of the main subfamilies and tribe of the Oligochaeta family observed at Balma Lake in both seasons (S = summer; A = autumn).

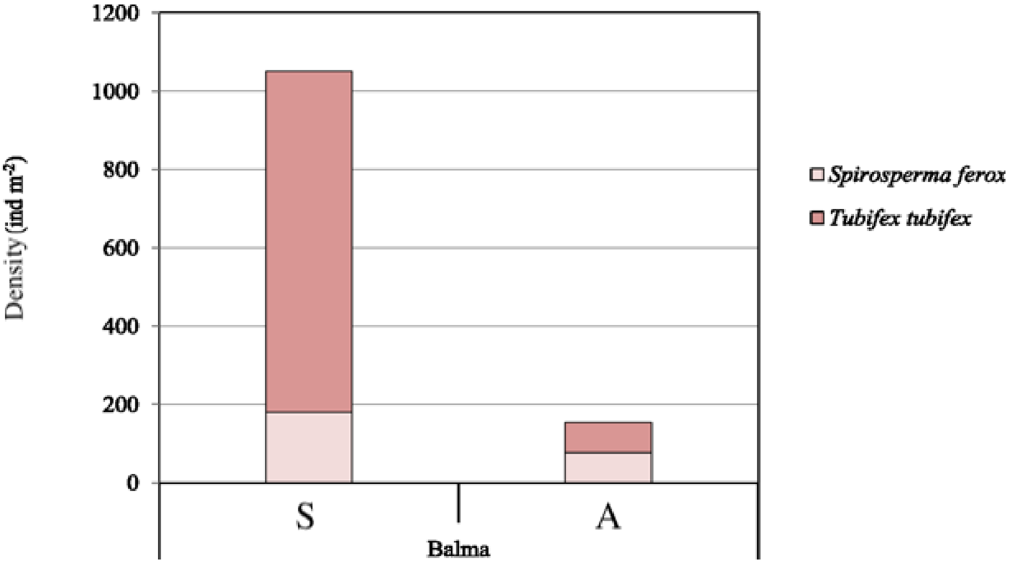

Figure 29. Average density (ind m^{-2}) of the main macrobenthic invertebrates observed at the deep sites at Balma Lake in summer (S) and autumn (A).

Dimon Lake

In the sampling sites of the littoral zone, the number of genera ranged from 6 to 12 in summer and from 4 to 11 in autumn, for a total of 3585 and 2338 individuals, respectively. During the summer, the community (Fig. 30) was mostly composed of Hexapoda belonging to the Diptera Chironomidae, with a density between 1116.7 ind m^{-2} (site 5) and 3089.5 ind m^{-2} (site 1), and four subfamilies (Prodiamesinae, Chironominae, Orthocladiinae, and Tanypodinae) (Fig. 31). The most abundant

Chironomid subfamilies of were Prodiamesinae and Chironominae. The Prodiamesinae subfamily was represented by the single species *Prodiamesa olivacea* (density 170.0 ind m^{-2} - 1263.3 ind m^{-2}), while the subfamily Chironominae was almost entirely represented by the tribe of the Tanytarsini genus *Paratanytarsus* (density 403.3-1120.0 ind m^{-2}). The Orthocladiinae subfamily was represented only by the genus *Cricotopus* (*Isocladius*) (density 330.0-613.3 ind m^{-2}), the Tanypodinae subfamily by the genera *Macropelopia* (density 10-173.3 ind m^{-2}) and *Zavrelimyia* (density 6.7-149.1 ind m^{-2}), and by specimens of the Thienemannimyia series (density 3.3-133.3 ind m^{-2}).

The taxon second highest in density was the Oligochaeta (Fig. 32). The family Lumbriculidae was represented by *Lumbriculus variegatus* (density 6.7-453.3 ind m^{-2}) and *Stylodrilus heringianus* (density 13.3-73.3 ind m^{-2}), the subfamily Tubificinae by the genera *Limnodrilus* (density 33.3-120.0 ind m-*2*) and *Tubifex* (density 13.3 -93.3 ind m^{-2}). The Naidinae family was present, with the species *Nais alpina* at site 3. The Hirudinea was present with a single species, *Helobdella stagnalis* (density 10.0-603.3 ind m^{-2}).

During autumn the community was still mainly composed of Hexapoda and Oligochaeta (Fig. 30). The density of Oligochaeta was higher than in summer. The Hexapoda was almost entirely attributable to Diptera Chironomidae, with four subfamilies present (Fig. 31): Orthocladiinae was numerically the most abundant, followed by the Tanypodinae. The Orthocladiinae was represented by the genus *Cricotopus* (density 126.7-840.0 ind m^{-2}) and the Tanypodinae by the genus *Zavrelimyia* (density 6.7 to 470.0 ind m^{-2}). Lower densities were recorded for Prodiamesine (*Prodiamesa olivacea* 16.7-90.0 ind m^{-2}) and the Tanytarsini (*Paratanytarsus* 13.3-336.7 ind m^{-2}).

Among the most abundant Oligochaeta recorded were the Lumbriculidae, with the species *Lumbriculus variegatus* (density 70.0-663.3 ind m^{-2}) and the Tubificinae, with the genera *Limnodrilus* (density 6.7-103.3 ind m^{-2}) and *Tubifex* (density 30.0-333.3ind m^{-2}) (Fig. 32). Also recorded were Naidinae of the species *Nais barbata* and *Nais bretscheri*.

The Hirudinea were the taxon third highest in density, with the species *Helobdella stagnalis* (density103.3-313.3 ind m^{-2}). The presence of *Nemoura cinerea* (Plecoptera) (density 40.0-53.3 ind m^{-2}) was also recorded.

Samples from the deep sampling sites (Fig. 33) in both seasons revealed the presence of Chironomidae, with the genus *Chironomus* (gr. *Anthracinus* and *Prodiamesa olivacea*) and Oligocheta, with the species *Tubifex tubifex* and *Spirosperma ferox*.

The average density (ind m^{-2}) and functional feeding guilds (FFG) of taxa recorded in littoral sites of Dimon Lake are reported in Appendix 2.

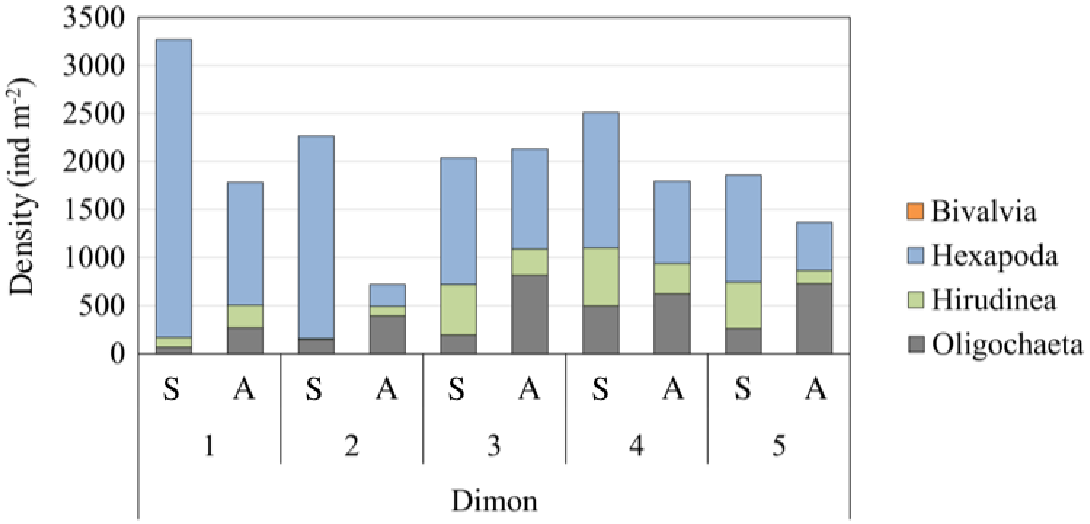

Figure 30. Average density (ind m^{-2}) of the main class of macrobenthic invertebrates in Dimon Lake in both seasons (S = summer; A = autumn).

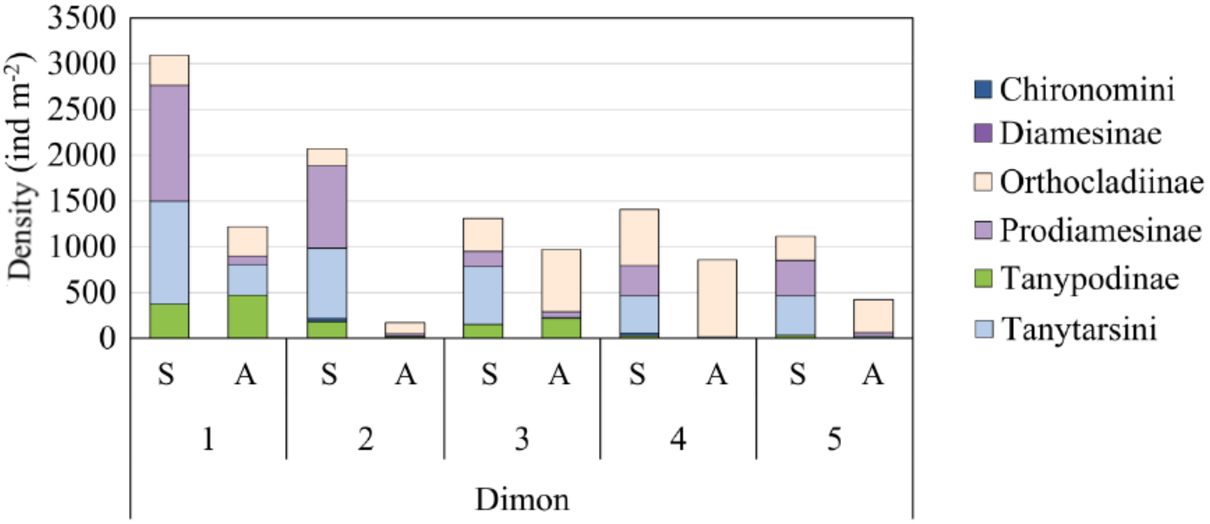

Figure 31. Average density (ind m^{-2}) of the main subfamilies and tribe of Dipera Chironomidae in Dimon Lake in both seasons (S = summer; A = autumn).

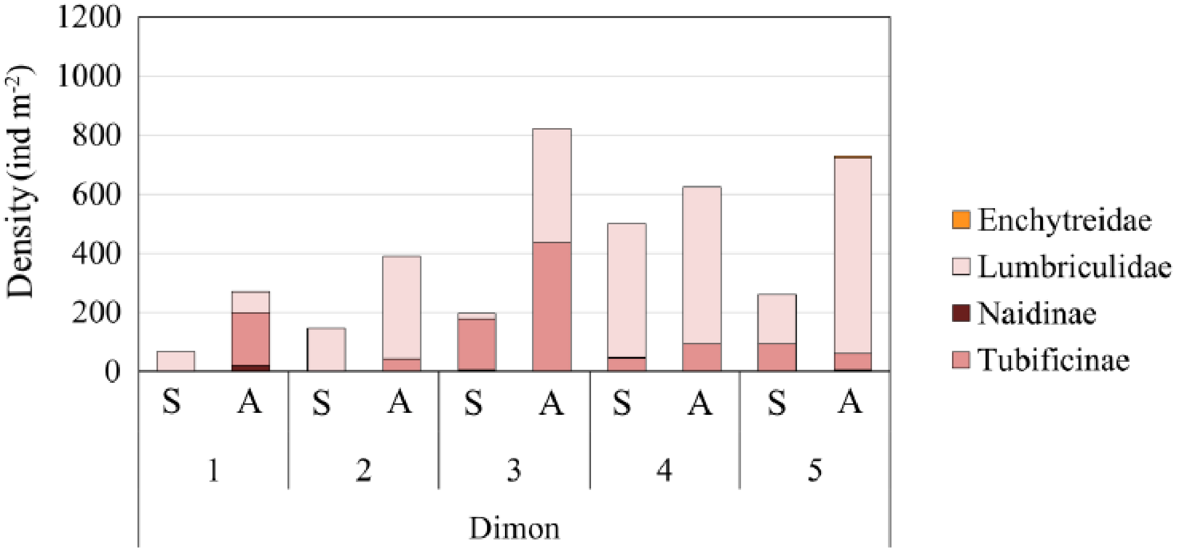

Figure 32. Average density (ind m^{-2}) of the main subfamilies and tribe of Oligochaeta in Dimon Lake in both seasons (S = summer; A = autumn).

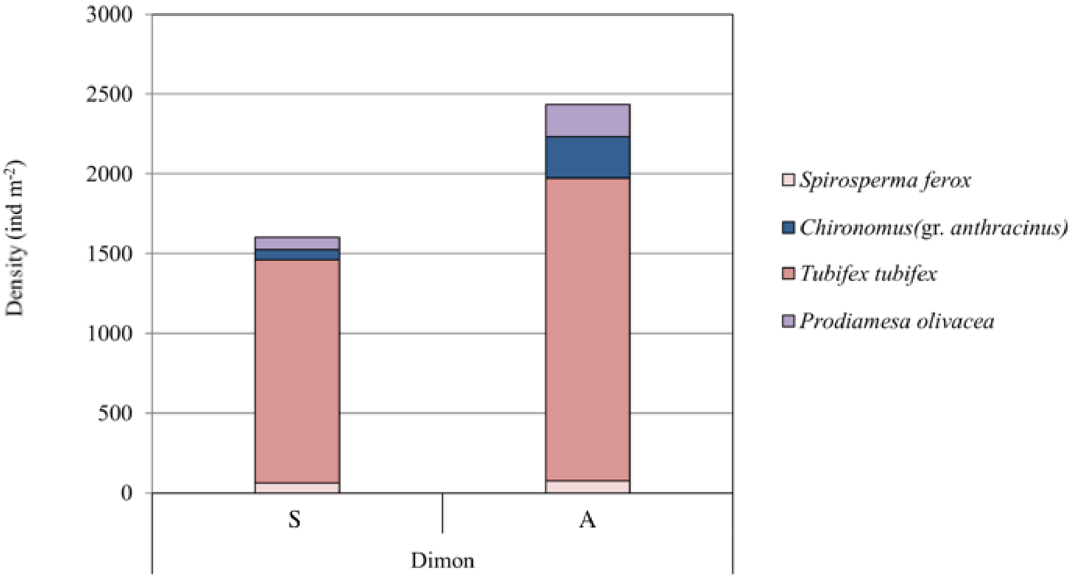

Figure 33. Average density (ind m^{-2}) of the main macrobenthic invertebrates at the deep sites in Dimon Lake in both summer (S) and autumn (A).

Comparisons between macrobenthic communities

PERMANOVA highlighted significant differences in macrobenthic communities from the littoral sampling sites of the two lakes. The SIMPER test (Table 10) revealed that the taxa that most differentiated the communities were *Cricotopus* (*Isocladius*), *Helobdella*, *Prodiamesa*, *Tubifex*, *Lumbriculus* (higher density at Lake Dimon) and *Psectrocladius* gr. *psilopterus*, *Spirosperma* and *Zavrelimyia* (higher density at Balma Lake). Other taxa contributed less than 3% to the variability.

Table 10. Results of SIMPER test. In the table are present only taxa that significantly contributed to at least the 4% of variability.

Taxon	**Av. Dissim**	**Contrib. %**	***p*-value**
Cricotopus (*Ospcladius*)	0.082	11.500	< 0.001
Psectrocladius gr. *psilopterus*	0.075	10.600	< 0.002
Helobdella	0.063	8.877	< 0.003
Prodiamesa	0.060	8.421	< 0.004
Spirosperma	0.046	6.504	< 0.005
Zavrelimyia	0.044	6.259	< 0.006
Tubifex	0.036	5.031	< 0.002
Pisidium	0.030	4.182	< 0.05
Paratanytarsus	0.028	3.998	< 0.05
Lumbriculus	0.028	3.984	< 0.05

The CCA is shown in Figure 34. The first two axes explain 56.3% of the observed variability and the first axis identifies a gradient correlated to pH, and water nutrient concentrations (P and NO_3^-). The Dimon Lake sites are shown on the central-left side of the plot, where the values of these variables were higher. The Balma Lake sites are shown on the right side of the plot, where the values of these variables were lower and the total organic carbon (TOC) in the sediments was higher.

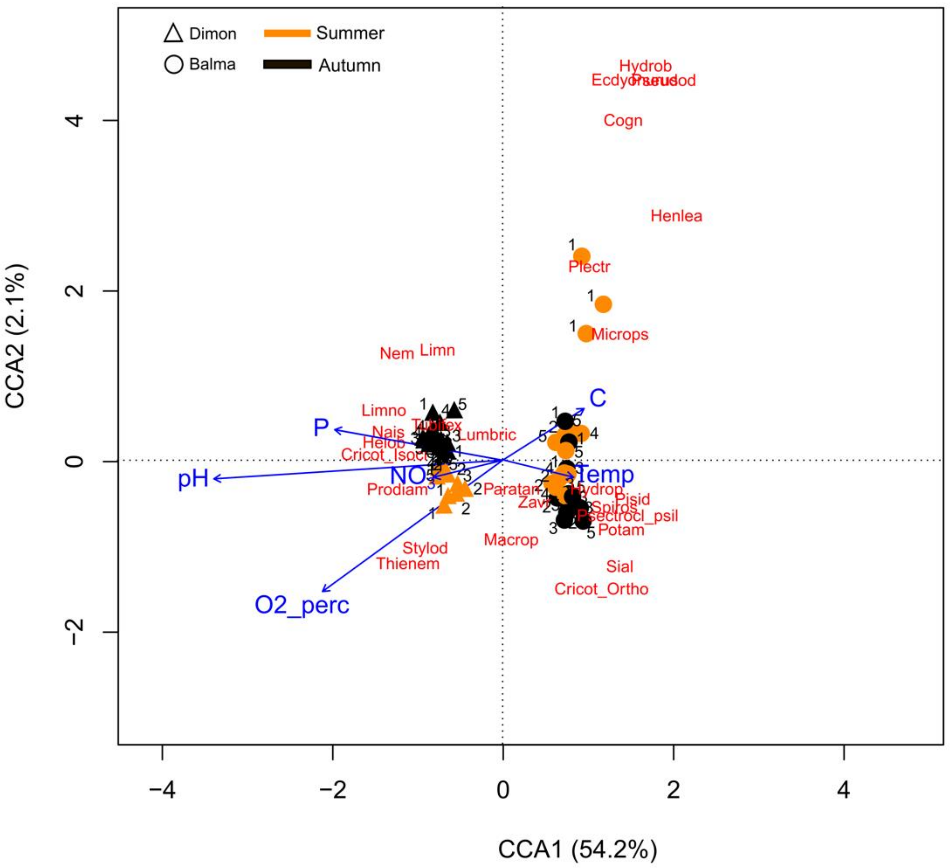

Figure 34. CCA analysis: relationship between physicochemical parameters of water, nutrients, content of total organic carbon, and taxa densities observed at the two lakes during summer and autumn sampling. O2_perc = oxygen saturation, C = percentage of total organic carbon-TOC in the sediment, P = PO_4^{3-}, Temp = temperature. Abbreviations of taxa: Cogn = *Cognettia*, Cricot_Isocl = *Cricotopus* (*Isocladius*), Cricot_Ortho = *Cricotopus*/*Orthocladius*, Ecdyon = *Ecdyonurus*, Helob = *Helobdella*, Henlea = *Henlea*, Hydrob = *Hydrobaenus*, Hydrop = *Hydroporus*, Limn = *Limnodrilus*, Lumbric = *Lumbriculus*, Macrop = *Macropelopia*, Microps = *Micropsectra*, Nais = *Nais*, Nem = *Nomoura*, Paratan = *Paratanytarsus*, Pisid = *Pisidium*, Plectr = *Plectrocnemia*, Potam = *Potamothrix*, Prodiam = *Prodiamesa*, *Psectrocl. psilopterus*, Pseudod = *Pseudodiamesa*, Sial = *Sialis*, Spiros = *Spirosperma*, Stylod = *Stylodrilus*, Thienem = *Thienemannymia*, Tubifex = *Tubifex*, Zavr = *Zavrelimyia*.

6.1.4 Discussion

The number of genera recorded for the two lakes are in line with data reported by Čiamporova-Zat’ovičová et al. (2010) in their study conducted in the Tatra Mountains (Slovakia, 1724 and 2157 m a.s.l.) where number of genera ranged from 5 to 18. The values of equality (Evenness) indicated a fair equipartition of organisms within the genera observed, albeit with a predominant trend for some taxa in some cases. The littoral community biodiversity was comparable with other high-altitude environments, where Diptera Chironomidae and Oligochaeta generally predominate (Bretschko, 1974; Kownacki et al., 2000; Füreder et al., 2006; Kownacki et al., 2006; Oertli et al., 2008; Fjellheim et al., 2009; Čiamporová-Zaťovičová et al., 2010; Dumnicka et al., 2015; Novikmec et al., 2015). PERMANOVA and the SIMPER test revealed differences in the littoral communities of the two lakes. *Prodiamesa olivacea* was the most often recorded species among Diptera Chironomidae in Dimon Lake, as reported for other high-altitude environments (Füreder et al., 2006; Fjellheim et al., 2009). Because the species is sensitive to low pH levels (Boggero et al., 2006; Moller Pillot, 2009c), this would explain its higher abundance in Dimon Lake (pH range 8.57-9.50) compared to Balma Lake (pH range 6.5-7.90), where the pH values are closer to the tolerance limit of the species (pH 6.06). The genus *Zavrelimyia*, together with *Paratanytarsus*, are the most widespread Chironomidae in Alpine lakes (Boggero et al., 2006; Füreder et al., 2006). The genera were found in both Dimon Lake and Balma Lake, with a higher density of *Zavrelimyia* in Balma Lake. This may be related to the tolerance of *Zavrelimyia* to low pH. Mollet Pillot (2009a) also reported finding individuals of this genus in freshwater environments with similar pH (pH 5.5-6.5). *Cricotopus* (*Isocladius*) was the only genus of Chironomidae of the Orthocladiinae subfamily at Dimon Lake. This is likely related to the trophic conditions of the lake (supported by results of physicochemical analysis) and to the pH. Boggero et al. (2006) reported this taxon as being frequent in Alpine lakes characterized by high trophic conditions. Also, Moller Pillot (2009c) reported that *Cricotopus* (*Isocladius*) prefers waters with pH > 7.5. This preference is generally reported also for *Psectrocladius gr. psilopterus*, which is characteristic of environments with low conductivity and low phosphorus concentration, however (Moller Pillot, 2009c). These conditions were present at Balma Lake, where *Cricotopus* (*Isocladius*) was observed in both sampling seasons, while it was absent at Dimon Lake. Some studies indicated *Psectrocladius gr. psilopterus* as being frequent in coastal environments rich in organic load (Langton, 1991; Boggero et al., 2006; Lods-Crozet et al., 2012), in agreement with the analysis of total organic carbon in the littoral sediments. As regards Hirudinea, the species *Helobdella stagnalism* was observed only at Dimon Lake, consistent with the results of water nutrient analysis (Elliot and Mann, 1979; Toro et al., 2006). The genus *Pisidium* was observed only at Lake Balma and this contrasts with observations by Nardi (2014) who reported the absence of this genus in environments with pH < 7. However, Boggero et al. (2005) reported the presence of *Pisidium* in Alpine lakes with pH < 7 (pH 6.21 and 6.74), in line with the values observed in summer at Balma Lake. Nardi (2014) also reported that the organism is abundant in systems characterized by sediments rich in organic matter, in line with the concentration of TOC and TN measured in Balma Lake. Regarding Oligochaeta, the species *Lumbriculus variegatus* was present in both lakes, although at significantly higher densities in Dimon Lake especially in autumn (temperature range 3.20-3.85°C) due to its low tolerance to higher temperature.

The CCA highlighted that site 1 (located near the inlet) in Balma Lake is clearly different from the other sites due to the presence of certain taxa. The genus *Ecdyonurus* (Ephemeroptera), a typical inhabitant of clear and well-oxygenated watercourses (Campaioli et al., 1994) was recorded. The genera *Pseudodiamesa* and *Micropsectra* (Chironomidae), which are also recorded in high-altitude oligotrophic lakes and in Alpine lakes (Pinder and Reiss, 1983; Moller Pillot, 2009c), were also recorded at this site. The genera *Cognettia* and *Henlea* (Enchytreidae) were recorded at two other sites in Balma Lake and are characteristic of acidic water (pH <6.3) (Campaioli et al., 1994; Dumnicka et al., 2015). The genus *Nais* was present in Dimon Lake (pH >7). The species *Nemoura cinerea* was observed at both lakes. This eurytherm species is widespread in Europe and Italy, where it can be found from the plains to the Alps. In Friuli Venezia-Giulia its distribution was reported in a study by Desio and Dorigo (2013), which recorded a maximum altitude of 1692 m a.s.l. To our best knowledge, our findings for Dimon Lake (1872 m a.s.l.) are the first for this species at a high-altitude environment.

Finally, *Gammarus lacustris* was absent from Lake Dimon, since first reported in the 1950s (Ruffo, 1952; Iannilli et al., 2004). Its absence is likely due to the introduction of non-native fish for recreational fishing. Since *G. lacustris* is known to be a fundamental component of the diet of salmonids (Iannilli et al., 2004), the introduction of alien species such as brook trout (*Salvelinus fontinalis*) in the past, probably led to the local extinction (Milardi et al., 2016; Perrine et al., 2017).

6.2 Consequences of fish introduction on macrobenthic invertebrates: a paleolimnological approach

6.2.1 Preface

Recent studies have identified fish fauna as a pivotal factor in changes within macrobenthic invertebrate communities of lentic environments, with special regard for Diptera Chironomidae assemblages: Tiberti et al. (2014b) showed the impact of fish introduction on high altitude lake invertebrate communities in the European Alps area; Milardi et al. (2016) observed how fish introduction contributed to decrease the macroinvertebrate abundance in the pelagic areas and to increase it in the littoral zone of some Finnish lakes; also in Finland, Perrine (2017) observed how chironomid assemblages in lakes with abundant fish populations are distinct from the assemblages in those without fish; Raposeiro et al. (2017) showed changes in the chironomid community composition of Lake Azul (Azores Islands) after fish introductions occurred over a period of about 220 years. In particular, the latter studies used a paleolimnological approach: subfossil chironomids are frequently abundant and well preserved in lake sediments, including older ones, due to the larval chitinous head capsules, and are an excellent material for paleolimnological investigations (Walker, 1995; Heiri and Lotter, 2003; Lencioni and Lazzara, 2004; Skov et al., 2010; Cao et al., 2014). Moreover, the study of chironomid head capsules could be a useful tool in order to detect past introductions, changes in fish populations (Raposeiro et al., 2017) and to rebuild past abundances of fish species in lentic environments (Perrine, 2017).

The aims of the present study were (a) to characterize the chironomid paleo-community through paleolimnological analyses and (b) to asses changes in chironomid community in relation to alien fish introduction. This study represents the first attempt to investigate the consequences of fish introduction on macrobenthic invertebrate communities of alpine lakes through paleolimnological analyses for the European Alps area. In this study, we analyzed the results only for Balma Lake, since the number of chironomid head capsule recorded in each core section of Dimon Lake were lower than the minimum number (n=50) indicated for obtaining a representative sample (Brooks et al., 2007).

6.2.2 Material and Methods

Chironomid head capsule identification

Head capsule extraction from core sediment samples from both lakes was performed following the indications reported by Lang et al. (2003) and Brooks et al. (2007). Samples were deflocculated in distilled water warmed at 40°C for 20 minutes and then sieved using a 100 µm mesh. The sieve residues were then sorted and chironomid head capsules were extracted under the stereomicroscope (at least 25x magnification). Samples were processed in order to obtain at least 100 head capsules for each section, to produce significant results (Brooks et al., 2007). Collected head capsules (HC) were then progressively dehydrated in 80% and 100% ethanol (5 minutes for each step) and mounted on microscope slides, ventral side up, in Euparal® essence.

The collected head capsules (HC) were identified to genus or species level (when possible) using an optical microscope at 60x –100x magnification, with reference to Oliver (1983), Pinder and Reiss (1983), Sæther (1983), Lencioni et al. (2007), Brooks et al. (2007), Moller Pillot (2009a, b, c) and Andersen et al. (2013). For each core section three subsamples were considered in order to perform statistical analyses, with each subsample containing a number of HC always higher than the minimum value ($n > 50$) for representative samples (Brooks et al., 2007).

Statistical analyses

Results of age-depth modelling previously present in section 5 were used to define four age groups (A-D), with particular attention to the period including fish introduction.

Non-metric Multidimensional Scaling (NMDS) was performed in order to summarize variations among age-grouped sections, with particular regard to the period of the fish introduction. For this purpose, head capsule densities in analyzed core sections (HC g^{-1}) were considered, data were transformed prior to analyses ($\log(x+1)$) in order to reduce the influence of very abundant taxa (Clarke and Gorley, 2006), and a resemblance matrix was subsequently obtained using the Bray–Curtis measure. One-way PERMANOVA (Anderson, 2001; McArdle and Anderson, 2001) was performed in order to check significant differences in chironomid assemblages among identified age groups, which were pairwise compared. Multivariate homogeneity of group dispersions was checked using PERMDISP (Anderson, 2006) before PERMANOVA application. SIMPER analysis (Clarke, 1993) was applied to the data matrix in order to identify the main taxa contributing the most to observed significant differences highlighted by the PERMANOVA. Finally, dominance and Shannon-Wiener diversity were calculated for each subsample section and Kruskal-Wallis test was used to check for significant differences among values observed for different section groups. The Conover–Iman test (Conover and Iman, 1979; Conover, 1999) was used as post-hoc test, to assess the presence of significant differences between groups. All analyses were performed using RStudio version 3.4.3. Figures are produced with RStudio and processed with software Inkscape version 0.92.

6.2.3 Results

Subfossil chironomid assemblages in Dimon Lake

Analysis of the Dimon Lake core samples allowed for the identification of 96 chironomid head capsules (HC) distributed among 4 subfamilies and 6 genera (Table 11). The maximum number of HC (n = 26) was found at 17-19 cm, while the minimum (n = 2) at 2-4 cm. However, in six sections (21-23; 23-25; 29-31; 31-33 and 33-35 cm) were not possible to observe any HC. Among the core sections, mean head capsule density was equal to 0.28 ± 0.17 HC g^{-1}. The number of HC observed at each section was lower than the minimum number indicated in literature to obtain a representative sample (n = 50). Therefore, only the results from Balma Lake were analyzed in detail. The low number of HC recorded in Dimon Lake may be due to the calcareous nature of the basin. In fact, carbonate sediments tend to embed the HC, making difficult the extraction and identification (Lang et al., 2003; Lencioni and Lazzara, 2004; Brooks et al., 2007).

Table 11. Genera and number of collected head capsules (HC) in each section of core sample from Dimon Lake.

Depht	*Macropelopia*	*Chironomus*	*Corynocera oliveri*	*Paratanytarsus*	*Prodiamesa olivacea*	*Cricotopus/ Orthocladius*
0-2				2	2	
2-4					2	
4-6	2			2	7	
6-8					3	1
8-10					1	3
10-12				2	2	
12-14.5		5			2	2
14.5-17				8	12	
17-19				12	8	6
19-21				3	3	
21-23						
23-25						
25-27				2	2	
27-29			2			
29-31						
31-33						
33-35						

Subfossil chironomid assemblages in Balma Lake

Analysis of the Balma Lake core samples allowed for the identification of 7480 chironomid head capsules (HC) distributed among 5 subfamilies and 11 genera. In agreement with Brooks et al. (2007), the number of collected HCs was significant in all sections and was always higher both than the minimum ($n > 50$) and then the recommended value ($n > 150$) for representative samples. Among the core sections, mean head capsule density was equal to 11.2 ± 4.5 HC g^{-1}. The total number of chironomid taxa observed along the core sections ranged from 4 to 8, respectively detected in the top of the cores (depth of 0-2 and 2-4 cm) and in the 24-26 cm depth section. Percentage frequencies of the observed genera and species are reported in Figure 35. *Paratanytarsus austriacus* type and *Micropsectra* were the most frequent (41.3-88.1% and 4.7-39.4% respectively) and were found in all sections except at the top (depth of 0-2 and 2-4 cm), where *Micropsectra* was absent. *Corynocera oliveri* showed frequencies ranging between 1.9 and 17.6% and was present in all sections except in the top, at a depth of 22-24 cm and in the bottom of the core (depth of 28-30 cm). Along the core, subfamily Orthocladiinae showed the higher richness (5 genera) than other subfamilies: *Heterotrissocladius marcidus* type (0.4-3.5%) and *Metriocnemus* (0.4-2.6%) were observed in 11 sections, found to be absent only in the top samples and in the bottom, while *Psectrocladius* gr. *Psilopterus* (0.4-15.7%) and *Cricotopus*/*Orthocladius* group (0.9-4.27%) were observed within 6 sections. Among Tanypodinae, genus *Zavrelimyia* was detected only in the two top samples and was absent in the rest of the core. Other taxa showed percentage frequencies lower than 2% and were not considered in further analyses. In order to summarize results, percentages of head capsules HC, values of total lead Pb (µg g^{-1}), organic carbon TOC (%), nitrogen TN (%) and C/N ratio (previously presented in section 5) observed in each section obtained from the core sample collected in Balma Lake are also reported in Figure 35.

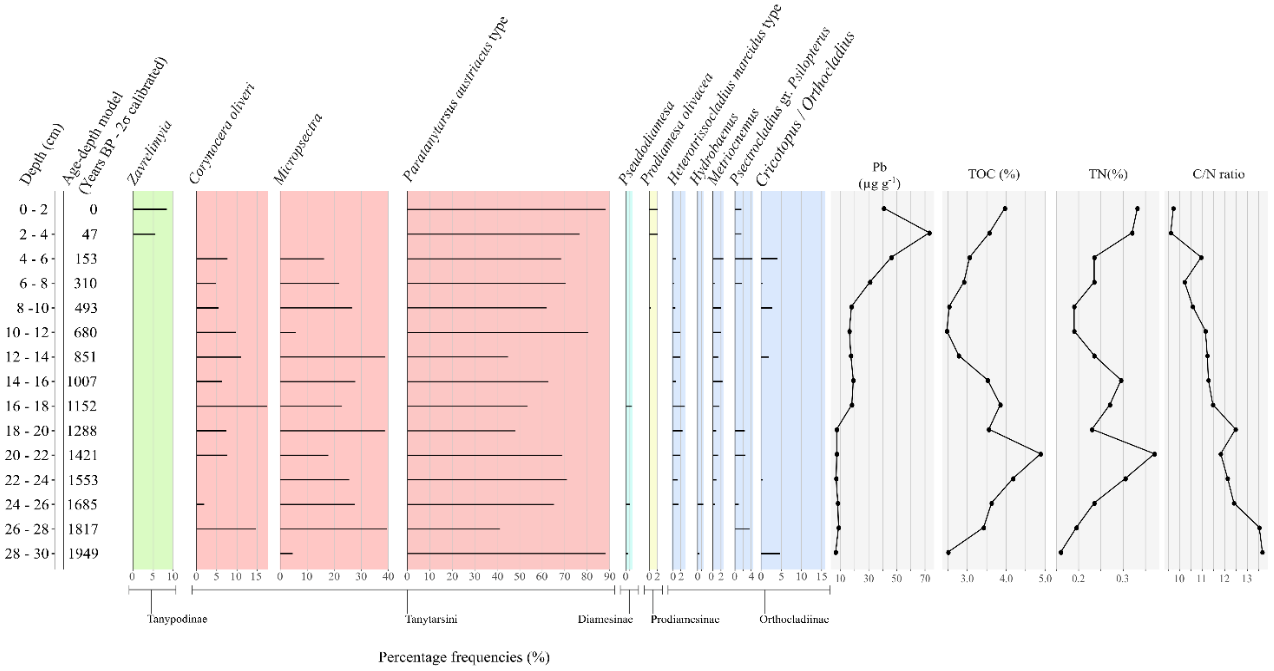

Figure 35. Percentages of head capsules HC and values of total lead Pb (µg g^{-1}), organic carbon TOC (%), nitrogen TN (%) and C/N ratio observed in each section obtained from the core sample collected in Balma Lake.

Non-metric Multidimensional Scaling is showed in Figure 36, and groups of core section subsamples are highlighted by ellipses. Groups including older sections (B: 310-851 years BP; C: 1007-1421 years BP; D: 1553-1949 years BP) generally showed a good overlap, while the upper group (A: 0-153 years BP) is slightly separated from the others and in particular the top sections (0-2 and 2- 4 sections, 0-47 years BP), dated after the fish introduction, which occurred during the seventies. These top sections were characterized by the exclusive presence of *Zavrelimyia* (absent elsewhere within the core) and by the absence of *Micropsectra*, *Heterotrissocladius marcidus* and *Metriocnemus* which were widely present across the other sections (Fig. 36).

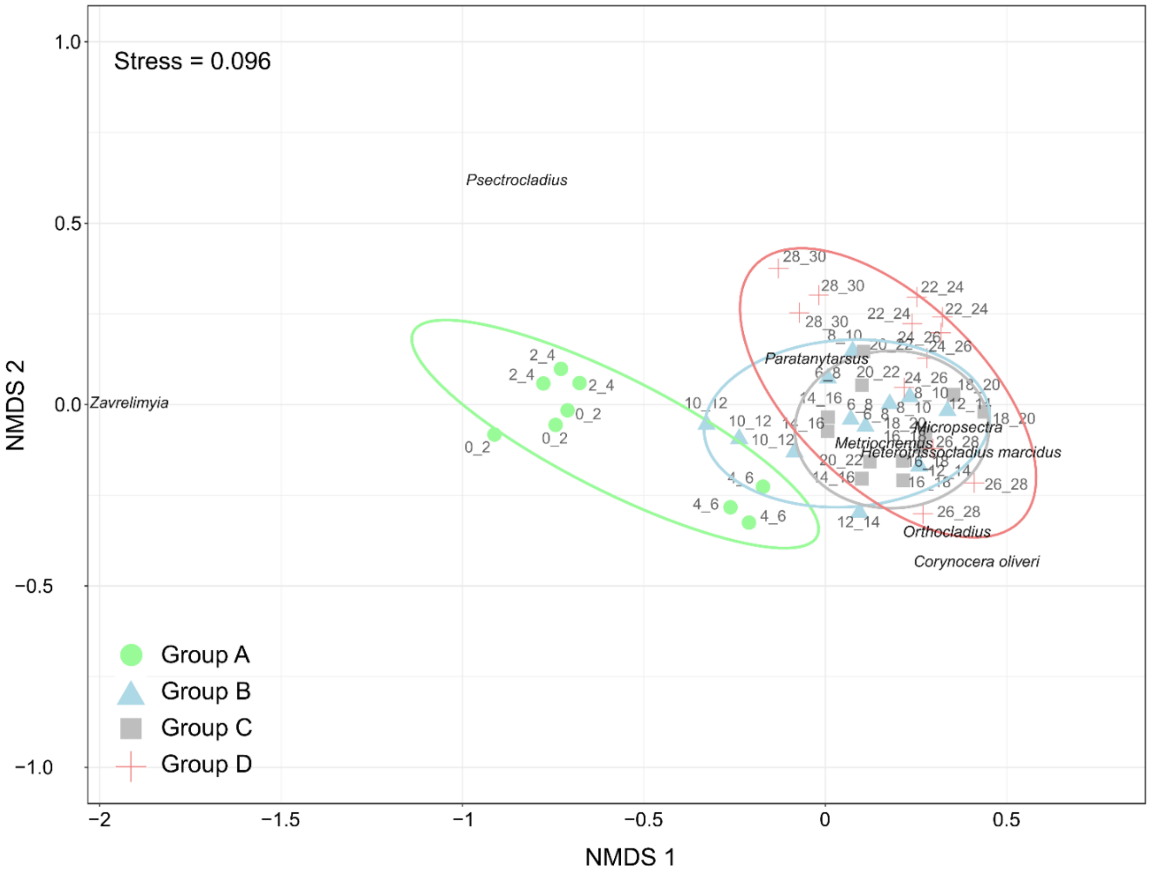

Figure 36. Non-Metric Multidimensional Scaling (NMDS) performed on the chironomid data obtained from the sections of the Balma Lake core. Symbols represent subsamples of each section; numbers near the symbols indicate the section depth.

As PERMDISP application did not show significant differences in the multivariate homogeneity of group dispersions (F=2.083, p=0.117), PERMANOVA comparisons (Table 12) allow for the detection of significant differences between the top group (A) and the other ones, while no significant difference was observed among the groups B, C and D. SIMPER test highlighted that observed dissimilarity between communities ranged between 40.58% and 48.38% and were mainly related to *Corynocera oliveri*, *Zavrelimyia*, *Micropsectra*, *Metriocnemus*, *Psectrocladius* gr. *Psilopterus* and *Heterotrissocladius marcidus* (Table 12).

Table 12. Results of PERMANOVA and SIMPER tests based on chironomid assemblage observed in the Balma core sample during the time period identified by the indirect dating method.

PERMANOVA	Df	Sum of Squares	Mean Squares	*F*	*p*-level	Overall average dissimilarity (%)	SIMPER results: significant taxa and relative percentage contribute to dissimilarity	
Global test	3	1.423	0.474	17.475	0.001			
	41	1.113	0.027					
	44	2.535						
A vs B	1	0.593	0.593	19.041	0.001	40.58	*Micropsectra*	36.54
	19	0.592	0.311				*Corynocera oliveri*	14.76
	20	1.185					*Psectrocladius* gr. *psilopterus*	10.27
							Zavrelimyia	5.68
							Metriocnemus	3.45
							Heterotrissocladius marcidus type	3.07
A vs C	1	0.876	0.876	33.054	0.001	45.55	*Micropsectra*	37.30
	19	0.503	0.026				*Corynocera oliveri*	17.11
	20	1.379					*Psectrocladius* gr. *psilopterus*	9.39
							Heterotrissocladius marcidus type	4.83
							Zavrelimyia	4.65
A vs D	1	0.880	0.880	21.946	0.001	48.38	*Micropsectra*	36.89
	19	0.762	0.040				*Psectrocladius* gr. *psilopterus*	8.17
	20	1.642					*Corynocera oliveri*	8.08
							Zavrelimyia	4.15

The Shannon-Wiener diversity was significantly lower in the top group A (Fig. 37) (Kruskal-Wallis test: $H_{2,42}>7.73$, p-level < 0.05; Conover-Iman test: $p < 0.05$ for all comparisons) while it was not different among the other groups. Dominance was significantly higher for the top group (A) (Kruskal-Wallis test: $H_{2,42} = 9.308$, p-level < 0.05; Conover-Iman test: $p < 0.05$ for all comparisons) (Fig. 37).

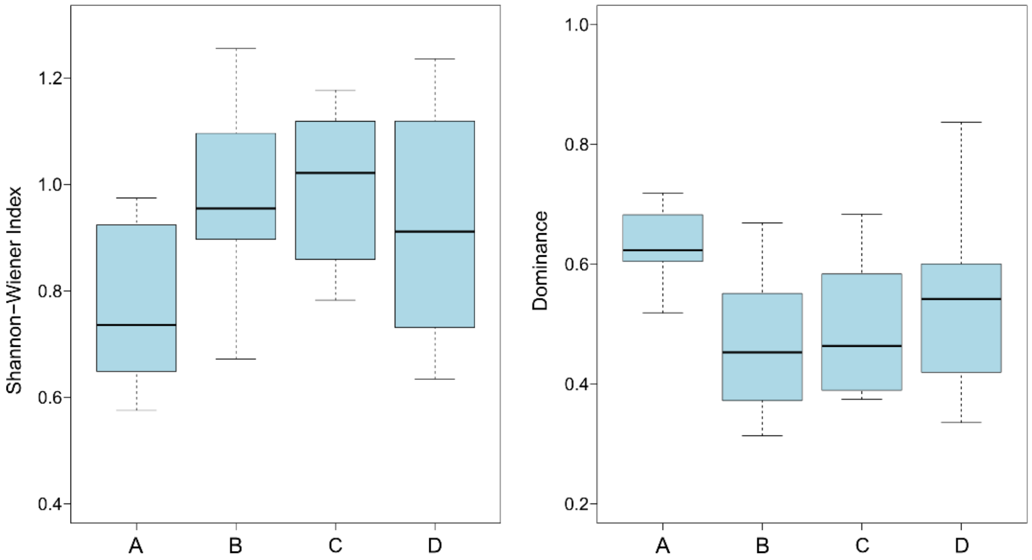

Figure 37. Trends of Shannon Wiener diversity and dominance calculated from the Balma core section groups. based on indirect dating (black line = median; box = first and third quartile; whisker = min-max).

6.2.4 Discussion

As already reported in section 6.1, chironomid littoral community in Balma Lake was well diversified. The most frequent taxa were *Zavrelimyia* and *Paratanytarsus austriacus*, which are two of the most widespread chironomids in high-mountain lakes (Boggero et al., 2006; Füreder et al., 2006). In particular, *Paratanytarsus austriacus* could be one of the most abundant (Boggero et al., 2006; Hamerlík et al., 2017). Among the subfamily Orthocladiinae, *Psectrocladius* gr. *psilopterus* was always present in all sampling sites and is commonly associated with low conductivity and phosphate poor freshwater environments (Moller Pillot, 2009c), as observed in Balma Lake. However, several taxa were present almost exclusively in the inlet area at the site 1. Among these genera, *Parorthocladius* is related to small streams and shallow margin of standing waters (Brooks et al., 2007) while *Micropsectra* also inhabits spring water and small streams (Pinder and Reiss, 1983). On the other hand, *Micropsectra* is a cold stenothermic organism associated with oligotrophic conditions (Brooks et al., 2007), which could dominate oligotrophic environments (Frossard et al., 2013; Frossard et al., 2014) and it is also widespread in alpine lakes (Boggero et al., 2006; Fjellheim et al., 2009; Novikmec et al., 2015; Hamerlík et al., 2017). It is a good water quality indicator, especially of oxygen-rich conditions (Oliver, 1971; Mousavi et al., 2002) and low total organic carbon content (Brodersen and Quinlan, 2006; Brooks et al., 2007). The limited presence of *Micropsectra* in Balma Lake modern community could likely be explained by the presence of allochthonous brook trout

Salvelinus fontinalis, as species belonging to the genus *Micropsectra* were found to be highly sensitive to fish predation after introduction (Raposeiro et al., 2017). As reported by Tiberti et al. (2016a) in a study regarding the diet of *Salvelinus fontinalis* introduced in Alpine lakes, the brook trout is an opportunistic predator which feeds commonly adopting visual predation strategy, and which in these environments shows a diet based mostly on small invertebrates such as chironomids, especially in the first years of life. In this context, high pigmentation or much visible color patterns could favor predation of certain taxa (Armitage, 1995; Perrine, 2017). This situation could regard the genus *Metriocnemus*, which shows alternate white and violet bands coloration pattern, and which was present only in the inlet site. The fish presence could also explain the extremely poor and reduced benthic community at the deep sites, consisting only of strictly endobenthic Oligochaeta. In fact, some burrowing taxa, such as Oligochaeta (but also Diptera larvae) could take advantage of the presence of fish in high altitude lakes (Carlisle and Hawkins, 1998; Knapp et al., 2001a, 2001b; Tiberti et al., 2014b). In addition, fish introductions could cause a severe decrease in macroinvertebrate abundances of the pelagic area, favoring the littoral zone (Milardi et al., 2016; Perrine et al., 2017).

The impact of alien fish introduction on the chironomid community could be also noticed analyzing the results of paleolimnological investigations, as changes in subfossil community composition were observed and highlighted by the application of NMDS, PERMANOVA and SIMPER tests. Subfossil community observed across the core sections showed the presence of cold stenothermic taxa generally associated to oligotrophic conditions, such as *Micropsectra*, *Corynocera oliveri*, *Heterotrissocladius marcidus* type and *Paratanytarsus austriacus* type. The presence of these organisms and the absence of the tribe Chironomini suggest that the Balma Lake kept an oligotrophic status (Gandouin and Franquet, 2002) during time period identified by the age-depth model. However, composition of the subfossil community was fairly constant along the core and changed dramatically in the top samples (0-4 cm), approximately since the fish introduction period, when some taxa disappeared (*Micropsectra*, *Corynocera oliveri*, *Metriocnemus* and *Heterotrissocladius marcidus* type), *Zavrelimyia* appeared (Fig. 35), and Shannon-Wiener diversity significantly decrease while the dominance increase (Fig. 37). The presence of brook trout could have a severe impact on the abundances of macrobenthic invertebrate communities, as part of its diet (Tiberti et al., 2014b, 2016a). As top predator introduction alters chironomid assemblages, changes in diversity could occur, as observed by Raposeiro et al. (2017). Moreover, several authors observed a reduction in abundance, diversity and richness in relation to fish predators (Lammens and Hoogenboezem, 1991; Miller and Crowl, 2006; Rieradevall et al., 1995; Weber and Brown, 2009).

The genus *Zavrelimyia* was one of the most abundant in the modern community and was observed in the whole littoral zone. *Micropsectra* was present in all core sections except in the most recent and was the second taxon in terms of percentage frequencies (Fig. 35). As mentioned above, *Micropsectra* is sensitive to fish predation (Raposeiro et al., 2017) and is also a prey item for third and fourth instars of *Zavrelimyia* (Moller Pillot 2009a, c), which is a littoral dweller. *Zavrelimyia* preys also on *Heterotrissocladius marcidus*, which is also widespread in alpine lakes (Boggero et al., 2006; Novikmec et al., 2015; Hamerlík et al., 2017) and it is commonly associated with cold waters and oligotrophic conditions (Brooks et al., 2007; Moller Pillot 2009c). However, it disappears from the core in the top sections and it is absent in the littoral community such as the cold stenothermic *Corynocera oliveri* (Brooks et al., 2007), which was the third taxon in terms of percentage frequencies observed within the core sections (Fig. 35). Fish introduction could hardly alter trophic webs (Sánchez-Hernández et al., 2015), with a decrease in the percentages for predator and/or detritivore taxa and an increase in grazer organisms (Raposeiro et al., 2017), in our case respectively represented by *Corynocera oliveri*, *Micropsectra*, *Heterotrissocladius marcidus* (Serra et al., 2017) and by *Paratanytarsus* (Heiri and Lotter, 2003).

In the core samples, Orthocladiinae was the subfamily with the greatest number of genera, including species which are well adapted to the low temperature characteristic of high-altitude environments (Bretschko, 1974; Wathne et al., 1995; Franceschini and Lencioni, 2002; Boggero et al., 2006; Füreder et al., 2006). It also cannot be excluded that brook trout introduction affected nutrient load in the lake in the top sections via execration, as an increase in nutrient availability has consequences on primary production and on food webs (Du et al., 2015; Raposeiro et al., 2017).

On the other hand, temperature is the main factor affecting Chironomidae assemblages (Füreder et al., 2006; Brooks et al., 2007; Moller Pillot, 2009a) which could also be influenced by pH variations, nutrient changes (Brooks et al., 2007). The trends of C/N ratios observed In Balma Lake core indicate a higher contribute of non-vascular aquatic plants in the top sections (Klug et al., 2009). This could favor Orthocladiinae taxa related to the presence of aquatic plants, algae or submerged parts of macrophytes (*Psectrocladius* gr. *psilopterus*, *Cricotopus*/*Orthocladius* and *Parorthocladius*).

Changes in chironomid taxa across the core sections could also be due to potentially different drivers, such as variations of nutrient concentration and water temperature. In fact, *Zavrelimyia*, a shallow dweller and warm well adapted taxon, has been found in sediments with increasing organic matter content (Ilyashuk et al., 2011). The presence of taxa adapted to warmer and shallow habitats could be related to an increase in the lake productivity, especially of in the shallow zone, which could be related to warm summers. This situation was observed in a paleolimnological study carried out in the Schwarzsee ob Sölden Alpine lake (2792 m a.s.l.) in Austria by Ilyashuk et al. (2011). In addition, *Psectrocladius* is generally associated to macrophyte presence and/or productive lakes (Bordersen et al., 2001; Langdon et al., 2010; Axford et al., 2017). It could respond to changes in macrophyte abundance or other aspects of lake productivity (Axford et al., 2017). Moreover, *Paratanytarsus austriacus* type is also often related to macrophyte presence (Buskens, 1987; Brodersen et al., 2001; Brooks et al., 2007). No aquatic macrophytes were observed at the Balma Lake during the present study but the decreasing C/N ratio values in the top sections of the Balma core are related to the presence of non-vascular aquatic vegetation (Klug et al., 2009). Moreover, the Secchi Disk remained visible at 3.25 m from the surface in summer and 2.80 m in autumn, never reaching the bottom.

However, *Zavrelimyia* and *Paratanytarsus austriacus* are two of the most widespread chironomids in high-mountain lakes (Boggero et al. 2006; Füreder et al. 2006) and the latter was always present along the core. As we cannot exclude the influence of a warming period in shaping the midge community, the effect of the fish introduction is surely significant.

6.3 Macrobenthic invertebrates: bioindicators of trace elements

6.3.1 Preface

Macrobenthic invertebrates accumulate trace elements based on their functional feeding guilds (FFG) (Goodyear and McNeill, 1999; Santoro et al., 2009; Pastorino et al., 2019). Uptake is also governed by water temperature, morphology, physiology, and tolerance to environmental contaminants (Goodyear and McNeill, 1999). The structure and functional characteristics of macroinvertebrate communities is influenced by local (hydrochemistry, nature of the substratum, vegetation cover, presence of tributaries or emissaries) and regional (biogeographic history of the lake, climate, and transport of long-range air pollution) factors (Fjellheim et al., 2009). Trace elements contamination can reduce macrobenthic invertebrate richness, as well as density, growth, and production (Maret et al., 2003; Gray and Delaney, 2008). Macrobenthic invertebrates are closely associated with sediments and constitute an important link to transfer contaminants to higher organisms such as fish or birds.

Pastorino et al. (2019) showed that detection of trace elements in macrobenthic invertebrates is a useful analysis for obtaining information about the status of freshwater watercourses, since sites classified as ‘‘Moderate’’*sensu* Water Framework Directive also had the highest trace elements amount. Starting from this point, the main aim of this study was to detect the amount of As, Cd, Pb and Zn in surface sediment and of As, Cd, Cr, Cu, Fe, Ni, Pb, Se, Zn in the tissues of macrobenthic invertebrates in two Alpine lakes (Balma Lake and Dimon Lake) that differ in geomorphology and hydrochemistry. The choice of these elements was made in accordance with the available literature on trace elements in surface sediment from Alpine lakes (Camarero et al., 2009a) and in freshwater macrobenthic invertebrates (Santoro et al., 2009; Pastorino et al., 2019).

6.3.2 Material and Methods

Analysis of surface lake sediment

Integrated surface sediment (0-2 cm) samples from each station of the littoral zones of the two lakes were collected with a plastic spatula and tested for four trace elements (As, Cd, Pb, and Zn); the concentrations were detected by inductively coupled plasma-mass spectrometry (ICP-MS NexION 350, PerkinElmer Inc., Waltham, MA, USA) after microwave-assisted acid digestion (Multiwave PRO reaction system, Anton Paar GmbH, Graz, Austria). An adaptation of the EPA Method 3052 (Environmental Protection Agency, 1996) was used. Freeze-dried samples (0.3 g) were reduced to powder with an agate mill and placed in vessels where a mixture of 5 mL of nitric acid and 1 mL of hydrogen peroxide was added. Mineralization was performed. The samples were then centrifuged and MilliQ water was added to a volume of 25 mL. Concentrations of As, Cd, Pb, and Zn were determined by ICP-MS using calibration curves obtained from analysis of five standard solutions.

Macroinvertebrates selection and detection of trace element

The most abundant taxa (Chironomidae and Oligochaeta in Balma Lake and Chironomidae, Oligochaeta, and Hirudinea in Dimon Lake) by dry weight recorded during the sampling campaigns carried out in 2017, were chosen to investigate the amounts of 9 trace elements (As, Cd, Cr, Cu, Fe, Ni, Pb, Se, Zn). The samples were oven-dried at 70°C for 72 h to obtain a total (summer plus autumn) dry weight of 300-1000 mg (depending on taxa) and for trace element detection. A pool of each taxon collected during summer and autumn was prepared for analysis, since the dry weight of the samples collected during a single season was insufficient for single trace element analysis.

Trace elements were detected by inductively coupled plasma-mass spectrometry (ICP-MS Xseries II, Thermo Scientific, Bremen, Germany). Determination of As, Cd, Cr, Cu, Fe, Ni, Pb, Se, and Zn was performed following the protocols reported in Squadrone et al. (2016b). Multi-elemental determination was performed by means of ICP-MS after daily optimization of instrumental parameters and using an external standard calibration curve. Analytical performance was verified by processing certified reference materials (Oyster Tissue - SRM 1566b from the National Institute of Standard and Technology), along with blank reagents in each analytical session. The analytical method was validated according to ISO/IEC 17025 (general requirements for the competence of testing and calibration laboratories).

Statistical analysis and calculation of bioaccumulation factor (BAF) values

Normality and homogeneity of variance was tested using the Kolmogorov-Smirnov test. Differences in concentration of the physicochemical features between the two lakes were checked using the Wilcoxon test. Differences in trace element concentration in the taxa collected from each lake were assessed using the Wilcoxon test (Balma Lake) or the non-parametric Kruskal-Wallis test followed by post-hoc pairwise comparisons based on the Dunn test (Dimon Lake). Spearman’s rank correlation coefficient was calculated to determine the relationship between trace element concentration in the taxa and (a) water physicochemical parameters, (b) respective concentration in sediment for each lake. Results were considered statistically significant at *p*-values < 0.05. Statistical analysis was performed using Graph Pad Statistics Software Version 6.0 (GraphPad Software, Inc., USA). Principal component analysis (PCA) was performed using the trace element concentrations measured in the taxa to check for trends in trace elements between the two lakes. The PCA results were plotted using open source data analysis software RStudio® version 1.1.463 (RStudio, Inc.). To evaluate patterns of trace elements bioaccumulation, bioaccumulation factors (BAF) were calculated as follows (Klavinš et al., 1998; Ruus et al., 2005):

$BAF = M_{tissue} / M_{sediment}$

whereby M_{tissue} is the trace elements concentration in the tissue of a given macroinvertebrate and $M_{sediment}$ is the trace elements concentration in the sediment.

6.3.3 Results

Macrobenthic invertebrate communities in Balma Lake and Dimon Lake

Macrobenthic invertebrates recorded both in Balma Lake and Dimon Lake are already described in section 6.1. The most abundant taxa (Chironomidae and Oligochaeta in Balma Lake; Chironomidae, Oligochaeta and Hirudinea in Dimon Lake) were chosen for trace elements detection. Table 13 presents the dry weight for the three main taxa collected in this study.

Table 13. Dry weights (mg) measured for the three main macrobenthic invertebrates collected from the two lakes during the study period.

	Balma Lake		**Dimon Lake**	
	Summer	Autumn	Summer	Autumn
Oligochaeta	148.00	162.00	615.50	397.10
Hirudinea	0.30	0.30	115.10	243.00
Chironomidae	70.90	206.00	55.70	54.20

Trace elements in sediment

Table 14 presents the trace elements concentration values in sediment from the two lakes. The concentration in decreasing order for the two lakes was: Zn > Pb > As > Cd. Furthermore, the total average trace elements content was higher in Dimon Lake than Balma Lake (287.62 mg kg^{-1} vs. 94.94 mg kg^{-1}). The mean concentration of each trace elements (obtained from the mean of the two seasons) was used to calculate the BAF values in Diptera Chironomidae and Oligochaeta from Balma Lake and in Diptera Chironomidae, Oligochaeta, and Hirudinea from Dimon Lake. No correlation was found between trace elements concentration in sediment and in the macrobenthic invertebrates.

Table 14. Concentration of trace elements (mg kg^{-1}) in surface sediment from Balma Lake and Dimon Lake. (n = summer and autumn samples).

	Balma Lake (n=2)		**Dimon Lake (n=2)**
	Mean ± SD		Mean ± SD
As	1.3 ± 0.4	**As**	39 ± 0.3
Cd	0.10 ± 0.07	**Cd**	0.62 ± 0.01
Pb	41 ± 1.2	**Pb**	110 ± 1.1
Zn	52 ± 1.7	**Zn**	138 ± 1.4

Trace elements in macrobenthic invertebrates

Table 15 and Figure 38 present the mean concentration and the relative standard deviation of each element detected in the two main taxa from the two lakes. The mean concentration in decreasing order in the Oligochaeta from Balma Lake was: Fe (892) > Cu (644) > Zn (337) > Ni (85) > Pb (68) > Cr (62) > Se (12) > Cd (2.4) > As (1.5) mg kg^{-1}; the mean concentration in decreasing order in the Chironomidae was Fe (1158) > Cu (728) > Zn (418) > Ni (113) > Cr (81) > Pb (66) > Se (5.5) > Cd (2.8) > As (1.2) mg kg^{-1}. The total amount of elements was higher in the Chironomidae than the Oligochaeta (2573.5 mg kg^{-1} vs. 2103.9 mg kg^{-1}).

The mean trace element concentration in decreasing order in the Oligochaeta from Dimon Lake was: Fe (1837) > Cu (457) > Zn (293) > Pb (46) > Cr (26) > Ni (18) > As (17) > Se (7.1) > Cd (3.8) mg kg^{-1}; the mean concentration in decreasing order in the Chironomidae was Fe (1831) > Zn (606) > Cu (487) > Pb (49) > Ni (24) > Cr (14) > As (10) > Se (4.4) > Cd (3.2) mg kg^{-1} wet weight. The mean concentration in decreasing order in the Hirudinea was Fe (1733) > Zn (374) > Cu (247) > Ni (24) > Pb (11) > As (6.4) > Se (4.1) > Cr (2.4) > Cd (2) mg kg^{-1}. The total amount of elements was higher in the Chironomidae than the Oligochaeta (3028.6 mg kg^{-1} vs. 2704.9 mg kg^{-1}).

Considering the sum of the two main taxa (Oligochaeta and Chironomidae), the total amount of trace elements was higher in Dimon Lake than Balma Lake (5733.5 mg kg^{-1} vs. 4677.4 mg kg^{-1}). In particular, the amount of Cr, Ni, Pb, and Se was higher in Balma Lake, while the amount of As, Cd, Cu, Fe, and Zn was higher in Dimon Lake. Comparison of the total amount of each element detected in the two main taxa (Oligochaeta and Chironomidae) from the two sites showed the highest values for Fe, Cu, and Zn.

The Kruskal-Wallis test evidenced significant differences in trace element concentration among the taxa from Dimon Lake ($p < 0.05$); Dunn test: $p < 0.05$ only for Chironomidae vs. Hirudinea. The Wilcoxon test revealed no significant differences in trace element concentration in the two taxa from

Balma Lake ($p > 0.05$). Spearman's rank correlation coefficient did not highlight any relationships between trace element concentration in macrobenthic invertebrates and water physicochemical parameters.

Table 15. Concentration of trace elements (mg kg^{-1}) in macrobenthic invertebrates from Balma Lake and Dimon Lake (n = number of technical replicates).

	Balma Lake (n = 3)		**Dimon Lake (n = 3)**		
	Chironomidae	**Oligochaeta**	**Chironomidae**	**Oligochaeta**	**Hirudinea**
	Mean ± SD		Mean ± SD		
As	1.2 ± 0.1	1.5 ± 0.5	10 ± 1.3	17 ± 1.3	6.4 ± 0.2
Cd	2.8 ± 0.15	2.4 ± 0.2	3.2 ± 0.1	3.8 ± 0.26	2 ± 0.17
Cr	81 ± 1.0	62 ± 2.2	14 ± 1.2	26 ± 0.87	2.4 ± 0.1
Cu	728 ± 1.0	644 ± 1.3	487 ± 0.87	457 ± 1.5	247 ± 0.43
Fe	1158 ± 2.8	892 ± 1.6	1831 ± 1.3	1837 ± 1	1733 ± 1.3
Ni	113 ± 2.0	85 ± 1.3	24 ± 1.5	18 ± 1.5	24 ± 0.3
Pb	66 ± 2.6	68 ± 0.5	49 ± 0.5	46 ± 1.5	11 ± 0.17
Se	5.5 ± 1.0	12 ± 2	4.4 ± 0.09	7.1 ± 0.1	4.1 ± 0.36
Zn	418 ± 2.0	337 ± 3	606 ± 1.8	293 ± 1.3	374 ± 0.2

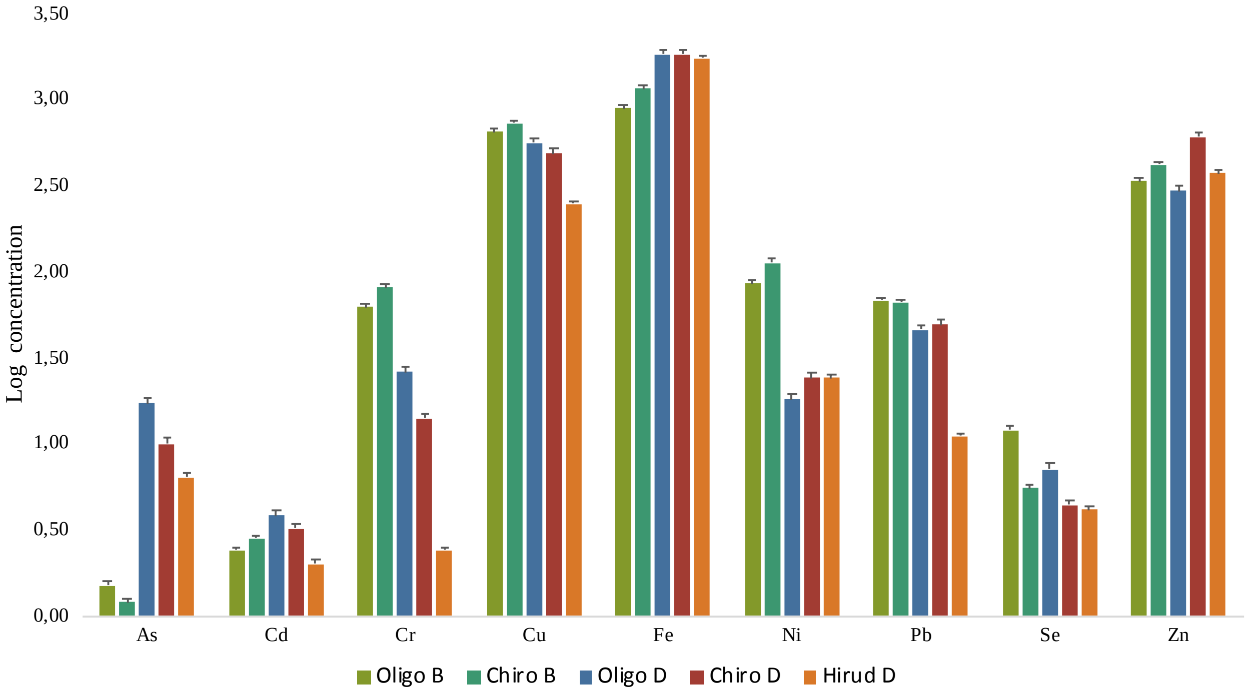

Figure 38. Bars of trace elements (Log concentration; mg kg^{-1}) detected in Chironomidae (Chiro) and Oligochaeta (Oligo) from Balma Lake (B), and in Chironomidae, Oligochaeta, and Hirudinea (Hirud) from Dimon Lake (D).

With regard to PCA (Fig. 39), the first two principal components (PCA1; PCA2) accounted for meaningful amounts of the total variance (79.2%), while the other components accounted for a relatively smaller fraction of the variance. In particular, PCA1 explained little more than 57.3% of the total variance and was positively correlated with the variables Cr, Cu, Ni, Pb, Se, and Zn and negatively correlated with As, Cd, and Fe. PCA2 explained 21.9% of the total variance and was positively correlated with the variables As, Cd, Cr, Cu, Fe, Pb, and Se, and negatively correlated with Ni and Zn. The biplot of loadings (variables) and score (observations) shows which taxon of macroinvertebrates is closest to them, and which variables (trace elements) contribute to this grouping in the coordinate of PCA1 and PCA2. Moreover, the taxa are arranged according to trace element concentration measured at the two sites. The separation between the macrobenthic invertebrates from the two lakes (Balma on the right, Dimon on the left) suggests a difference in trace elements accumulation capacity. Furthermore, the Chironomidae and Oligochaeta from Balma Lake are much closer to them compared to all taxa from Dimon Lake, which are separated from each other, suggesting a different level of trace element concentration, as confirmed by the Kruskal-Wallis test.

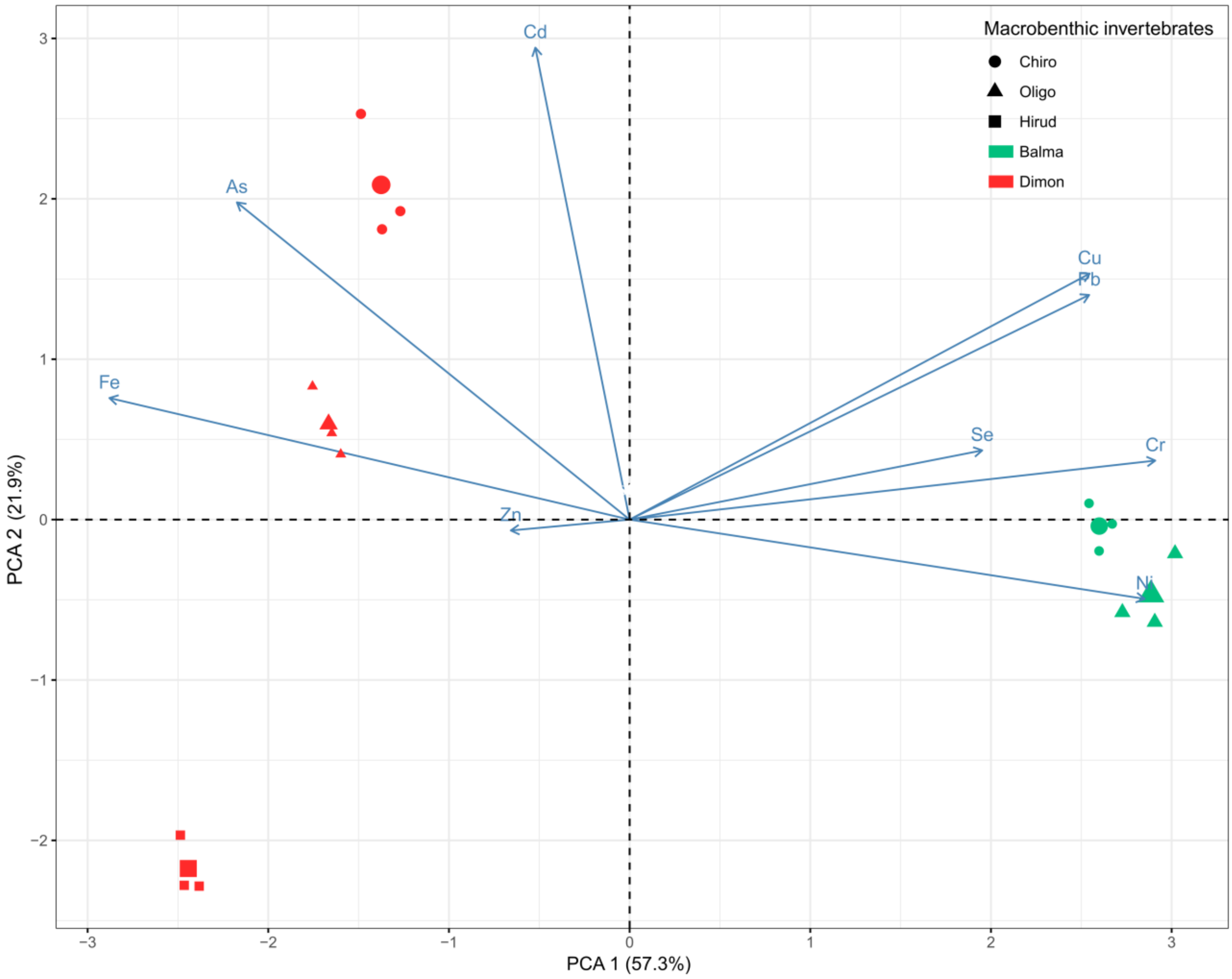

Figure 39. Biplot of score and loadings from principal component analysis. The scores of each taxon are denoted by a symbol (largest symbol = average value); each lake has a different color (Balma Lake in green; Dimon Lake in red).

Bioaccumulation factors (BAF)

Table 16 presents the BAF values. Among the trace elements, Cd and Zn were found to score higher BAF values in all taxa, and the highest values (28 and 8.04, respectively) were observed in the Chironomidae from Balma Lake. The BAF values were higher for all elements in Balma Lake than Dimon Lake.

Table 16. Bioaccumulation Factor (BAF) values for macrobenthic invertebrates from Balma Lake and Dimon Lake.

	Balma Lake			**Dimon Lake**		
	Chironomidae	**Oligochaeta**		**Chironomidae**	**Oligochaeta**	**Hirudinea**
	BAF			**BAF**		
As	0.92	1.15	**As**	0.26	0.43	0.16
Cd	28	24	**Cd**	5.16	6.13	3.22
Pb	1.61	1.66	**Pb**	0.45	0.42	0.1
Zn	8.04	6.48	**Zn**	4.39	2.12	2.71

6.3.4 Discussion

For this study we used the level of trace element accumulation in macrobenthic invertebrates to highlight trace elements concentration in two Alpine lakes, since they have proprieties that make them useful sentinel organisms in freshwater monitoring programs (Santoro et al., 2009; Pastorino et al., 2019).

Trace elements in essential and non-essential forms are naturally persistent in the environment and accumulate in nature, especially in sediments (Hosseini Alhashemi et al., 2012). Their concentration in sediment depends mainly on the geological structure of the catchment area and the intensity of material transport from the catchment to the lake (Boyle, 2001; Tylmann et al., 2011). The two elements with the highest concentration in the sediment in both lakes were Pb and Zn; they are present at the highest contamination level on the European scale (Renberg et al., 2000), also in remote areas (Köck and Hofer, 1998; Camarero et al., 2009a). In particular, Catalán et al. (2009) showed that an enrichment in Pb and Zn are higher in southern than in central areas of the Alps, suggesting the main source of these pollutants is from the industrial areas of the south. We observed a remarkable concentration of arsenic in the sediment of Dimon Lake (39 ± 0.3 mg kg^{-1}) probably due to the rock composition of the area, since volcanic rocks naturally contain high amounts of arsenic (Fuganti et al., 2005). Generally, As, Pb and Cd concentrations were much higher for Dimon Lake than those reported for surface sediments by Camarero et al. (2009a) for Alpine lakes located in Piedmont-Ticino, Central, and Tyrol Alps, suggesting that atmospheric deposition plays an important role in the transport of trace elements in this site. Klavinš et al. (1998) reported values for Pb (range 12.75-83.21 mg kg^{-1}) and Cd (0.41-2.75 mg kg^{-1}) in surface sediments from 11 lakes in Latvia, suggesting that the highest values recorded for Pb and Cd (83.21 mg kg^{-1} and 2.75 kg^{-1}, respectively) result from the anthropogenic impact. Our results are in line with those reported by Camarero (2003) who studied the trace elements concentration in 75 lakes in the Pyrenees mountains and described the effect of atmospheric pollution on mountain areas.

Comparison of the trace elements concentration found in the sediment from Balma Lake and Dimon Lake with the recommended levels of sediment quality targets for the protection of sediment-dwelling organisms (Crane et al., 2006a, 2006b) shows that the concentration of the majority of the trace elements is within the limit, except for Pb in both lakes and Zn in Dimon Lake, which exceeded the level I limit: the level of contaminant concentrations below which harmful effects on sediment-dwelling organisms (i.e., macrobenthic invertebrates) are unlikely to be observed. Of note, As in Dimon Lake exceeded the level II limit: the contaminant concentration above which harmful effects on sediment-dwelling organisms are likely to be observed.

But overall, Dimon Lake was found to contain higher total amounts of trace elements in surface sediment and in macrobenthic invertebrates than Balma Lake, suggesting a flux of pollutants from the lowland promoted by the high amount of average precipitation (2500 mm yr^{-1}in 2017) in this area (ARPA FVG, 2017), which generally occurs when the wind blows from the south (Camarero et al., 2009a). Differently, the area of Balma Lake has lower average precipitation (1296 mm yr^{-1} in 2017) (ARPA Piemonte 2017), explaining the lower values.

The few studies on trace element accumulation in biota from high-altitude lakes in general and in Alpine lakes in particular have mostly focused on fish. Köck et al. (1996) studied the concentrations of Cd, Pb, Zn, and Cu in Arctic char (*Salvelinus alpinus*) from five oligotrophic Alpine lakes in northern Tyrol (Austria) and reported that trace elements concentrations were comparable with those in fish from waters polluted by direct discharge of trace elements, suggesting that the lakes are an import source and accumulation site for inorganic contaminants due to transport from industrialized areas.

No previous study on trace element accumulation in macrobenthic invertebrates has been performed in Alpine lakes; the amount found in the invertebrates from both lakes indicates an origin of anthropogenic and pedogenic sources. Fe was found at the highest concentration in all taxa analyzed. Fe mainly originates from the soil and rocks of watersheds, which explains the highest content in our samples, as reported previously for a similar environment (Batifol and Boutron, 1984), yet it cannot be excluded that the values we recorded also result from emissions from combustion sources. Nevertheless, Fe is an indispensable element for life because present in many metabolic pathways, also in aquatic invertebrates (Rainbow and Dallinger, 1992). Its solubility in water depends both on the pH and its state of oxidation, which is linked to the redox potential of the environment (Davison, 1993). The Fe concentration is an example of how the concentration of oxygen regulates the redox potential of a chemical species in the aquatic environment (Davison, 1993). The reduction and therefore the solubilization of Fe at the water-sediment interface is also accompanied by the release of phosphorus from the sediment, with possible negative effects for the lake (Jensen et al., 1992). Several Fe and P compounds, such as ferric phosphate ($FePO_4$), can indeed co-precipitate with ferric hydrate. In shallow Alpine lakes, since no thermal stratification occurs (Tiberti et al., 2010), Fe concentration is low throughout the water column because all the Fe is oxidized, forming the insoluble hydrate (Bertoni, 2006).

Trace elements ad Fe, Cu, and Zn are essential for life at low concentration, they can over-accumulate in contaminated aquatic environments. Furthermore, non-essential elements like As, Pb, Cd, and Cr are toxic for organisms even at low levels of exposure (Esposito et al., 2018). Macrobenthic invertebrates can take up and accumulate these contaminants from the sediment (being in close contact) that represents the major sink (Chen et al., 1996). The uptake of trace elements from sediment occurs through permeable body surfaces, as well as through the gut of organisms that feed on contaminated substrates (Bat et al., 1998; Cardoso et al., 2009). Santoro et al. (2009) found that the major uptake of As, Cd, Cr, Cu, Pb, and Zn in collector-gatherers was due to their close contact with sediment. Recently, Pastorino et al. (2019) also provided evidence for a greater accumulation of trace elements in collector-gatherers, suggesting that the tropic level of organisms influences trace elements accumulation.

Chironomids are excellent bioindicators (Wiederholm, 1984) to investigate the trophic state of lakes over the course of several years (Sæther, 1979, 1893; Hofmann, 1988). The survival of Chironomids in an environment depends on diverse environmental factors, including water temperature, pH, and dissolved oxygen (Oliver, 1983; Lencioni and Lazzara, 2004). The ecological achievement of this family in a wide range of trophic conditions is a consequence of their physiological adaptability, which allows individuals to live in extreme conditions. Chironomids show a diversity of feeding habits and functions, although most are considered omnivorous (Nessimian and Sanseverino, 1998; Nessimian et al., 1999; Henriques-Oliveira et al., 2003) belonging to the FFG of collectors, shredders, and predators. Among other taxa recorded, Oligochaeta can also be found in littoral, sublittoral, and deep zones, where they generally prefer fine substrates (Dumnicka, 1994, Verdonschot, 2001). Oligochaeta are classified as collectors-gatherers (Merritt and Cummins, 2006). In fact, most of the aquatic Oligochaeta burrow through sediment in lotic and lentic habitats and ingest organic particles, though some are important algal feeders or predators (Dodds and Whiles, 2010).

In Dimon Lake we also analyzed Hirudinea belonging to the Glossiphoniidae. This family occurs in both lotic and lentic habitats such as creeks, rivers, lakes, swamps and ponds and are classified in the FFG of predators. Their trace element concentration was lower than in either Chironomidae or Oligochaeta, suggesting that the biological uptake from immediate contact with the sediment or solid substratum (collector-gatherers), instead of biomagnification along the biotic food webs (predators), is the more effective biological sequestering pathway for trace elements (Santoro et al., 2009).

As compared with our data, Klavinš et al. (1998) detected lower concentrations of Pb (average range 3.21-28.71 kg^{-1}), Cu (3.21-8.32 kg^{-1}) and Cd (0.09-1.02 kg^{-1}) in chironomids from 11 sites in Latvia (Baltic Region), whereas Santoro et al. (2009) and Pastorino et al. (2019) reported values in macrobenthic communities similar to our study for sites located near industrialized or urbanised areas. In Balma Lake the trace elements concentration in the macrobenthic invertebrates was higher than in the sediment. Except for Zn, the elements concentration in the macrobenthic invertebrates from Dimon Lake was lower than in the sediment. These findings are supported by the BAF values that were consistently higher in the macrobenthic invertebrates from Balma Lake for all trace elements.

These findings are in line with those reported by Klavinš et al. (1998): BAF values decreased with increasing trace elements concentration in sediment, indicating mechanisms of elements excretion in biota in areas of environmental contamination. In particular, organisms have evolved mechanisms to regulate the concentration of trace elements in their tissues when metal pollution in water, sediment or food is present (Casado-Martinez et al., 2009; Kalantzi et al., 2014). In fact, the accumulation of trace elements in macrobenthic invertebrates is regulated by the input and output of metals by the organism, which closely depend on its morphology (Kiffney and Clements, 1993), physiology (Hare, 1992), and the metal distribution in cells (Luoma, 1989). Barka et al. (2010) determined the distribution of bioaccumulated metals, both essential and nonessential, in aquatic invertebrates with a view to assessing their availability to the next trophic level. They described three adaptive strategies that invertebrates may implement: 1) limit the entrance of metals into the body; 2) balance the uptake by increasing excretion; 3) detoxify and store metals that enter the cells. Aquatic invertebrates can excrete the metals or retain them in tissues also in function of their larval development (Rainbow and Dallinger, 1992; Pastorino et al., 2019). Our results revealed different concentrations of trace elements in Chironomidae and Oligochaeta taxa, albeit belonging to the FFG of collector-gatherers. This depends on several abiotic and biotic factors, such as absorption properties which differ between species or even among individuals of the same species (Arslan et al., 2010). These considerations are strengthened by the results of PCA analysis that evidenced a clear separation in trace element concentration between taxa and sites.

For example, Timmermans and Walker (1989) reported that Zn, Cu, and Cd are excreted by *Chironomus riparus* after experimental exposure to high concentrations of dissolved metals. It should also be remembered that interspecific variation in metal accumulation is present among midge larvae. For example, *Stictochironomus histrio* showed considerable losses of Zn, Cu, and Cd upon metamorphosis, whereas *Chironomus anthracinus* did not (Timmermans and Walker 1989). Trace elements may be sequestrated and detoxified by metallothioneins, mitochondria, and lysosomes in the gut system and elsewhere in the body, resulting in apparent resistance to metal contaminants (Ahearn et al., 2004; Schaller et al., 2010).

7. FISH

7.1 Assessment of fish assemblages

7.1.1 Preface

Mountain lakes are usually isolated from streams by physical barriers that have prevented natural colonization of fish (Knapp et al., 2001a; Miró and Ventura, 2013), and the introduction of fish species is mainly related to recreational fishing (Pister, 2001; Schindler and Parker, 2002).

These originally fishless lakes have low resilience to disturbances and can be especially sensitive to the introduction of alien fish, which can significantly reduce or eliminate native organisms such as macrobenthic invertebrates, zooplankton, and amphibians. Furthermore, the introduction of alien species can cause the spread of new pathogenic agents (e.g., parasites, bacteria, viruses, fungi) that may be more virulent to new hosts due to the lack of innate immunity in the native species (Sheath et al., 2015; Pastorino et al., 2017b). In the Italian Alps, stocking mountain lakes with salmonids began in the 1960s, when recreational angling became popular (Cantonati et al., 2006). Among the salmonid species, brook trout *Salvelinus fontinalis*, native to eastern North America, has an almost worldwide distribution (MacCrimmon et al., 1971), and it has been one of the most widely used alien species also for stocking high-mountain lakes (Savini et al., 2010). It is appreciated by anglers for its readiness to take baits and by fishery managers for its ability to maintain itself in such marginal situations as are frequently found in high-mountain lakes. The main difficulties involved in studying introduced fish populations in such remote environments are access to the sites (most often only on foot), the absence of information about the introduction/release (origin and date of fish stock), and the angling impact. The aim of the present study was to assess the biological and sanitary condition of fish introduced in Balma Lake and Dimon Lake.

7.1.2 Qualitative fish sampling

7.1.2.1 Material and Methods

During 2017 two sampling campaigns (July and October) were performed in each lake to obtain information about the fish populations. In Dimon Lake fish were sampled using an electrofishing boat, whereas since Balma lake is only accessible on foot, the fish were captured using gillnets (two multimesh gillnets 36 × 1.8 m). The gillnets are divided into 6 panels of different mesh size. They can capture indiscriminately all size classes except for very small fish (young of the year, 0+). The multimesh gillnets were held vertically close to the shore and perpendicularly to the shore. The panels with the smallest mesh sizes were placed close to shore. The gillnets were placed for about 3 hours and then recovered. The captured fish were suppressed by deep anesthesia with tricaine methanesulfonate MS-222 (200 mg L^{-1}). We then proceeded with identification of the species and morphometric measurement: weight (g) and total length (cm). The fishes were sectioned, and the organs sampled and stored until further analysis. The stomach contents were preserved in 70% alcohol to determine the frequency of prey's occurrence (Fi) (Tiberti et al., 2016a):

Fi = Ni/N x 100

where Ni is the number of fish with prey i in their stomach and N is the total number of analyzed stomachs).

Bacteriological analysis

Specimens were necropsied under aseptic conditions and examined for the presence of lesions (e.g., wounds, bleeding or other pathological alterations). Bacteriological analysis was performed on kidney and brain and the inoculum was directly plated out on first isolation media (Columbia Blood Agar or Triptic Soy Agar). The samples were incubated at 22 ± 2 °C for 24-72 h; the colonies were cloned and identified by phenotypical and biochemical tests (API System, bioMèrieux, France). Phenotypic bacteria identification was confirmed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) technology on a VITEK MS system (bioMérieux).

Histological analysis

Gills, heart, liver, spleen, and gonads were fixed in 10% neutral-buffered formalin for histological analysis. The samples were analyzed at the Histopathology Laboratory of the Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta. Single samples were processed by standard paraffin wax techniques. Samples were cut in sections 4±2 mm thick with a microtome (Leica SM 2000R, Leica Biosystem) and stained with hematoxylin-eosin (H&E) staining; periodic-acid Schiff reaction (PAS) was carried out on livers.

For H&E staining, the sections were put in an oven (Duroni s.a.s., Turin) at 37 °C and then rehydrated in an automated stainer:

- 2 changes in xylene substitute (Kaltek, Padua) for 15 min each;
- 2 changes in 100% alcohol (Bio Optica, Milan) for 4 min each;
- 1 change in 95% alcohol solution for 4 min;
- 1 change in 80% alcohol solution for 4 min;
- 1 change in 50% alcohol solution for 4 min;
- 1 wash in distilled water for 4 min.

The sections were stained with hemalum solution acidic according to Mayer for 1 min, washed in running tap water for 10 min, then dehydrated as follows:

- 1 change in 50% alcohol solution for 3 min;
- 1 change in 80% alcohol solution for 3 min;
- 1 change in 95% alcohol solution for 3 min;
- 1 change in 100% alcohol for 6 min.

After two changes in xylene substitute for 15 min, the sections were cover slipped using a synthetic mounting medium (Ecomount-k, Kaltek, Padua). The slides were viewed under a microscope at increasing magnification (10x, 20x, 40x) to evaluate degenerative and/or inflammatory changes.

7.1.2.2 Results

A total of 35 individuals of bullhead (*Cottus gobio*) (15 in the first campaign, 20 in the second) and four individuals of minnow (*Phoxinus phoxinus*) were captured in Dimon Lake. The minnows were not included in the analysis. A total of 40 individuals of brook trout (*Salvelinus fontinalis*) (20 specimens for each campaign) were sampled in Balma Lake. The average total length for *C. gobio* captured during the two sampling campaigns was 13.52 ±1.29 cm and the average weight was 28.63 ± 7.42 g. The average total length for *S. fontinalis* captured during the two sampling campaigns was 18.81 ± 4.57 cm and the average weight was 79.20 ± 50.75 g.

Only total weight and total length for fish captured in July 2017 were reported, since positive for bacteriological test (Tables 17 and 18).

Analysis of stomach contents showed that the *C. gobio* from Dimon Lake fed chiefly on Chironomidae larvae in both summer (Fi: 96.18%) and autumn (Fi: 98.30%). Other taxa were present at extremely low frequencies (Chironomidae pupae, Fi: 3.18% in summer and 0.79% in autumn; Trichoptera, Fi: 0.64% in summer and 0.39% in autumn). Instead, in Balma Lake we identified 5 prey groups in stomachs of *S. fontinalis*: Hymenoptera (Fi, summer: 73.86%; autumn: 73.81%), Coleoptera (Fi, summer: 20.33%; autumn: 7.23%), Formicidae (Fi, summer: 4.14%; autumn: 0%), Chironomidae larvae (Fi, summer: 0.41%; autumn: 17.6%), Chironomidae pupae (Fi, summer: 1.24%; summer: 0.41%). A descriptive summary of the diet with relative Fi values for both *Cottus gobio* and *S. fontinalis* is provided in Table 19.

Table 17. Biometric measures and bacteriological test results of *Cottus gobio* sampled in Dimon Lake (July 2017).

Specimen	Length (cm)	Weight (g)	Test	Isolated bacteria	Gram
1	14.5	31	(-)		
2	15.5	33	(-)		
3	15.0	32	(-)		
4	15.5	39	(-)		
5	13.5	23	(-)		
6	14.5	34	(+)	*Aeromonas sobria*	(-)
7	13.0	26	(-)		
8	13.5	28	(+)	*Aeromonas sobria*	(-)
9	11.0	20	(+)	NSG	
10	12.5	24	(-)		
11	13.5	27	(-)		
12	11.8	19	(-)		
13	12.0	20	(+)	*Aeromonas sobria*	(-)
14	11.7	19	(-)		
15	12.0	23	(+)	*Aeromonas sobria*	(-)

Table 18. Biometric measures and bacteriological test results of *Salvelinus fontinalis* in Balma Lake (July 2017).

Specimen	Length (cm)	Weight (g)	Test	Isolated bacteria	Gram
1	22.0	104	(+)	*Yersinia ruckeri*	(-)
2	19.5	67	(+)	*Plesiomonas shigelloides*	(-)
3	18.5	61	(-)		
4	22.0	115	(-)		
5	17.0	56	(-)		
6	13.0	20	(-)		
7	19.0	69	(-)		
8	13.0	38	(-)		
9	11.5	18	(-)		
10	21.0	116	(-)		
11	24.0	146	(-)		
12	21.0	119	(-)		
13	18.5	61	(-)		
14	16.5	50	(-)		
15	22.0	123	(-)		
16	20.5	101	(-)		
17	12.0	17	(-)		
18	11.5	15	(-)		
19	16.0	43	(-)		
20	17.0	59	(-)		

Table 19. Frequency of occurrence of prey groups recorded in stomachs of *Salvelinus fontinalis* and *Cottus gobio* in both summer and autumn 2017.

	Salvelinus fontinalis		***Cottus gobio***	
Season	Summer	Autunm	Summer	Autunm
Hymenoptera	73.86%	73.81%	-	-
Formicidae	4.14%	0	-	-
Coleoptera	20.33%	7.23%	-	-
Chironomidae larvae	0.41%	17.6%	96.18%	98.30%
Chironomidae pupae	1.24%	0.41%	3.18%	0.79%
Trichoptera	0	0.41%	0.64%	0.39%
Arachnida	0	0.27%	0	-
Odonata	0	0.14%	0	-

None of the fish captured in the lakes during the two-sampling campaign showed external lesions or clinical signs. Results of bacteriological analysis of bullhead sampled in Dimon Lake during the first sampling campaign are reported in Table 17. Bacteriological analysis was positive in 5 fish (33.33%) from the first sampling campaign. In one specimen (no. 9) we isolated not significant germs (NSG), which are environmental bacteria found ubiquitously in nature or widespread in aquatic environments; they were not significant for the aim of the present study. Four fish (nos. 6, 8, 13, 15) were positive for *Aeromonas sobria*. The bacteriological analysis of fish collected during the second campaign was negative for all fish sampled. For Balma Lake the results are shown in Table 18. During the first campaign, only two fish (nos. 1 and 2) were positive (10%). One (no. 1) was infected by *Yersinia ruckeri*, and the other (no. 2) by *Plesiomonas shigelloides*. During the second sampling campaign, three fish (15%) showed only NSG bacteria. Histological examination revealed no alterations in the organs of *S. fontinalis* from Balma Lake, whereas histological examination of 33 livers of *Cottus gobio* caught from Dimon Lake during both seasons showed mild-to-severe cytoplasmic vacuolization of hepatocytes, with multifocal to diffuse localization. Only two showed no hepatic alterations. A specific session (see 7.2) was designed to clarify the interpretation of these results.

7.1.2.3 Discussion

With this preliminary study we wanted to investigate the effects of fish stocking in remote ecosystems not from an ecological point of view (in fact, we did not perform a quantitative analysis of fish populations) but rather from a health point of view, due to the lack of data about the health conditions of alien fish. We knew that individuals of brook trout (*Salvelinus fontinalis*) were introduced in Dimon Lake in the 1980s for recreational fishing (Ente Tutela Patrimonio Ittico FVG, personal communication). We assume that *S. fontinalis* is no longer present in the lake, since no individuals were caught during the two sampling campaigns. This forced us to think about the reasons why they disappeared from Dimon Lake, knowing that no eradication actions had been undertaken over the years. Interestingly, we found that a small population of minnow (*Phoxinus phoxinus*) inhabits the lake. Since the minnow is usually used as live bait by fisherman, we believe it was probably introduced in Dimon Lake in this way or accidentally with fish stocking. Mirò and Ventura (2013, 2015) reported that in lakes where minnow and brook trout are introduced together, *S. fontinalis* starts to decrease, and eventually disappears completely in some cases, whereas in lakes where only brook trout are present, the population remains stable. The study explained that this phenomenon is due to the ecological strategy of minnow that feed on brook trout eggs, reducing or eradicating the brook trout population when no further individuals are released. We hypothesized that a sum of effects led to a not direct human-mediated eradication of *S. fontinalis* in Dimon Lake: the absence of new releases in recent years, the presence of fishing pressure, and the minnow that feed on brook trout eggs.

The implications of stocking mountain lakes derive primarily from the fact that fish occupy a higher trophic level that was previously inexistent, leading to some severe ecological changes to native aquatic communities. Non-native fish introduction in original fishless lakes is commonly associated with the reduction of native biodiversity (e.g., invertebrates and amphibian) and can have indirect effects on the whole aquatic ecosystem (Eby et al., 2006). The bulk of studies investigating the ecological effects of fish introduction in high-mountain lakes have focused attention on salmonids as *S. fontinalis*. Nonetheless, *P. phoxinus*, *C. gobio*, and other small fish pose a threat to biodiversity conservation, too. These species are native to northeast Italy (Padano-Veneto, Friuli-Venezia Giulia) (Forneris et al., 1990) but were introduced accidentally many years ago in Dimon Lake. For this reason, they are considered a fully-fledged alien species. In Dimon Lake these fish species are, in fact, at the top of the food chain and are responsible for the same cascade-effects on the whole ecosystem, including the terrestrial habitat. Salmonids are visual predators, and most of the direct impacts are attributable to their size selective predation strategy, affecting only larger non-fossorial taxa (Tiberti et al., 2016b).

Previously published studies have focused on the effects of trout introduction on native species, especially amphibians, since they are among the most threatened animal groups worldwide (Beebee and Griffiths, 2005). The effects of fish introduction are also related to the terrestrial habitat, especially on macroinvertebrate during their emergency phase when they pass through the water column, which results in a substantial alteration of their emergency rates (Epanchin et al., 2010) or on terrestrial insects, as observed in Balma Lake. Fish stocking is a widespread practice worldwide, also in high-mountain lakes including Alpine lakes. Anglers usually release the fish for fishing. But in order to restore the natural conditions, remediation actions should be adopted. The most effective way seems to be the eradication of non-native fish. In this context, anglers can play an active role in eradication (Tiberti et al., 2016c). But because recreational anglers can have an effect only on adult populations, other measures (gillnets and electrofishing) should be taken in order to obtain complete eradication and reestablish original conditions.

As regards bacteriological analysis, we isolated environmental bacteria and also pathogenic bacteria, demonstrating that alien species may introduce pathogens even in Alpine lakes, contributing to the deterioration of these pristine ecosystems. In Dimon Lake we isolated motile Aeromonads. *Aeromonas sobria* is a gram-negative bacterium widespread in aquatic environments. It is an opportunistic bacterium and is usually not pathogenic though it can cause disease in certain stress conditions (Noga, 2010). Various species of Aeromonads are linked to a variety of diseases in different fish species, and motile aeromonads are often involved in fish disease (Roberts, 1993). Among the motile Aeromonads, *Aeromonas hydrophila*, *A. sobria,* and *A. caviae* are most commonly associated with fish (Austin et al., 1989; Carnahan and Altwegg, 1996; Abbott et al., 2003). The aeromonads can produce a wide range of potential virulence factors including extracellular hemolysins, cytotoxins, and proteases (Chacòn et al., 2003; Wang et al., 2003; Xia et al., 2004). In fish, *A. sobria* can act as a primary pathogen, depending on the different strains present and on the general health status of the population. There are virulent and avirulent strains of this bacteria (Wahli et al., 2005). The virulent strain has hemolytic and cytotoxic effects (Wahli et al., 2005). The literature reports wild fish mortality events caused by *A. sobria* (Manfrin et al., 2004; Wahli et al., 2005; Fichi et al., 2013). However, we found no clinical signs in the fish sampled and we assume that the bullhead population of Dimon Lake presented the non-virulent strain of *A. sobria* or that the stress or scarce environmental conditions that can lead to the onset of the disease were not present, as evidenced by physicochemical parameters measured the same year (see section 5).

We found only environmental bacteria in Dimon Lake, whereas in Balma Lake we isolated a primary fish pathogen: *Yersinia ruckeri*, the etiological agent of enteric red mouth disease (ERMD) in salmonids. This gram-negative bacterium belongs to the Enterobacteriaceae family. The disease causes significant economic losses every year in aquaculture facilities worldwide (Tobback et al., 2007). ERMD can affect fish of all ages but is most acute in small fish. In larger fish, the disease appears as a chronic condition. Changes in fish behavior may be observed, often including lethargy and swimming near the surface. Hemorrhages on the fish body surface are common, with reddening at the base of the fins and along the lateral line, as well as in the head region. The characteristic hemorrhages in and around the oral cavity have led to the name "red mouth" disease. Although these reddened areas are not apparent in some fish, the absence of a classic "red mouth" does not rule out infection with *Y. ruckeri*. Petechial hemorrhages on the surface of the liver, pyloric caeca, swim bladder and in the lateral musculature can be noted (Austin and Austin, 2007). The spleen is often enlarged. The intestine is inflamed and filled with a thick, opaque, and purulent fluid. The abdomen is distended as a result of fluid accumulation. *Yersinia ruckeri* is usually absent in pristine alpine lakes. The presence of this primary fish pathogen in Alpine lakes results from alien fish introduction. In fact, it is often present in aquaculture facilities that raise salmonids. The sampled fish showed no clinical signs, which can be easily explained because the disease appears as a chronic condition in older/larger fish (Kumar et al., 2015).

In Balma Lake we also isolated *Plesiomonas shigelloides*. This oxidase-positive, gram-negative, motile bacterium has been implicated as an agent of human gastroenteritis (Miller and Koburger, 1985) and can be found in freshwater and marine ecosystems in tropical and temperate climates (Levin, 2008). It is not a primary pathogen in fish and the occurrence of disease is often related to the increase of water temperature, especially in summer. Moreover, there is a pronounced increase of prevalence in waterways with high organic matter (Cruz et al., 1986). Furthermore, *P. shigelloides* may be normally resident in the gastrointestinal tract of fish (Austin and Austin, 2007), where it can serve as a reservoir of infection. The low water temperature throughout the year and the absence of water nutrient pollution (see section 5) in Balma Lake explain the absence of clinical signs. *A. sobria* and *P. shigelloides* are widely present in aquatic environment. During fish stocking, especially with non-native species, the introduced fish can act as carriers of these bacteria that could be more virulent or with an antibiotic resistance different from native aquatic bacteria and affect native aquatic fauna. For example, fish from aquaculture facilities can harbor bacteria that are dangerous for other species like amphibians. Alpine lakes are suitable breeding sites of the European common frog (*Rana temporaria*) (Tiberti, 2011). *R. temporaria* can be infected by *Aeromonas hydrophila*, one of the pathogenic agents of red-leg disease and one of the most commonly isolated bacteria in fish (Saikot et al., 2013). Fish stocking with individuals infected by *A. hydrophila* may introduce virulent strains that could threaten endangered native amphibians, with implications on their biodiversity conservation.

7.1.3 Quantitative fish sampling

7.1.3.1 Preface

Brook trout have been introduced in several Alpine lakes of Italy, establishing many reproductive populations in some large portions of the Cottian Alps, a mountain range located in the southwestern part of the Alps, which represent the natural border between France (Hautes-Alpes and Savoie) and Italy (Piedmont). To better understand the population structure of *S. fontinalis* recorded in Balma Lake during the first monitoring carried out in 2017, a quantitative fish sampling campaign was performed in August 2018.

7.1.3.2 Material and Methods

Fish sampling

A fish sampling campaign was performed on 3 August 2018 following the protocol used to assess the Lake Fish Index (Volta, 2009). The protocol was developed from a standardized method for fish sampling in European lakes EN 14757:2005 (CEN, 2005) that requires the use of benthic and pelagic gillnets in relation to the lake typology, surface area, and depth. Six benthic gillnets were used for sampling in Balma Lake. Since high-altitude lake fish usually prey on terrestrial insects and spend most of their time in the pelagic zone during summer (Tiberti et al., 2017), we employed 2 pelagic (P) and 4 benthic (B) gillnets. Benthic gillnets were 30 m long and 1.5 m high (total surface: 45 m^2) and were composed of 12 panels (2.5 m length) with variable mesh size, from 5 to 55 mm (Table 19). The pelagic gillnets were 27.5 m long and 6 m high (165 m^2) and had the same sequence of panels as the benthic gillnets but without panel 5 (11 panels in total). Gillnets placement was based on the lake bathymetry profile (Fig. 40). In the shallower zone, only benthic gillnets were used, while in the deeper zone both pelagic and benthic gillnets were placed. Gillnets were placed at 6 p.m. and recovered 12 hours later. Fish sampling was performed by permission of the Città Metropolitana di Torino (authorization n. 176-19040/2017), as required by local laws.

Table 19. Sequence and mesh size (mm) of panels which constitute the gillnets used for fish sampling in Balma Lake.

Panel (P)	Mesh size (mm)
1	43
2	19.5
3	6.25
4	5
5	55
6	8
7	12.5
8	24
9	15.5
10	10
11	35
12	29

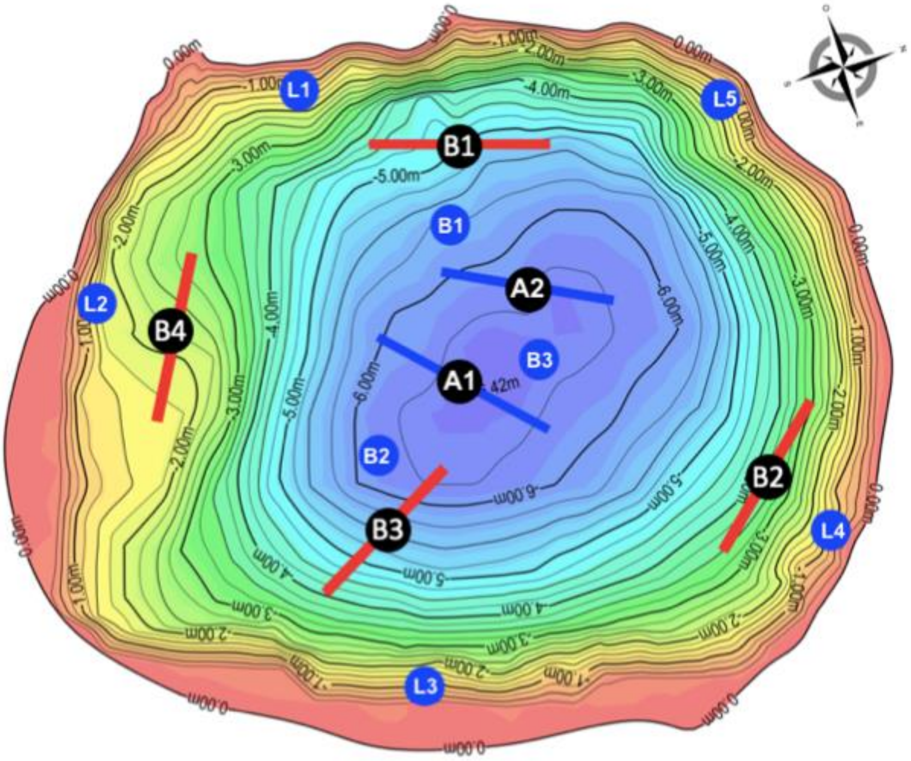

Figure 40. Placement of gillnets in Balma Lake. L1- L5 = littoral sites; B1-B3 = deep sites for physicochemical parameters (see section 5). Fish sampling: B1-B4 = benthic gillnets; A1-A2 = pelagic gillnets.

Stomach content analysis

Stomach content analysis was performed to obtain information about brook trout diet and diet preferences. The stomachs were sampled, preserved in 70% alcohol, and then identified in the laboratory using both a stereomicroscope and an optical microscope. We identified the ingested prey at the order or family level because a more precise identification was often impossible due to the digestion status of the prey. In order to describe the diet, frequency of occurrence (Fi) was calculated for the prey items (Tiberti et al., 2016a). Relative abundance (Ai) was also calculated (Tiberti et al., 2016a) as follow:

$Ai = Si/St \times 100$

where Si is the sum of the number of prey i in all the stomachs and St is the total sum of all the counted prey.

Sanitary evaluation

For sanitary evaluation, specimens were necropsied and evaluated for possible pathological alterations. For parasitological exam, tissue scrapings from skin and gill filaments were prepared with a drop of water, covered with a clean cover slip (wet mount preparation) and examined under a microscope at 10x to 40x magnification. The bacteriological exam was performed from kidney, brain and eye (vitreous humor) and the inoculum was directly plated out on first isolation media as Columbia Blood Agar or Tryptic Soy Agar. This procedure was performed immediately after fish recovery from gillnets. The colonies, eventually grown after 24-72 hours of incubation at 22 ± 2 °C, have been cloned and identified by Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) technology on VITEK MS system (bioMérieux, France). Finally, Pb and Cd concentrations in muscle of *S. fontinalis* were determined by inductively coupled plasma-mass spectrometry (ICP-MS Xseries II, Thermo Scientific, Bremen, Germany) after microwave digestion, following the protocol described by Squadrone et al. (2016b). Hg concentration was also determined by direct mercury analyzer (DMA-80 Analyzer from Milestone, Shelton, CT, USA). For heavy metals determination, 5 specimens of homogeneous size (total length: 20 ± 2 cm) were selected and muscle tissue samples were taken from each animal. Samples were homogenized with an electric mill and divided into two sub-samples: one to determine mercury, the other to determine cadmium and lead. The limit of quantification (LOQ) was 0.010 mg kg^{-1} for Hg and 0.020 mg kg^{-1} for both Cd and Pb.

Biometric measures and statistical analysis

Catch per unit effort (CPUE) and biomass per unit effort (BPUE) were calculated for both benthic and pelagic gillnets. CPUE was calculated as the number of fish per square meter of net per 12 hours (Cavalli et al., 1997). BPUE was determined as the weight (g) of fish per square meter of net per 12 hours (Alexander et al., 2015). Furthermore, the number and weight of each fish captured per net were also reported. The non-parametric Kruskal-Wallis test was used to compare distribution of the length of fish captured with panels (P) of different mesh size. The Conover-Iman test was used as a post-hoc test to assess the presence of significant differences between panels of different mesh sizes. Total length (L_t; cm) and total weight (W; g) were recorded for each specimen. Five scales above the lateral line were collected from each specimen to determine age. The relationship between total length and total weight was determined by non-linear regression for males and females (Ricker, 1975):

$W = a\, L_t^b$

where: W = fish weight (g); a = intercept on the x-axis; L_t = total fish length (cm); b = exponent of the arithmetic form of the weight-length relationship and the slope of the regression line in the logarithmic form. Coefficient of determination (r^2) value was obtained by curve interpolation and used to assess whether the curve values fit the data (Treer et al., 2008). The curve is more reliable as the r^2 is closer to the theoretical value of 1. ANCOVA was used to compare b-values as slopes of linear form of the standard length-weight regression equations. Differences in biometric parameters (L_t and W) between males and females of different age classes were checked using the non-parametric Mann-Whitney U test. Fish health condition was checked using the mean condition factor (K_{mean}), which is the average condition factor for a given length obtained from the respective weight-length relationship (Froese, 2006), making it suitable for comparison of different populations of the same species. The mean condition was calculated as follows:

$K_{mean} = 100\ a\ L_t^{b-3}$

where K_{mean} = mean condition factor; L_t = total length (cm); a = coefficient of the arithmetic weight-length relationship and the intercept of the logarithmic form; b = exponent of the arithmetic form of the weight-length relationship and the slope of the regression line in the logarithmic form. Non-parametric Kruskal-Wallis test followed by post-hoc Conover-Iman test was used to detect significant differences in K_{mean} values between age classes of males and females. Results were considered statistically significant at p value < 0.05. All analyses were performed using software RStudio version 1.1.463.

7.1.3.3 Results

Fish population

A total of 90 specimens of brook trout (*Salvelinus fontinalis*) were collected. The number of fish captured ranged from 12 to 17 per benthic gillnet. Sixteen individuals were captured with both pelagic gillnets. The CPUE for benthic gillnets ranged from 0.267 (B1) to 0.378 (B4) fish per m^2 per 12 h. The CPUE was 0.100 fish per m^2 per 12 h for both the pelagic gillnets (A1 and A2). BPUE ranged from 21.39 to 24.05 g m^{-2} per 12 hours for benthic gillnets and from 7.73 to 8.85 g m^{-2} per 12 hours for pelagic gillnets (Table 20).

Table 20. Number of fish (n) captured with pelagic (A) benthic (B) gillnets. It is also reported the CPUE (n of fish per m^2 per 12 hours), the weight (W; g) of each fish and the BPUE (weight of fish per m^2 per 12 hours).

Gillnet	**A1 (n = 16)**	**A2 (n = 16)**	**B1 (n = 12)**	**B2 (n = 14)**	**B3 (n=15)**	**B4 (n = 17)**
CPUE	0.100	0.100	0.267	0.311	0.333	0.378
W1	78.26	67.53	58.72	116.53	18.41	18.45
W2	146.86	79.55	73.58	98.35	17.29	15.12
W3	120.16	53.16	64.59	45.81	58.75	17.34
W4	92.80	94.46	71.24	50.49	14.75	20.87
W5	114.85	96.58	44.52	54.76	11.70	38.23
W6	109.4	61.10	128.00	65.32	6.07	43.02
W7	93.45	50.64	133.41	50.90	14.70	50.46
W8	97.53	45.44	122.89	61.75	102.2	56.54
W9	73.02	62.71	107.24	60.65	138.63	59.87
W10	52.64	58.78	100.94	92.05	131.99	61.67
W11	113.44	111.75	105.23	49.63	83.74	69.98
W12	77.91	71.39	72.06	62.25	91.99	67.43
W13	89.88	133.82		59.32	117.30	61.07
W14	69.64	101.88		104.17	112.74	101.4
W15	47.85	68.02			42.17	116.32
W16	82.21	118.96				123.25
W17						146.7
BPUE	8.85	7.73	24.05	21.60	21.39	23.73

The mean fish biomass in Balma Lake was 17.89 g m^{-2} (considering the BPUE of both pelagic and benthic gillnets). The distribution of the length of fish differed across panels (P) with different mesh sizes (Kruskal-Wallis test; $p = 0.0013$), and pairwise comparisons showed significant differences in fish length distribution between P3 and P12 ($p = 0.0011$), P5 and P12 ($p = 0.0004$), P8 and P12 ($p = 0.0010$), P10 and P12 ($p = 0.0001$), P4 and P10 ($p = 0.0008$) (Fig. 41). No fish were captured in panels 1 (43 mm) and 11 (35 mm). Specimens smaller than 8 cm were not captured in the gillnets. These very small individuals inhabit shallow areas near the shoreline. These findings derived from visual observations carried out during gillnet placement.

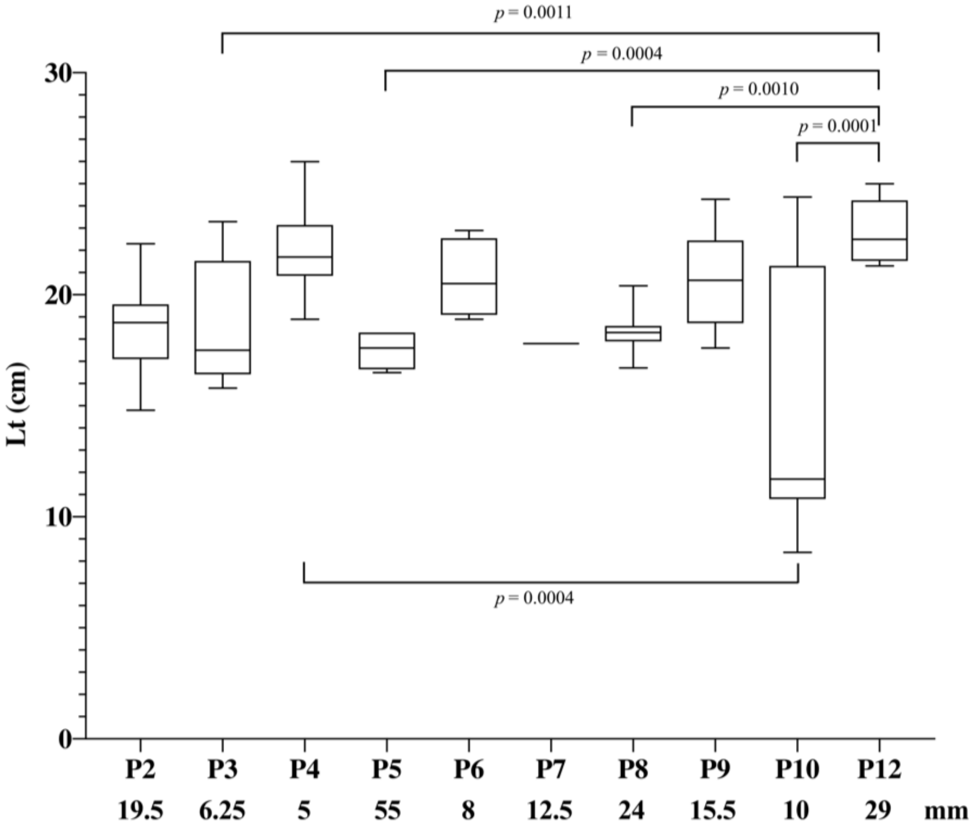

Figure 41. Length distribution of brook trout from Balma Lake in relation to different panel (P) mesh sizes.

Sex was determined for all 90 specimens (59 females and 31 males) belonging in age class 0+ to 4+. The highest number of individuals were recorded for classes 1+ and 2+ (Fig. 42).

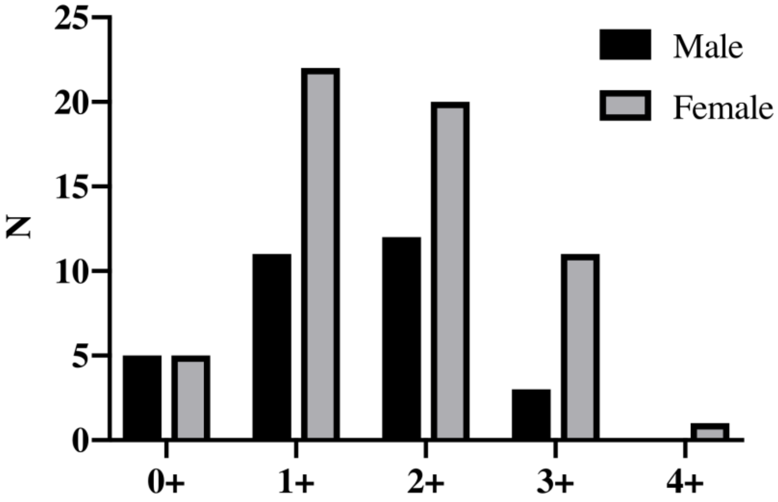

Figure 42. Distribution of age classes of *S. fontinalis* males and females from Balma lake.

There were more females in all classes (except for age 0+, which was equal to males). It is remarkable that the oldest recovered specimen (class 4+) was a female. Within each age class, L_t and W values did not differ significantly between males and females (Table 21), as confirmed by length-weight regression, which showed clearly overlapping curves with a similar trend (Fig. 43). Regression analysis between W and L_t did not differ significantly between males and females (ANCOVA: F = 0.453; p = 0.954). Generally, the K_{mean} values decreased with increasing age for both males and females (Table 22). K_{mean} values differed significantly among age classes for females (Kruskal-Wallis test; $p < 0.0001$; Conover-Iman test: $p < 0.0001$ for all comparisons), while they differed for males except between classes 2+ and 3+ (Kruskal-Wallis test; $p < 0.0001$; Conover-Iman test: $p < 0.001$ for all comparisons except between classes 2+ and 3+, $p > 0.05$).

Table 21. Values of total length L_t (cm) and weight W (g) of *S. fontinalis* males and females for each age class observed in Balma Lake.

		Females (F)		**Males (M)**		**Mann-Whitney test (F *vs* M)**	
Age class		**L_t (cm)**	**W (g)**	**L_t (cm)**	**W (g)**	**L_t**	**W**
0+	**Mean**	11.58	16.46	10.70	14.48	*p* = 0.3016	*p* = 0.0659
	Median	11.50	17.29	11.10	14.75		
	SD	1.12	3.37	1.33	5.05		
	Min	9.90	11.70	8.40	6.07		
	Max	11.50	20.87	11.70	18.41		
	CV %	9.68	20.45	12.00	34.24		
	n	5		5			
1+	**Mean**	17.46	58.38	17.14	55.69	*p* = 0.4333	*p* = 0.4625
	Median	17.40	58.73	17.00	53.16		
	SD	1.45	9.93	1.36	12.14		
	Min	13.00	38.23	14.80	42.17		
	Max	19.50	79.55	19.00	78.26		
	CV %	8.28	17.00	7.99	22.83		
	n	22		11			
2+	**Mean**	20.48	97.41	19.92	86.38	*p* = 0.2589	*p* = 0.1821
	Median	20.55	99.70	19.90	84.95		
	SD	1.38	18.84	1.87	22.68		
	Min	18.30	62.25	17.60	58.78		
	Max	22.90	133.41	23.30	122.89		
	CV %	6.76	19.34	9.39	26.25		
	n	20		12			
3+	**Mean**	23.06	119.64	23.07	122.33	*p* = 0.9725	*p* = 0.999
	Median	23.30	118.96	22.20	123.25		
	SD	1.14	21.50	1.68	10.15		
	Min	21.30	83.74	22.00	123.25		
	Max	24.40	146.86	25.00	131.99		
	CV %	4.96	17.97	7.27	8.30		
	n	11		3			
4+	**Mean**	26.00	100.94	-	-		
	Median	-	-	-	-		
	SD	-	-	-	-		
	Min	-	-	-	-		
	Max	-	-	-	-		
	CV %	-	-	-	-		
	n	1		0			

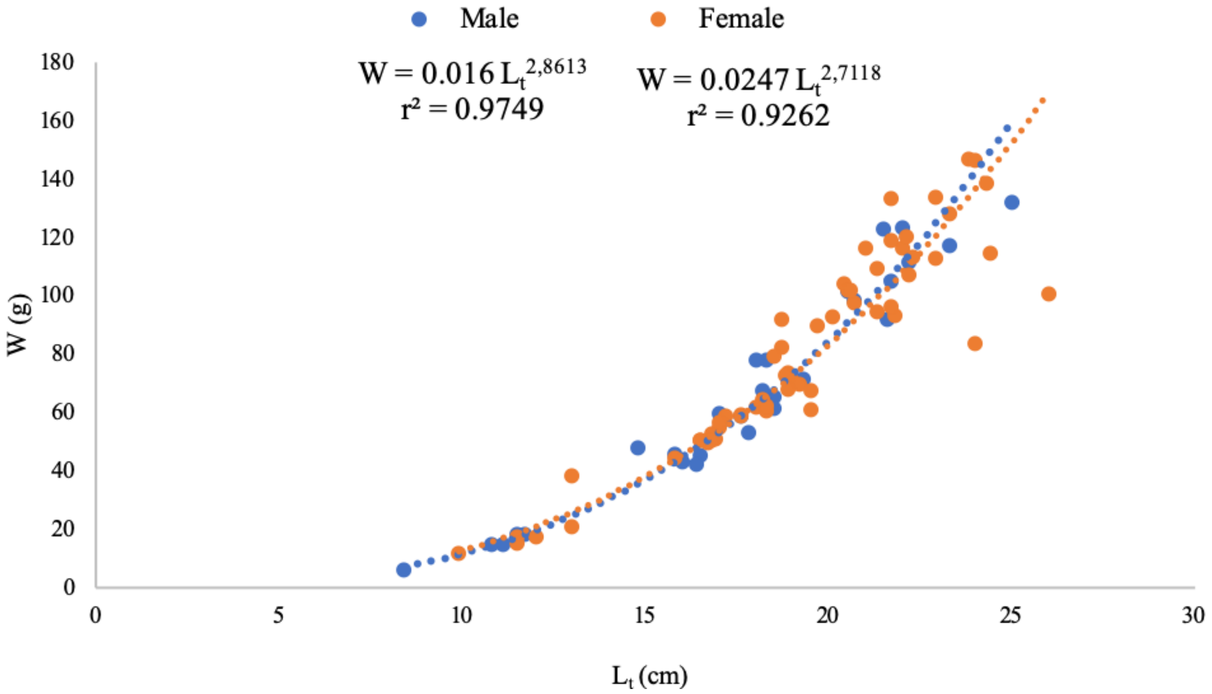

Figure 43. Relationship between total length (L_t) and total weight (W) obtained from *S. fontinalis* specimens (males and females) collected in Balma Lake.

Table 22. Age classes, sex and relative K_{mean} (mean ± SD) for *S. fontinalis*.

Age	**Gender**	**K_{mean}**
0+	M	1.03 ± 0.04
	F	1.14 ± 0.01
1+	M	0.89 ± 0.02
	F	1.08 ± 0.01
2+	M	0.86 ± 0.02
	F	1.05 ± 0.01
3+	M	0.82 ± 0.02
	F	1.03 ± 0.01
4+	M	-
	F	1.01

Stomach content analysis

The mean number of prey items per stomach was 14.63 ± 14.71 (range 2-70). We identified four prey groups (Diptera Chironomidae, Imenoptera and Coleoptera) in the diet of *S. fontinalis* from Balma Lake which we grouped into two macrogroups (benthic invertebrates and terrestrial invertebrates). A descriptive summary of the diet of *S. fontinalis* is provided in Table 23.

Table 23. Diet of *Salvelinus fontinalis* from Balma Lake. Fi = frequency of occurrence; Ai = relative abundance.

Prey groups	Fi (%)	Ai (%)
Benthic invertebrates	**6.66**	**1.65**
Diptera Chironomidae	6.66	1.65
Terrestrial invertebrates	**83.33**	**94.19**
Hymenoptera	66.67	73.86
Formicidae	1.33	4.14
Coleoptera	15.55	20.33

Sanitary examination

None of the fish showed any clinical signs or pathological alterations. Parasitological examination was negative for all specimens, while bacteriological test was positive in 33%. All bacteria were isolated from the eye and belonged to *Carnobacterium maltaromaticum* and *C. divergens*. Concentrations (mean ± standard deviation SD) of total mercury, cadmium, and lead in muscle tissue of *S. fontinalis* are summarized in Figure 44. Cd was < LOQ in all samples. Pb concentration ranged from 0.041 to 0.12 mg kg^{-1}, Hg ranged from 0.013 to 0.015 mg kg^{-1}.

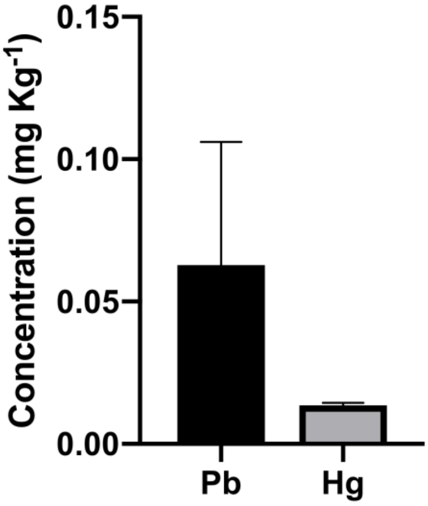

Figure 44. Concentration values (mean ± SD) of Pb and Hg (mg kg $^{-1}$) in muscle of *S. fontinalis* from Balma Lake.

7.1.3.4 Discussion

Gillnets of smaller mesh size are not very selective for fish, P10 (mesh size 10 mm) captured specimens from 8.4 cm to 24.4 cm. The capture efficiency was dictated by the ability of the smaller mesh size to entangle the fish. These observations are shared by Tiberti et al. (2017) who showed how gillnets with mesh size from 10 to 25 mm entangled brook trout of different size in four high-altitude lakes in the Italian Alps. In contrast, larger mesh size (35, 43, 55 mm) were too large to efficiently capture the relatively small brook trout of the study population. Cavalli et al. (1997) observed in Plan Vianney Lake (French Alps, 2250 m a.s.l.) that the CPUE of the surface and bottom nets for *S. fontinalis* was 0.02 individuals m^{-2} h^{-1}. If we take our CPUE values and compare them hourly, we obtained similar results: 0.008 individuals m^{-2} h^{-1} for both pelagic gillnets and 0.02-0.03 individuals m^{-2} h^{-1} for benthic gillnets.

In their study on a brook trout population from Grand Mesa, Uncompahgre and Gunnison National Forests (USA) Adams et al. (2008) sampled both juveniles and adults, but fish larger than 20 cm were observed only in 16% of the total fish sampled, with most ranging between 7.5-25.0 cm, thus similar to our results. Changes in mean length reflected the trophic condition of the environment. For example, the brook trout population in an ultraoligotrophic high-altitude lake (2703 m a.s.l.) in the Western Alps reached a total length of 15-18 cm at age 3+ and 20-22 cm at age 5+ (Alessio et al., 1987). Differently, brook trout in Lake Nero (Western Alps, 2007 m a.s.l.) reached 18-20 cm at age 2+, as this is a more productive environment (Forneris, 1984).

The biomass of brook trout in Balma Lake (17.89 g m^{-2}) was similar to those recorded in Lake Dres (18 g m^{-2}) in Gran Paradiso National Park, Italy (Tiberti et al., 2017). Lake Dres is similar in hydrochemistry (P = 0.04 mg l^{-1}; pH = 7 ± 0.5), altitude (2087 m a.s.l.), and depth (7.4 m) to Balma Lake. The biomass of the brook trout population is related to their abundance, phosphorus concentration, and altitude, which are related to the trophic and thermal state of Alpine lakes (Tiberti et al., 2017). Balma Lake lies at 2100 m a.s.l and is characterized by oligotrophic conditions (PO_4^{3-} < 0.02 mg l^{-1}) without thermal stratification; under such conditions brook trout cannot reach a considerable biomass.

In our study, the scalimetric method for age determination was found to be reliable for small specimens, which have a relatively stable growth rate. For larger specimens (standard length > 15 cm), however, in which growth rates vary and can rapidly decrease, this method may underestimate specimen age (Šanda et al., 2015). This problem was reported by Dutil and Power (1977) for brook trout. Kozel and Hubert (1987) found that neither scales nor otoliths produce reliable age estimates for brook trout from a high-elevation environment, and that age estimates based on otoliths tended to be higher than that based on scales. Accordingly, we did not analyze otoliths in this study. Nevertheless, the age structure of the brook trout population from Balma Lake was confirmed by previous results obtained by Šanda et al. (2015) who reported a maximum age of 4+ for brook trout in two high-mountain lakes. Our findings are shared by other studies that the majority of brook trout populations reach their maximum age of 3+ or 4+ in high-altitude environments (McFadden, 1961; Wydoski and Cooper, 1966; Flick and Webster, 1976; Quinn et al., 1994).

Regarding sex determination, several mechanisms may influence the sex ratio, which constitutes a parameter of pivotal importance for population viability (Ospina-Álvarez and Piferrer, 2008). In our study, the higher number of females than males may be explained by the low water temperature throughout the year. Pavlidis et al. (2000) showed that temperature during very early developmental stages is a crucial factor affecting sex differentiation in fish, with low rearing temperatures (13 or 15 °C) resulting in sex proportions consistently skewed in favor of females. Furthermore, epigenetic mechanisms can work with genomic and environmental factors to modify gene activity that generates a particular phenotype and sex determination consequently (Piferrer, 2019).

The mean condition factor (K_{mean}) showed a declining trend from age 0+ to 4+ in line with the changes in body length, indicating a steady loss of weight in relation to length. Compared to females, the K_{mean} for males was lower in all age classes, which could suggest differences in the use of resources for growth. Unfortunately, there are no K_{mean} data for brook trout from mountain lake available for possible comparison. Nevertheless, Reimers (1958) showed a downward trend for Fulton’s condition factor (K) values in brook trout from a small high-altitude lake in the eastern Sierra Nevada. Cavalli et al. (1997) showed that the condition factor of brook trout from Plan Vianney Lake (French Alps) gave a minimum value of 0.86 in May and was indicative of poor food availability, whereas the condition factor was close to 1.1 in July-August, with the increased availability of prey.

Summer food of brook trout consists mainly of terrestrial insects, constituting more than 80% of the food intake (Dawidowicz and Gliwicz, 1983). We found that the brook trout in Balma Lake feed on mostly terrestrial invertebrates. This result is consistent with the two-year study by Utz and Hartman (2007) on brook trout diet in the Appalachian Mountains of Randolph County (West Virginia). They found that during warmer months the fish feed on terrestrial invertebrates, whereas aquatic animals and macrobenthic invertebrates contributed less to sustaining the brook trout populations. In our study, we found that the benthic component accounted for only a limited portion of the diet of the fish. The large number of taxa found in the stomachs reveals, however, that *Salvelinus fontinalis* is an opportunistic and visual predator, as reported in the literature (Lacasse and Magnan, 1992; Tiberti et al., 2016a). The finding of taxa belonging to the Formicidae family showed that brook trout also feed on organisms that accidentally fall into the water, confirming the opportunistic dietary behavior of this salmonid. The observed prevalence of terrestrial invertebrates in the diet of brook trout in Balma Lake is shared by previous studies on the same species in other Alpine lakes (Sotiropoulos et al., 2006; Tiberti et al., 2016a). The aquatic communities of invertebrates in mountain lakes are composed of few taxa: Diptera Chironomidae (Hexapoda) and Oligochaeta may make up more than 70% of the whole community (Boggero et al., 2006; Kownacki et al., 2006; Dumnicka and Boggero, 2007). Only members of the Chironomidae family were found in the fish stomachs, but at low frequencies. Hyslop (1980) pointed out that Oligochaeta are underrepresented in the diet of fish due to their poor resistance to digestion which can hamper their occurrence. Nevertheless, our results showed that the brook trout population has a negative pressure on terrestrial insects. Stocking fish into fishless lakes is known to trigger a series of effects that cascade through the food web (Eby et al., 2006). For example, stocking arctic char *Salvelinus alpinus* into streams can reduce the emergence of benthic insects eaten by spiders and birds in the riparian zone (Nakano and Murakami, 2001; Baxter et al., 2005), potentially decreasing the flux of carbon from aquatic to surrounding terrestrial ecosystems (Baxter et al., 2004). Furthermore, a reduced flux of terrestrial invertebrates can indirectly affect riparian communities by reducing the pollination of terrestrial plants (Knight et al., 2005).

Finally, regarding the evaluation of sanitary conditions, bacteriological exam was positive in the specimens in which we isolated *Carnobacterium maltaromaticum* and *C. divergens*. *Carnobacterium* is a genus of Gram-positive bacteria within the family Carnobacteriaceae. These bacteria are frequently isolated from natural environments and foods (Leisner et al., 2007). Furthermore, C. *maltaromaticum* (previous *C. piscicola*) was also isolated in diseased fish and is considered an opportunistic pathogen (Ramia et al., 2019). The presence of this bacterium has been associated with splenomegaly, renal and splenic congestion, thickening of the swim bladder wall with accumulation of a mucoid exudate in the lake whitefish (*Coregonus clupeaformis*) from Lake Michigan (Loch et al., 2008). We observed no clinical signs in the brook trout from Balma Lake. This isolation leads us to hypothesize that this opportunist pathogen may remain in a sort of latent state in the eye (Trojan horse effect *sensu* Zlotkin et al., 2003) but then exert pathogenic action when immune defenses are reduced due to stress caused by adverse environmental conditions. This assumption needs to be better studied and investigated in the future to implement the knowledge about its pathogenesis.

As regards heavy metal accumulation, the mercury level was < 0.015 mg kg^{-1} in all samples, which is far below the maximum limit of 0.5 mg kg^{-1}set by the Regulation 1881/2006 (European Commission, 2006) and subsequent amendments. The cadmium content was also below the LOQ (< 0.02 mg kg^{-1}) in all samples and within the limit (0.05 mg kg^{1}) for cadmium in fish muscle (European Commission, 2006). The mean lead level was 0.064 mg kg^{-1}, also below the limit of 0.30 mg kg^{-1} (European Commission, 2006).

7.2 Hepatic steatosis in bullhead from Dimon Lake

7.2.1 Preface

Published data on fish adaptation to high-altitude environments are scarce. For example, some studies have focused on metabolic rate, as reported in highland westslope cutthroat trout (*Oncorhynchus clarkii*) as compared with introduced lowland rainbow trout (*Oncorhynchus mykiss*) in the Oldman River basin (Canada): fish at high altitudes have a lower metabolic rate (Rasmussen et al., 2012). Kang et al. (2017) investigated the genetic mechanism of high-altitude adaptation in *Creteuchiloglanis macropterus* (Siluriformes) and found that many genes were involved in energy metabolism and hypoxia, which might be an important resource for studying the adaptations to high-altitude environment. Li et al. (2013) characterized the mitochondrial genomes of two species ("morphologically primitive" clade and "morphologically specialized" clade) of schizothoracine fishes. They reported evidence for positive selection acting on the protein-encoding genes in the mitochondrial genomes of the "morphologically specialized" clade, implying a possible genetic basis for high-altitude adaptation in this derived lineage of cypriniform fish. Other studies on macrobenthic invertebrate in streams have showed a decrease in species richness with increasing altitude due to lower oxygen saturation rather than low temperature (Jacobsen, 2008). These factors may reduce productivity in high-altitude ecosystems, meaning there will be less energy available for consumption, growth, and activity, which provides a survival advantage to fish with lower metabolic demands. During 2017, two fish sampling campaigns were conducted in Dimon Lake. Liver examination revealed alterations in specimens of bullhead (*Cottus gobio*). The aim of this study was to describe the liver alterations observed in *C. gobio* and discuss the possible causes of this abnormality.

7.2.2 Material and Methods

Fish sampling

In 2017 two fish sampling campaigns (July and October) were performed (see section). Fish were sampled using an electrofishing boat in both the littoral and the deep zones (max. depth 4.27 m). The fish were identified and measured for length (TL; cm) and weight (W; g). Fulton's condition factor (K) was also calculated for each specimen. K factor derived from the formula (Froese, 2006):

$K = (P / TL^3)\ 100$

where P is the weight (g), and TL is total length of fish (cm). A total of 35 fish (15 in the summer and 20 in the autumn campaign) were sampled, euthanized with an overdose of MS-222 (200 mg kg^{-1}) and then necropsied. The liver from each specimen was weighted to calculate hepatosomatic index (HSI) using the formula (Biney et al., 1994):

HSI (%) = (LW / FW) 100

where LW is the liver weight (g), and FW is whole weight of fish (cm). Liver was sampled for histological evaluation. Only larger fish were selected in order to have enough material for all histological analyses. Moreover, as control fish we also performed two sampling campaigns in the same periods (July and October) using electrofishing in Degano Creek. Degano Creek (46°26'21.9"N; 12°52'43.6"E) is a sub-alpine watercourse near the municipality of Raveo (Udine Province, 520 m a.s.l.), far about 40 Km from Dimon Lake, with scarce human impacts. The comparison of lake vs. stream populations was dictated by the absence of *C. gobio* in other high-altitude lakes in Northeast Italy. Only 10 individuals both for summer and autumn were captured to avoid putting pressure on the wild native population. W, TL, HSI and K were also determined. Fish sampling campaigns were performed by permission of Ente Tutela Patrimonio Ittico of Friuli Venezia-Giulia (authorization n. 11/DIR/17/01/2017).

Analysis of stomach contents

In both sampling campaigns the stomach contents of the fish from Dimon Lake were analyzed to obtain more detailed information about their diet. The stomachs were preserved in alcohol 70% and the content was identified by optical microscope (Olympus BX40) and stereomicroscope (Zeiss Stemis V8). The attempt was made to identify the ingested prey to the lowest recognizable taxa, but accurate identification was often not possible due to digestion of the prey. Stomach content analysis was also performed on the fish from Degano Creek. In order to describe the diet, prey frequency of occurrence (Fi) (Tiberti et al., 2016a) was calculated for both seasons.

Histological evaluation

A total of 35 liver samples were partly fixed in 10% neutral buffered formalin and partly frozen. The fixed tissues were dehydrated in a gradual series of alcohols and embedded in paraffin. The paraffin blocks were cut into 4 ±2 mm sections and stained with hematoxylin and eosin (HE) for microscopic evaluation. Histological changes (nuclear displacement and cytoplasm vacuolization) were evaluated and a semiquantitative severity score was assigned: 0-not observed; 1-mild; 2-moderate; 3-severe (Caballero et al., 2004). Histochemical stains, Periodic-acid Schiff (PAS) to detect mucopolysaccharides and Sudan III to detect lipids, were also performed (Luna, 1968). Slides were evaluated microscopically at increasing magnification (10x, 20x, 40x) on a Zeiss Axio Scope.A1 microscope. Samples were considered positive for the presence of red-purple glycogen or orange lipidic deposits in hepatocyte cytoplasm for PAS and Sudan III stain, respectively.

7.2.3 Results

Fish sampling and anatomopathological features

The average TL for *C. gobio* captured during summer campaign was 13.88 ± 1.12 cm, with an average W of 31.20 ± 7.54 g. The average TL of individuals captured during autumn was 13.25 ± 1.37 with an average W of 27.10 ± 12.98 g. K values ranged between 1.15 ± 0.13 in summer and 1.14 ± 0.15 in autumn. HSI ranged between 2.80 ± 0.38 in summer and 3.08 ± 0.39 in autumn (Table 23). At necropsy, the livers appeared variably pale to yellowish in color and friable in consistency (Fig. 45); no visible lesions in the other visceral organs were observed. Table 24 presents the measures of total length, weight, condition factor and hepatosomatic index for *C. gobio* from Degano Creek. The average TL of individuals captured during summer was 14.24 ± 0.86 cm with and average W of 33.60 ± 6.15 g, instead during autumn individuals showed a TL of 12.40 ± 1.76 cm with a W of 23.00 ± 9.14 g. K values ranged between 1.16 ± 0.14 in summer and 1.17 ± 0.13 in autumn. HIS ranged between 1.60 ± 1.16 in summer and 1.17 ± 1.60 in autumn. No lesions in organs were observed, liver included (Fig. 46). Statistical analysis showed no seasonal differences in liver alteration score in Dimon Lake (Wilcoxon test; $p = 0.6719$), but significant difference was found between Dimon Lake and Degano Creek in both summer (Mann-Whitney test; $p = 0.0001$) and autumn (Mann-Whitney test; $p < 0.0001$). As regard HSI, no seasonal differences were observed in Dimon Lake (Wilcoxon test; $p = 0.1173$), but significant difference were recorded between Dimon Lake and Degano Creek in summer (Mann-Whitney test; $p < 0.0001$) and also in autumn (Mann-Whitney test; $p < 0.0001$). No seasonal change in K values was recorded in Dimon Lake (Wilcoxon test; $p = 0.5696$), not even between Dimon Lake and Degano Creek in both summer (Mann-Whitney test; $p = 0.8589$) and autumn (Mann-Whitney test; $p = 0.6415$). A significant positive correlation between HIS and L was found (ρS 0.573).

Table 23. Total length (TL), weight (W), hepatosomatic index (HSI), Fulton's condition factor (K) and liver alteration score (L) of *Cottus gobio* (N) captured during summer and autumn in Dimon Lake.

Season	N	TL (cm)	W (g)	HSI	K	L
Summer (July)	1	14.0	29	2.77	1.06	2
	2	15.3	38	2.44	1.06	1
	3	15.3	37	2.50	1.03	2
	4	14.9	29	3.15	0.88	2
	5	13.1	27	3.21	1.20	3
	6	15.4	46	2.37	1.26	0
	7	14.5	41	2.23	1.34	2
	8	13.5	32	2.74	1.30	3
	9	13.2	26	3.36	1.13	3
	10	15.0	39	2.32	1,16	2
	11	13.4	31	3.11	1.29	3
	12	13.5	27	3.25	1.10	2
	13	12.2	20	2.97	1.10	3
	14	12.5	25	3.07	1.28	3
	15	12.4	21	2.57	1.10	1
Autumn (October)	16	14.3	37	3.85	1.27	3
	17	12.3	19	2.67	1.02	0
	18	14.5	37	2.39	1.21	3
	19	12.4	26	3.64	1.36	2
	20	12.0	17	3.12	0.98	3
	21	14.5	31	3.14	1.02	3
	22	15.5	33	2.61	0.89	2
	23	15.0	32	2.80	0.95	1
	24	15.5	39	3.07	1.05	3
	25	13.5	23	2.85	0.93	3
	26	14.5	34	3.62	1.12	3
	27	13.0	26	2.84	1.18	2
	28	13.5	28	2.56	1.14	2
	29	11.0	20	2.89	1.50	2
	30	12.5	24	3.49	1.23	3
	31	13.5	27	3.32	1.10	3
	32	11.8	19	3.19	1.16	3
	33	12.0	20	3.37	1.16	3
	34	11.7	19	3.10	1.19	3
	35	12.0	23	3.12	1.33	3

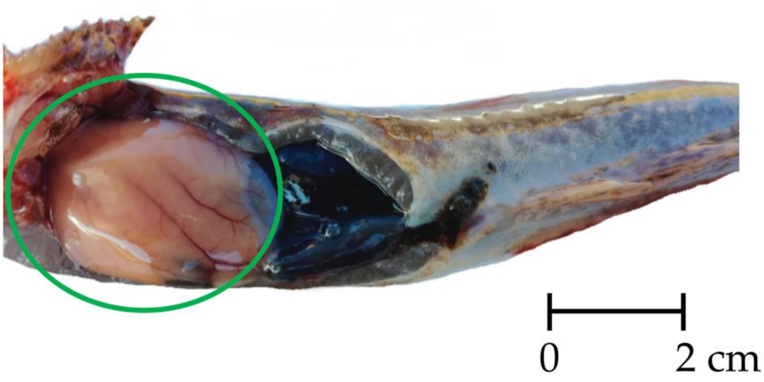

Figure 45. Macroscopical aspect of liver (green circle) from *Cottus gobio* captured in Dimon Lake. It appears pale to yellowish in colour and friable in consistence.

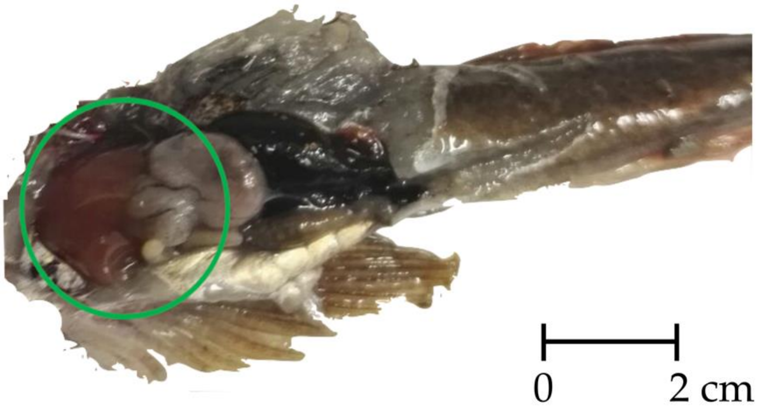

Figure 46. Macroscopical aspect of normal liver (green circle) of *Cottus gobio* captured in Degano Creek.

Table 24. Total length (TL), weight (W), hepatosomatic index (HSI), Fulton's condition factor (K) and liver alteration score (L) in individuals of *Cottus gobio* (N) captured during summer and autumn in Degano Creek.

Season	N	TL (cm)	W (g)	HSI	K	L
Summer (July)	1	13.5	27	1.509	1.10	0
	2	14.2	40	1.920	1.40	1
	3	15.4	38	1.490	1.04	0
	4	14.9	31	1.543	0.94	0
	5	15.1	37	1.789	1.07	1
	6	13.1	25	1.560	1.11	1
	7	14.5	41	1.458	1.34	0
	8	13.5	32	1.657	1.30	0
	9	13.2	26	1.652	1.13	1
	10	15.0	39	1.475	1.16	0
Autumn (October)	11	11.2	16	1.766	1.14	0
	12	13.2	26	1.547	1.13	0
	13	10.7	15	1.631	1.18	0
	14	14.1	38	1.639	1.36	1
	15	13.0	24	1.735	1.09	0
	16	10.6	14	1.542	1.14	0
	17	10.7	16	1.584	1.29	1
	18	14.4	28	1.651	0.93	0
	19	10.7	17	1.479	1.39	1
	20	15.0	37	1.437	1.10	0

Stomach contents analysis

Analysis of stomach contents showed that *C. gobio* from Dimon Lake fed chiefly on Chironomidae larvae in both summer (96.18%) and autumn (98.30%). Other taxa were present in extremely low proportion (Chironomidae pupae: 3.18% in summer and 0.79% in autumn; Trichoptera: 0.64% in summer and 0.39% in autumn; Trichoptera Limnephilidae: 0% in summer and 0.52% in autumn). Stomach content analysis of the Degano Creek fish revealed Trichoptera (42.63% in summer; 34.03% in autumn), Ephemeroptera (7.5% in summer; 5.8% in autumn) and Plecoptera (4.1% in summer and 2.5% in autumn).

Histological analysis

Histology of the liver samples of *C. gobio* from Dimon Lake showed a range of cytoplasm vacuolization of hepatocytes from mild (score 1) to severe (score 3) in 33 specimens, with multifocal to diffuse localization (Fig. 47). Only two specimens showed no hepatic lesions (score 0). Table 23 presents the scores for each subject examined. Sudan III resulted positive in all the samples with a high vacuolization score (2-3) (Fig. 48). PAS stain positivity was inversely proportional to histological score. Fish with grade 3 showed only rare residual glycogen deposits in hepatocytes (Fig. 48).

Finally, individuals used as control and sampled from Degano Creek (Table 24) showed no hepatocyte vacuolization (score 0; Fig. 47a) in both seasons, except few cases with score 1.

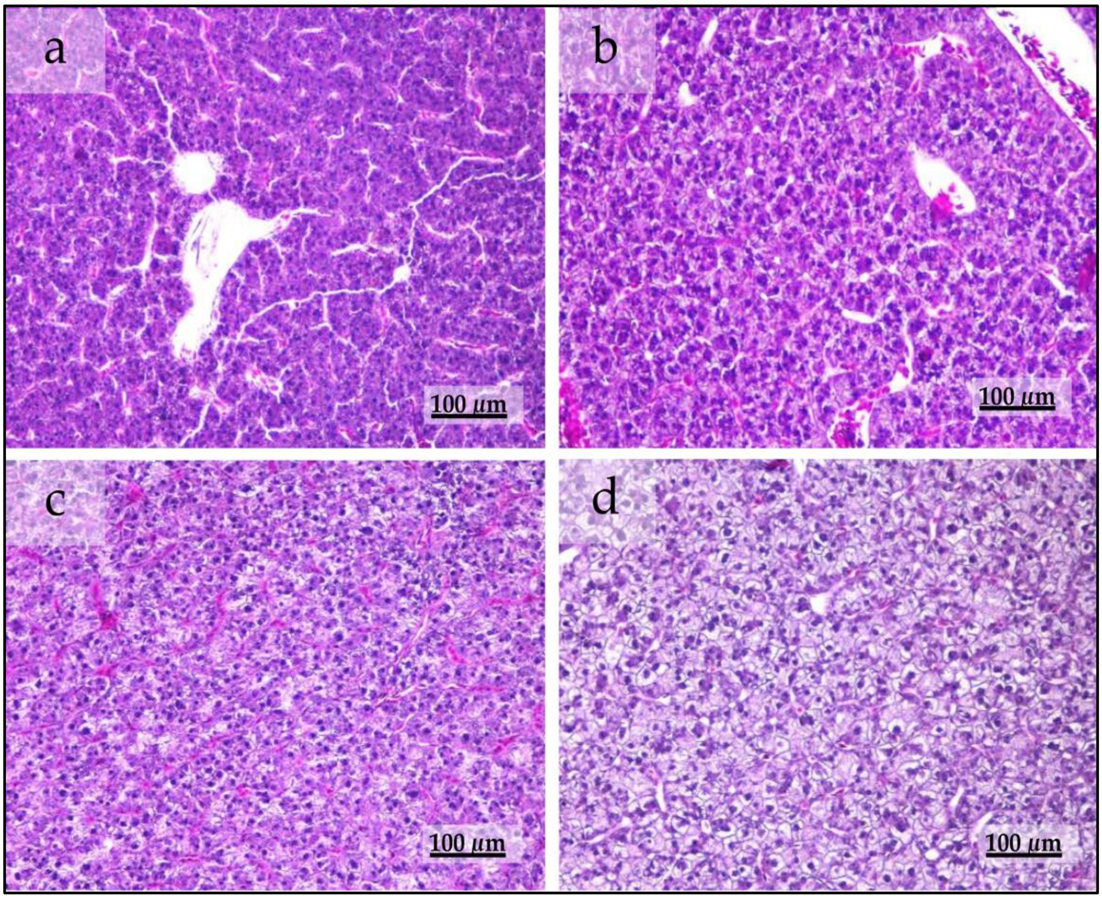

Figure 47. Liver sections of *Cottus gobio* (HE): (a) no vacuolisation observed in hepatocytes (control); (b) mild vacuolisation; (c) moderate vacuolisation; (d) severe, diffuse vacuolisation.

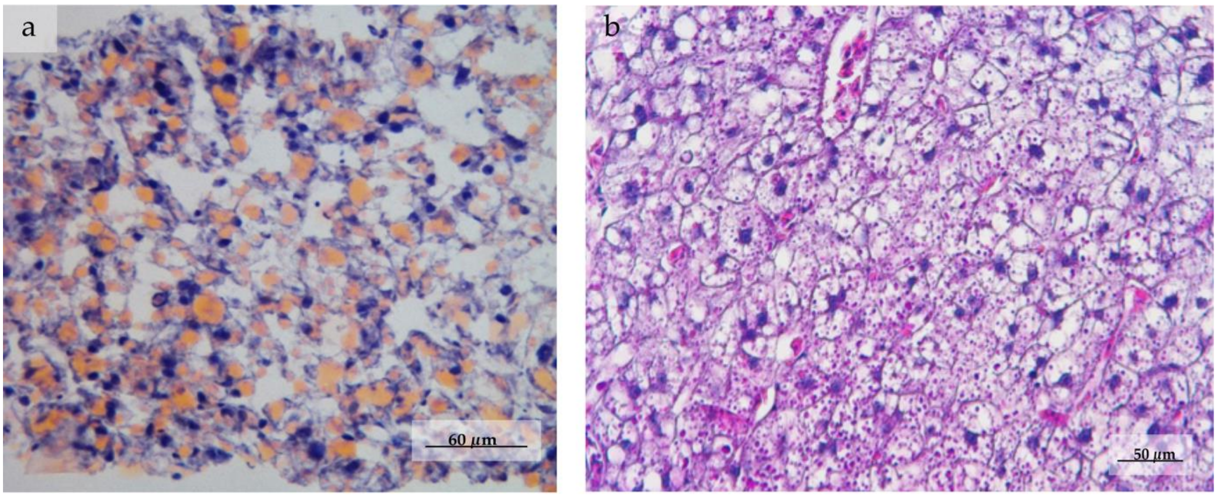

Figure 48. Liver sections of *Cottus gobio* from Dimon Lake. (a) Sudan III stain; orange lipidic deposits in hepatocyte cytoplasm of a subject with a high vacuolization score (3); (b) PAS stain: red-purple residual glycogen deposits in hepatocyte cytoplasm of bullhead classified as grade 3.

7.2.4 Discussion

Our study started with the aim to explore the fish community of the lake. After discovering bullhead specimens with pronounced steatosis, we decided to investigate this abnormal condition and pose a hypothesis for its cause. Stomach contents analysis was performed to understand nutritional requirements, trophic and energy dynamics, food webs, food chains, material and energy transfers between and within ecosystems (Buckland et al., 2017). This is an important part of studying feeding habits and, in general terms, a necessary step in research into the more complex questions of freshwater fish ecology. We analysed the stomach contents of *C. gobio* sampled during two seasons both in Dimon Lake and Degano Creek to determine whether there were differences in diet across the two seasons (Buckland et al., 2017). Stomach content analysis showed that *C. gobio* from Dimon Lake feed mostly on Chironomidae larvae (see section). This could be justified by the relative, great abundance, and almost exclusively presence of Chironomidae larvae in the lake (and it could also be explained by the feed ecology of this species, since this taxon is most easily to prey than other aquatic macroinvertebrates. In fact, *C. gobio* is a stationary benthic feeder which shows a well-developed homing instinct (Copp and Warrington, 1994) Indeed, Hirudinea and Oligochaeta taxa are also present in this lake (see section) but more endobenthic than Chironomidae, thus more difficult to hunt, justifying the bullhead preference for Chironomidae. Generally, *C. gobio* prey on a wide range of insect larvae as Plecoptera, Trichoptera, Ephemeroptera and Diptera (Copp et al., 2005b; Vlach et al., 2013) as recorded in Degano Creek. Nevertheless, bullhead was found to be predominantly food generalists, with ability to shift their feeding strategy towards specialization when exposed to environmental disturbances (Števove et al., 2019). Comparison of the histological evaluations on fish from the two different sites (lake vs. stream) showed that only *C. gobio* from Dimon Lake was affected by medium-severe (score 2-3) liver alterations. Steatosis occurs when hepatic infiltration by lipids exceeds the storage capacity typical of the species considered (Caballero et al., 2004). Lipid accumulation in the hepatocytes in this species or in fish living in other Alpine lakes has never been reported in the literature to date. Our study is the first report of this phenomenon. Two hypotheses for the causes of these histological alterations can be offered. The first posits for adaptation of the fish to the winter season, which in this type of environment lasts for 6 months of the year. Our results clearly reveal that HSI values were significantly lower in Degano Creek compared to Dimon Lake in both seasons. Furthermore, a slight increase in hepatosomatic index was recorded in autumn compared to summer in Dimon Lake. In this situation, it can be assumed that the bullhead from Dimon Lake accumulates lipids in the liver and then use them as fuel for energy in the adverse season (winter). Adaptive processes are often based on biochemical and physiological adaptations. Lipids play a key role in biochemical adaptation in animals, especially in aquatic organisms, and they are of pivotal importance for long-term energy storage. Adaptations provide an effective response to stressors (e.g., changes in water temperature or oxygen). In some cases, fish may be able to adapt by changing their behaviour, but more often it is through physiological adaptations, especially at the cell membrane level. Temperature, for instance, can be considered a stressor that acts to establish a new balance between the environment and the biochemical properties of cell membranes. Fish respond to environmental temperatures by activating a suite of compensatory mechanisms operating mainly at the cellular and subcellular levels of organization (Hochachka and Somero, 1984). For example, carps progressively cooled from 30 °C to 2 °C, 17 °C, and 10 °C showed substantial changes in fatty acid composition of the endoplasmic reticulum phospholipids, with increasing proportions of unsaturated fatty acids and changes in the activity of other biosynthetic enzymes (Cossins, 1994; Trueman et al., 2000). Animals develop a complicated metabolic system to adapt to changes in nutritional states. In nature, when food is plentiful, animals eat and store the excess energy as lipids. This a survival strategy for animals when food is not readily available (Marshall, 2010). Lipids are the most important biochemical compounds of fish (Akpınar, 1986). Fish store the lipids in muscles and liver (Kandemir and Polat, 2007) in contrast to mammals, which store lipids in adipose tissue. Most of these lipids are transferred to different parts of the body for various physiological actions (Yılmaz, 1995). In some fish species, extensive lipid infiltration into the liver is not considered pathological, since the liver works as a major lipid storage organ, especially in autumn (Roberts, 1989; Spisni et al., 1998). But in our study, we found no significant statistical difference in liver alterations between summer and autumn seasons. Kandemir and Polat (2007) investigated the seasonal and monthly variations in the amount of total lipid and fatty acids in the muscle and liver of reared rainbow trout (*Oncorhynchus mykiss*). Lipid levels were higher in summer, autumn, and winter than in spring. Furthermore, the amount of total lipid and fatty acid was higher in the liver than in muscle tissue. Active fish store their lipids in muscle tissues, but fish living at the bottom store their lipids in the liver (Castell, 1972) as seen in the *Cottus gobio* in our study. These considerations seem to corroborate our hypothesis. However, values of K did not show significant difference between fish from Dimon Lake and Degano Creek, indicating a robustness and a good state of nutrition of both populations (Murphy and Willis, 1996). In this case, the use of Fulton's condition factor, did not allow us to predict the lipid accumulation, as also previously suggested (Hards et al., 2019).

The second hypothesis rests on the diet of bullhead, and consequently the lipid content of chironomids, which are the only trophic resource available for this species. Bogut et al. (2007) showed how *Chironomus plumosus* larvae represent a potential suitable natural component of farm fish, with a crude fat content of 1.3% and 9.7% in fresh larvae and dry matter, respectively. Timon-David (1930) reported a similar percentage of lipids (8.3%) for adults of *Chironomus* spp. and larva. Lipids are high-energy nutrients and typically make up about 7-15% of fish diet (Craig and Helfrich, 2002). Nevertheless, an adult diet of Chironomid larvae may induce liver steatosis in reared male *Nothobranchius furzeri* (Di Cicco et al., 2011). For this reason, we cannot exclude that the presence of only chironomids as feed could be the cause of the steatosis observed in the bullhead samples.

Morphological modifications, such as hepatocyte vacuolization may be also interpreted as a response to pathological process caused by infections or parasites (Kohler et al., 1992), but we did not find bacteria or parasites in the fish during a survey performed in the same fish (see section 7.1).

Further investigations are needed to explore this phenomenon, for example by analyzing the composition of the total body fat in chironomids present in the lake, by determining the lipid profile in the livers of bullhead specimens and monitoring other populations in watercourses of North Italy.

7.3 Oxidative stress ecology in brook trout

7.3.1 Preface

As sentinel organisms, fish are used to detect environmental contamination (Squadrone et al., 2013, 2014, 2016) and provide a useful model for assessing the impact of pollutants on biological functions such as detoxification (Elia et al., 2010). Assessment of contaminants in aquatic organisms can estimate and quantify the bioavailable fraction which might have the potential to induce an effect. However, the determination of body concentrations alone does not provide valuable data about the effects. Therefore, quantification of some biological responses is required in order to evaluate the health state of contaminant-exposed organisms (Elia et al., 2010). Metals are well-known pro-oxidants that exert oxidative stress via reactive oxygen species (ROS) production, leading to critical changes of cellular biotransformation/detoxification pathways (Lushchak, 2016). Biomarkers level can be also influenced by abiotic factors such as pH, dissolved oxygen content and water temperature (Sroda and Cossu-Leguille, 2011). This latter is a major factor affecting physiological processes in fish and can induce the production of ROS (Lushchak, 2011). Oxidative stress results from an imbalance between pro-oxidants such as ROS, and protective antioxidant system. Mechanisms that involve antioxidant enzymes activity such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), selenium-dependent glutathione peroxidase (Se-GPx, EC 1.11.1.9), glutathione reductase (GR, EC 1.6.4.2), glutathione S-transferases (GST, EC 2.5.1.18) and of the rates of certain metal trapping molecules like reduced glutathione and metallothioneins (MTs), represent important protective metabolic pathways which are used as biomarkers related to pollutant-induced oxidative stress. The selected oxidative stress biomarkers have already proven to be useful to assess the impact of a range of metals in aquatic organisms (Al Kaddissi et al., 2014; Cozzari et al., 2015; Elia et al., 2006, 2007a, 2007b, 2010). However, levels of contaminants and of antioxidant enzymes activity in aquatic organisms may show seasonal changes related to biological and environmental pressures (Monserrat et al., 2007). This leads to a limitation when field studies are performed, because biochemical response might be linked either to fish physiology or exposure to contaminants.

Thus, the present study aims to investigate: a) trace elements accumulation in muscle, since this tissue is considered to represent the stable pool of trace elements for fish (Barwick and Maher, 2003); b) biomarkers of oxidative stress in muscle, liver, kidney, gills and spleen for both sexes of *S. fontinalis* captured in summer and autumn 2017 in Balma Lake.

7.3.2 Materials and Methods

Fish sampling campaigns

Both fish sampling campaigns were carried out in 2017 during summer (August) and autumn (October). The choice of this period was prescribed by the necessity to reach on foot the site during the ice-free period. The permission for sampling was released by the competent authority (Città Metropolitana di Torino; authorization n. 176-19040/2017). Fish were captured using 4 multimesh gillnets (36 x 1.8 m) divided into 6 panels with different mesh sizes reaching from 10 to 38 mm able to capture indiscriminately all size classes with the exception of the offspring. Gillnets were randomly placed in the lake for three hours and then recovered. For each sampling period, 20 individuals were sacrificed by deep anesthesia with a lethal concentration (200 mg kg^{-1}) of tricaine methanesulfonate (MS-222) dissolved in water. Fish were necropsied, sexed, weighed, and measured for their total length in the field. Immediately after, samples of gill, liver, spleen, kidney and muscle of each specimen were collected and stored in dry ice and transported to the laboratory.

Stomach contents analysis was also performed to have information about fish diet and to point out any variation during seasons. Stomachs were preserved in 70% alcohol, and the contents were identified by a stereomicroscope (Zeiss Stemis V8). The ingested prey was identified at order or family level, since a closer identification was not possible because of the digestion status of the organisms. In order to describe the diet, prey frequency of occurrence (Fi) (Tiberti et al., 2016a) was calculated for both seasons.

Physicochemical parameters and nutrients of lake water

During both sampling periods the main physicochemical parameters and nutrients of lake water were monitored in 5 sites of the littoral zone and in other 3 of the deep zone. Methods and results were already reported in section 5.

Trace elements in fish muscle

Trace elements in fish muscle for both males and females were analyzed by inductively coupled plasma - mass spectrometry (ICP-MS Xseries II, Thermo Scientific, Bremen, Germany). Determination of Al, As, Cd, Cr, Cu, Fe, Ni, Pb, Se and Zn was performed following protocols previously reported by Squadrone et al. (2016b). Hg concentration was also determined by direct mercury analyzer (DMA-80 Analyzer from Milestone, Shelton, CT, USA). Analytical performance was verified by processing certified reference materials (Oyster Tissue - SRM 1566b from the National Institute of Standard and Technology), along with blank reagents in each analytical session.

The analytical method was validated according to ISO/IEC 17025 (general requirements for the competence of testing and calibration laboratories).

Biochemical analyses

Forty specimens of *S. fontinalis,* 26 females (12 in August and 14 in October) and 14 males (8 in summer and 6 in autumn) were examined individually for oxidative stress biomarkers.

Liver, gills, kidney, spleen and muscle were analyzed for SOD, CAT, GPx, Se-GPx, GR, and GST activity, and MTs levels only for muscle. For enzymatic analysis samples were homogenized with an UltraTurrax homogenizer in 100 mM potassium-phosphate buffer, pH 7.5, added with 2.5% sodium chloride (NaCl), 0.008 TIU ml^{-1} aprotinin and 0.1 mg ml^{-1} bacitracin. Homogenates were centrifuged at 50,000 x g for 30 minutes at 4 °C. Cytosolic fractions were used to determine the activity of antioxidant biomarkers. Biochemical analyzes were performed according to the methods reported in Elia et al. (2017). Briefly, SOD activity was assessed at 550 nm in 50mM Na_2CO_3 buffer, pH 10, 0.1 mM EDTA, 500mM cytochrome C and 1mM hypoxanthine and xanthine oxidase. Cytochrome C reduction by the xanthine/hypoxanthine complex was evaluated by comparison with a standard SOD unit curve. CAT activity was measured at 240 nm following the decrease in absorbance following the consumption of H_2O_2. Assay was carried out in NaH_2PO_4 buffer + Na_2HPO_4 100mM pH 7 and H_2O_2 24mM. Total glutathione peroxidase (GPx) and selenium-dependent glutathione peroxidase (Se-GPx) activity were determined at 340 nm in NaH_2PO_4 + Na_2HPO_4 100mM buffer, pH 7.5, 1 mM EDTA, 0.12 mM NADPH (b-Nicotinamide adenine dinucleotide), 2 mM GSH, 1 U of GR (glutathione reductase), 1mM NaN_3 and H_2O_2 0.6 mM for Se-GPx or 1 mM DTT and 0.8 mM cumene hydroperoxides for GPx. Activity of GR was measured at 340 nm in NaH_2PO_4 + Na_2HPO_4 100 mM buffer, pH 7, 1 mM GSSG (oxidized glutathione) and 0.06 mM NADPH. GST activity was performed at 340 nm using CDNB (1-chloro-2,4-dinitrobenzene) as a substrate. The assay was measured 100 mM NaH_2PO_4 + Na_2HPO_4 100 mM buffer, pH 6.5, 2 mM GSH and 2 mM CDNB. The concentration of cytosolic proteins was determined according to the method of Lowry et al. (1951) and was used to normalize the activity of the evaluated biomarkers.

Metallothionein (MT) levels were measured in muscle of both sexes. Samples were homogenized (1:4) in a buffer of 0.02 M TRIS/HCl, 0.5 M sucrose, 0.1 mg ml^{-1} bacitracin, 0.008 tiu ml^{-1} aprotinin, 87 µg ml^{-1} phenylmethylsulfonyl fluoride (PMSF) and 0.1 µl ml^{-1} α-mercaptoethanol. The homogenates were centrifuged at 14,500 x g at 4 °C to obtain the cytosolic fraction. The supernatants were firstly purified using chloroform/ethanol solution and then HCl/ethanol to obtain the partially purified MT fraction. The pellets were washed with Ethanol/Chloroform/TRIS/HCl (87/1/12) solution and suspended in 0.25 M NaCl. A destabilizing solution (HCl 1N + EDTA 4 mM) and

Ellman's reagent (DTNB: 5,5 dithiobis-2- nitrobenzoic acid) were added to each sample. Sulphydril residue contents (-SH) were spectrophotometrically quantified. The absorbance was measured at 412 nm and compared with that obtained from a standard curve with reduced glutathione (1 mg ml^{-1} GSH). All biochemical analyses were performed in triplicate for each sample with a Varian spectrophotometer (Cary 50 Thermostat Cell Holder) at a constant temperature of 25 °C.

Statistical analyses

Spearman's rank correlation coefficient (ρS) was used to test for correlations between trace elements concentration in fish muscle, water physicochemical parameters, nutrients and oxidative stress biomarkers determined in different tissues and seasons. Differences in concentration of physicochemical parameters, nutrients and trace elements between seasons were checked using the Wilcoxon test. Data from biochemical analysis are reported as mean and standard deviation (SD), and to check significant differences among both sexes and months, one-way ANOVA followed by Tukey's Multiple Comparison Test were used. Homogeneity of variance was checked by Bartlett's test. The criterion for significance was set at $p < 0.05$. Principal component analysis (PCA) was performed to check for trends in trace elements, biomarkers and physicochemical values between two seasons (summer and autumn). Statistical analyses were performed using open source data analysis software RStudio® version 1.1.463 (RStudio, Inc.).

7.3.3 Results

Captured fish

The average total length and weight of males and females captured in Balma Lake during summer and autumn 2017 are reported in Table 25. Stomach contents analysis revealed the almost exclusively presence of terrestrial insect preys both in summer (94.19%) and autumn (81.04%). Preys belonged to the orders of Hymenoptera (77.74% and 66.39% in summer and autumn respectively) and Coleoptera (16.45% and 14.65% in summer and autumn respectively). Other taxa were present in extremely low proportions (Diptera Chironomidae larvae: 5.18% in summer and 2.79% in autumn).

Table 25. Biometric values of females and males of sampled brook trout from Balma Lake in summer and autumn 2017.

	Summer (August)		**Autumn (October)**	
Sex	Males	Females	Males	Females
Number of individuals	8	12	6	14
Weight - W mean ± SD (g)	69.9 ± 30.34	71.20 ± 28.34	88.47 ± 56.23	90.05 ± 60.03
W min. (g)	15.00	15.36	10.23	11.00
W max. (g)	146.00	150.00	187.00	193.00
Total Length - TL mean ± SD (cm)	17.77 ± 3.90	18.56 ± 4.20	19.23 ± 4.66	19.35 ± 5.72
TL min. (cm)	11.50	11.00	7.50	8.00
TL max. (cm)	23.00	24.00	25.50	26.00

Trace elements in fish muscle

Figure 49 represents the mean concentration and the relative standard deviation of each trace element detected in muscle of brook trout in summer and autumn. The mean concentration of trace elements ranked as follow: Zn (16.25) > Fe (8.78) > Al (1.49) > Se (0.67) > Cu (0.60) > Cr (0.14) > As (0.06) > Ni (0.06) > Pb (0.05) > Hg (0.01) mg Kg^{-1}. In autumn the mean concentration of trace elements ranked as follow: Zn (16.13) > Fe (10.63) > Al (2.03) > Se (0.49) > Cu (0.36) > Cr (0.16) > As (0.10) > Pb (0.10) > Ni (0.06) > Hg (0.02) mg Kg^{-1}. Cd was < LOQ (0.02 mg Kg^{-1}) in both seasons. The Wilcoxon test did not reveal significant differences in trace elements concentration between seasons (*p* > 0.05 for all elements).

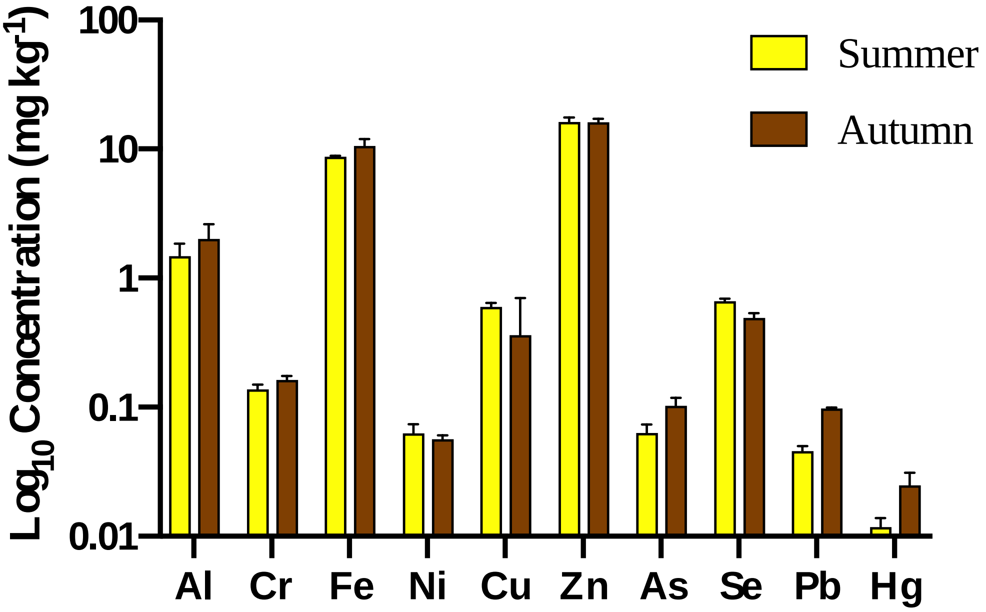

Figure 49. Bars of trace elements (Log concentration; mg kg^{-1}) detected in muscle of brook trout both in summer (August) and autumn (October) 2017.

Biochemical analyses

In liver, activity of SOD, CAT and GR was significantly lower (Tukey's test; $p < 0.05$) in autumn (up to 70%) compared to summer in both sexes (Figs. 50-52). In gills, GPx activity was significantly higher in autumn (90%) mainly in females (Tukey's test; $p < 0.05$) (Fig. 53), while Se-GPx and GST activity were significantly higher (up to 2-fold) (Tukey's test; $p < 0.05$) for both sexes in the same season (Figs. 54-55). In kidney, only Se-GPx activity was significantly different through the seasons (Tukey's test; $p < 0.05$), being the highest (up to 40%) in autumn (Fig. 54). In spleen, significantly lower activity (Tukey's test; $p < 0.05$) of Se-GPx (70%) was recorded in females (Fig. 54). Significantly higher activity of GR and GST (up to 70%) was measured in males sampled in autumn compared to summer (Tukey's test; $p < 0.05$) (Fig. 52, 55).

In muscle, SOD activity was significantly lower (Tukey's test; $p < 0.05$) (up to 50%) in autumn for females, whereas CAT activity showed an opposite trend through the seasons being significantly higher in autumn (up to one-fold) (Tukey's test; $p < 0.05$) (Figs. 51). For the same tissue MTs level was higher in autumn (up to onefold) compared to summer (Tukey's test; $p < 0.05$) (Fig. 56).

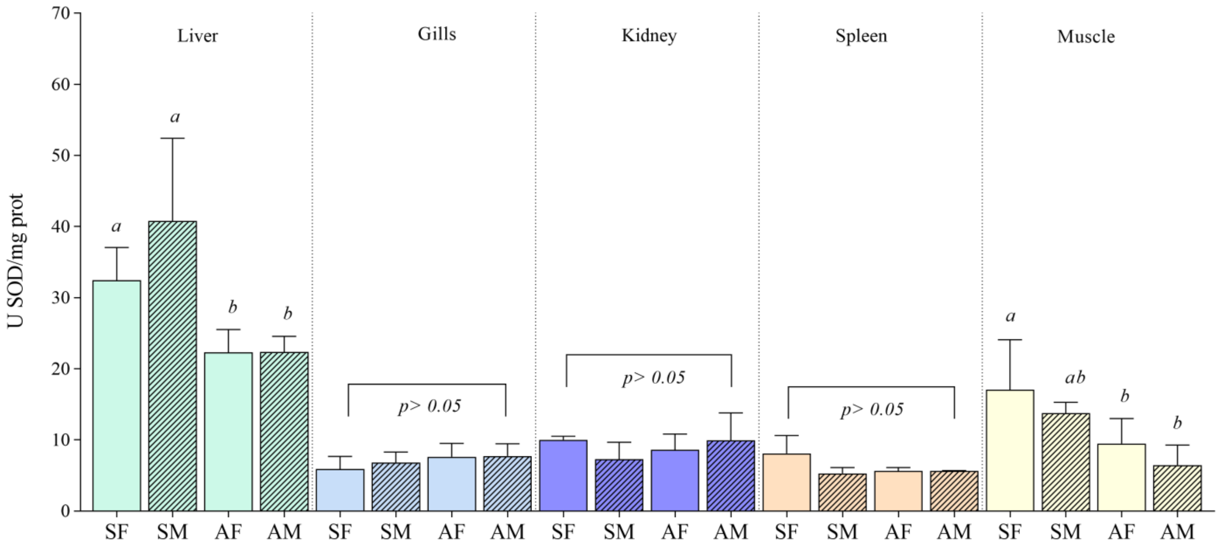

Figure 50. Superoxide dismutase (SOD) activity in liver, gills, kidney, spleen and muscle of female and male brook trout. Data are reported as mean ± standard deviation. Summer (S); Autumn (A); females (F); males (M). Different lowercase letters indicate statistically significant differences (Tukey's test, $p < 0.05$) between sexes and sampling months for each tissue.

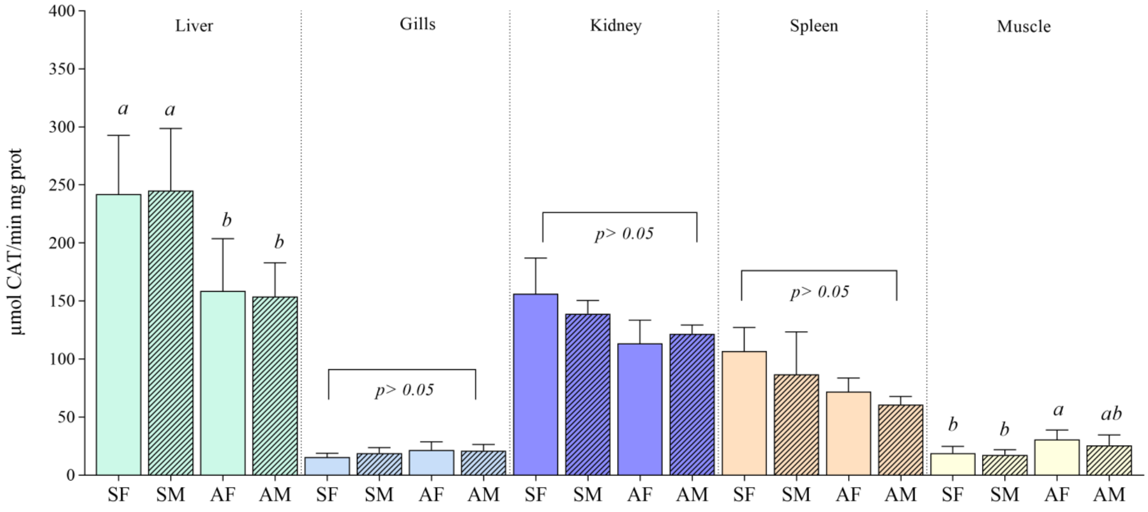

Figure 51. Catalase (CAT) activity in liver, gills, kidney, spleen and muscle of female and male brook trout. Data are reported as mean ± standard deviation. Summer (S); Autumn (A); females (F); males (M). Different lowercase letters indicate statistically significant differences (Tukey's test, $p < 0.05$) between sexes and sampling months for each tissue.

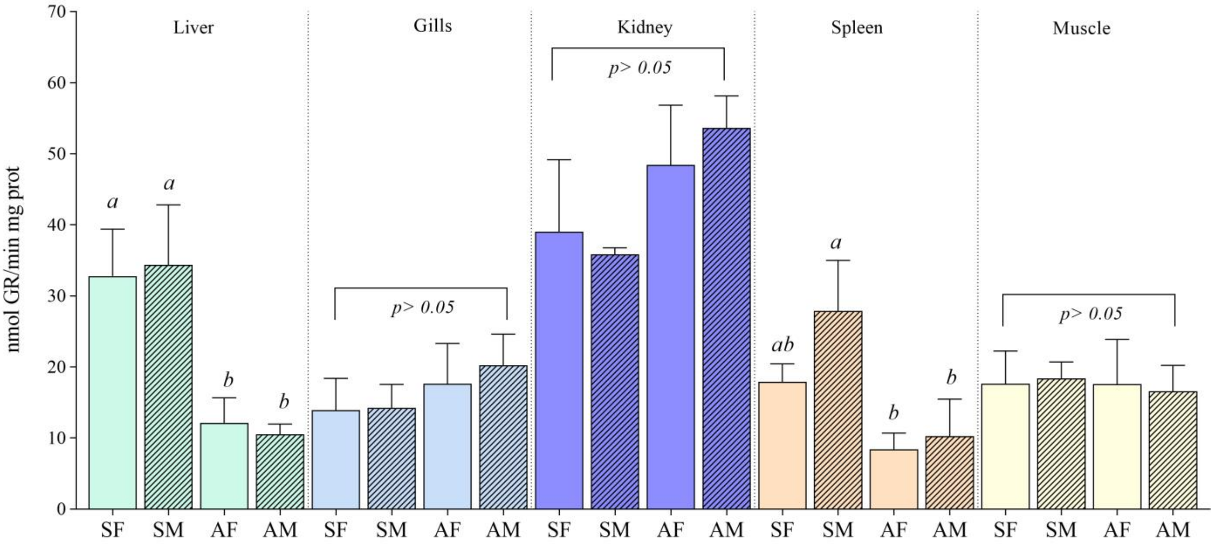

Figure 52. Glutathione reductase (GR) activity in liver, gills, kidney, spleen and muscle of female and male brook trout. Data are reported as mean ± standard deviation. Summer (S); Autumn (A); females (F); males (M). Different lowercase letters indicate statistically significant differences (Tukey's test, $p < 0.05$) between sexes and sampling months for each tissue.

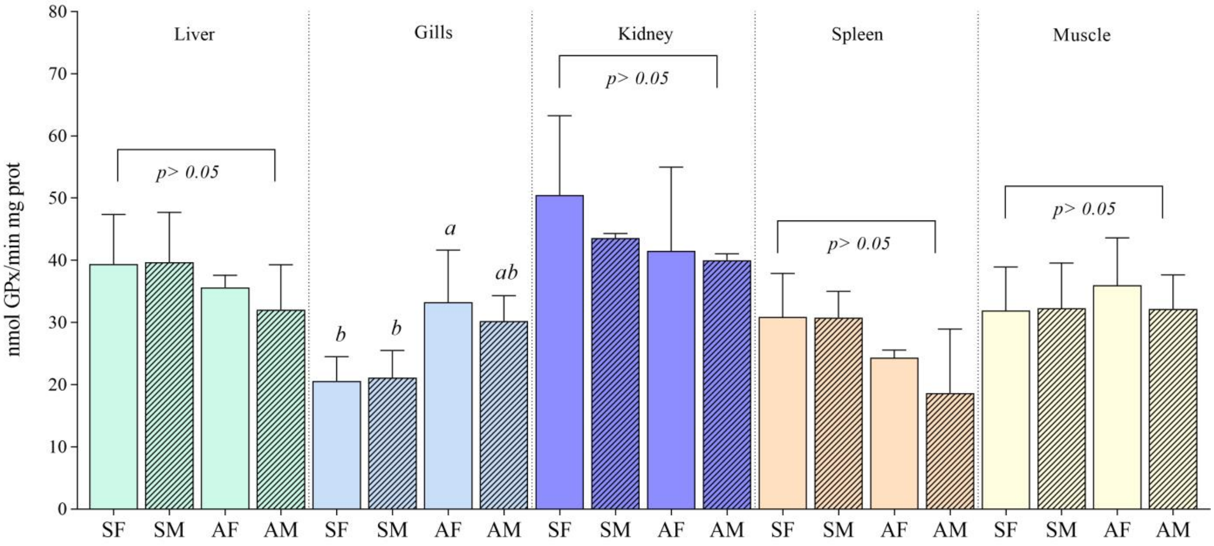

Figure 53. Total glutathione peroxidase (GPx) activity in liver, gills, kidney, spleen and muscle of female and male brook trout. Data are reported as mean ± standard deviation. Summer (S); Autumn (A); females (F); males (M). Different lowercase letters indicate statistically significant differences (Tukey's test, $p < 0.05$) between sexes and sampling months for each tissue.

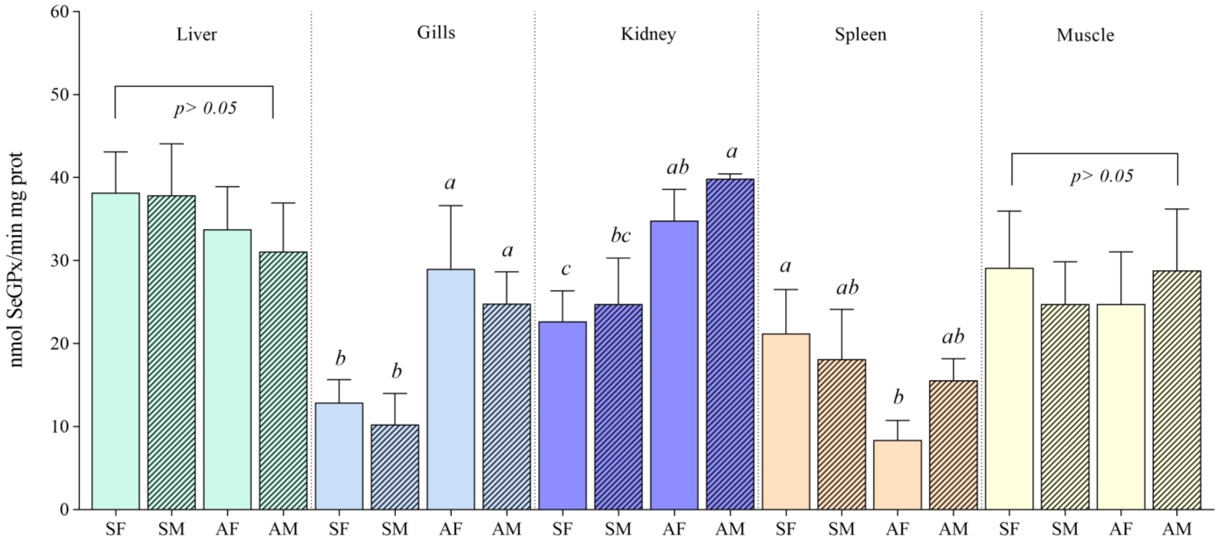

Figure 54. Selenium-dependent glutathione peroxidase (Se-GPx) activity in liver, gills, kidney, spleen and muscle of female and male brook trout. Data are reported as mean ± standard deviation. Summer (S); Autumn (A); females (F); males (M). Different lowercase letters indicate statistically significant differences (Tukey's test, $p < 0.05$) between sexes and sampling months for each tissue.

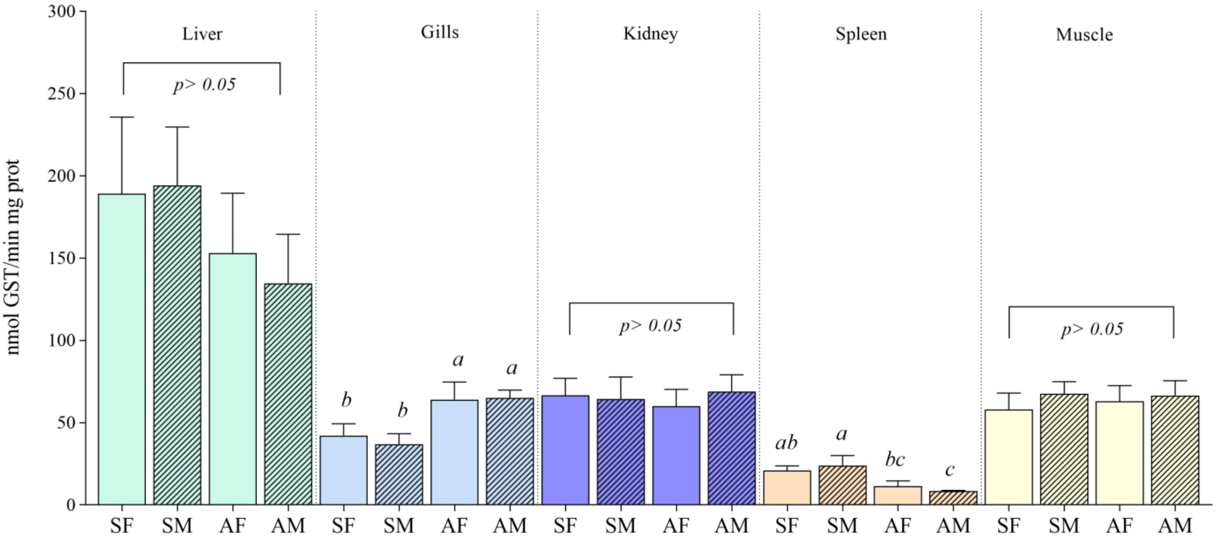

Figure 55. Glutathione S-transferase (GST) activity in liver, gills, kidney, spleen and muscle of female and male brook trout. Data are reported as mean ± standard deviation. Summer (S); Autumn (A); females (F); males (M). Different lowercase letters indicate statistically significant differences (Tukey's test, $p < 0.05$) between sexes and sampling months for each tissue.

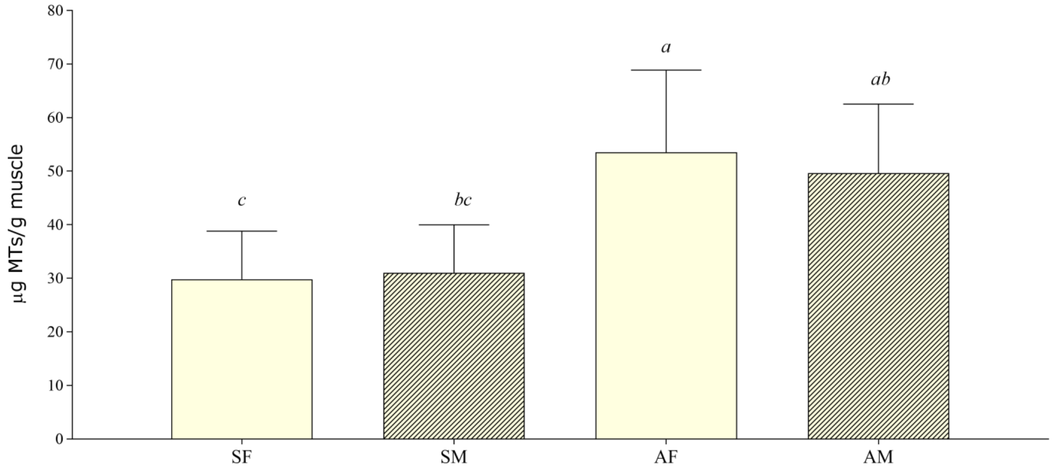

Figure 56. Metallothionein (MTs) concentration in muscle of female and male brook trout. Data are reported as mean ± standard deviation. Summer (S); Autumn (A); females (F); males (M). Different lowercase letters indicate statistically significant differences (Tukey's test, $p < 0.05$) between sexes and sampling months for each tissue.

Spearman correlation matrix

Spearman correlation matrix revealed several correlations between environmental parameters (trace elements, physicochemical parameters, nutrients) and oxidative stress biomarkers in muscle, gills, liver, spleen and kidney of *S. fontinalis* for both seasons. Only the most informative outcomes were presented and discussed enabling the reading of biological response of *S. fontinalis* to environmental parameters. In particular, significant positive correlation was found between MTs and Hg (ρS 0.787), MTs and Pb (ρS 0.787), MTs and Cu (ρS 0.683), MTs and pH (ρS 0.650), CAT and pH (ρS 0.737) in muscle of female captured in autumn; SOD and Cr (ρS 0.837) in liver of female captured in autumn; SOD and NO_3^- (ρS 0.750), Se-GPx and NO_3^- (ρS 0.750) in muscle and kidney respectively of female captured in summer.

Principal Component Analysis (PCA)

Regarding PCA (Fig. 57), the first two principal components (Dim1; Dim2) accounted for meaningful amounts of the total variance (58.2%), while the other components accounted for a relatively smaller fraction of the variance. In particular, Dim1 showed 35.2% of the total variance and was positively correlated with the variables Ni, Se, temperature, oxygen, conductivity and NO_3^- and negatively correlated with Al, Cr, Fe, As, Pb, Hg, and pH. Dim2 showed 23% of the total variance and was positively correlated with the variables GST, CAT, GPx, Se-GPx, SOD, GR. The biplot of loadings (variables) and score (observations) showed which organs (liver, gills, kidney, spleen and muscle) of brook trout is closest to them, and which variables (trace elements, biomarkers and physicochemical parameters) contributed to this grouping in the coordinate of Dim1 and Dim2. The separation between the organs from both seasons (summer on the right, autumn on the left) suggested a difference in biomarkers values. In particular samples of spleen, gill, muscle, kidney and liver from autumn are placed on the left in correspondence of an increasing values of Al, Cr, Fe, As, Pb, Hg, and pH. On the other hand, samples of spleen, gill, muscle, kidney from summer are placed on the right in correspondence of increasing values of NH_4^+, NO_3^-, conductivity, oxygen, temperature, Zn, Cu, Ni and Se. Remarkably, samples of liver in summer are well separated from other organs, following the trend in biomarkers values.

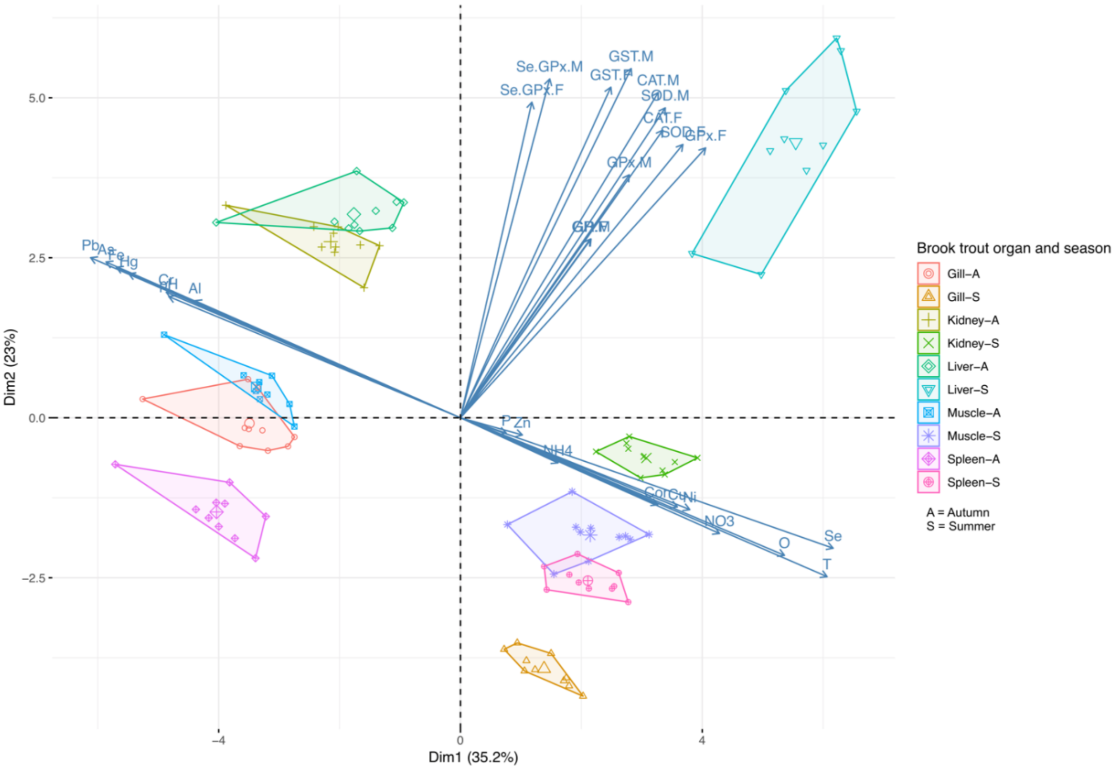

Figure 57. Biplot of score and loadings from principal component analysis. The scores of each organ (gill, kidney, liver, muscle and spleen) are denoted by a color and a symbol (largest symbol = average value). Confidence ellipses plot convex hull values of each organ.

7.3.4 Discussion

In the present study, a positive correlation between environmental parameters and biological responses occurred in *S. fontinalis* from Balma Lake. Antioxidant response of fish was tissue-specific, so that the aerobic tissues such as kidney, gills, spleen, and, mainly, liver have high potential for ROS production which is offset by protective mechanisms. Differently muscle, which has low content of mitochondria and low-intensive oxidative metabolism, did not show great response to oxidative/reductive conditions. Moreover, level of several biomarkers in different tissues have been related to the seasonal changes of some trace elements concentration.

The few studies on trace elements accumulation in biota from high-altitude lakes in general, and in Alpine lakes in particular, have mostly focused on fish. Köck et al. (1996) studied the concentrations values of Cd, Pb, Zn, and Cu in muscle of arctic char (*Salvelinus alpinus*) from five oligotrophic Alpine lakes located in northern Tyrol (Austria). Yang et al. (2007) studied the accumulation of trace elements in muscle of fish of the genus *Gymoncypris* (Cyprinidae) from high mountain lakes in the Tibetan Plateau. Results showed that Ni ranged from 0.094 to 0.12 mg kg^{-1}, Cu from 1.1 to 2.0 mg kg^{-1}, Zn from 4.4. to 6.9 mg kg^{-1}, As from 0.24 to 0.27 mg kg^{-1}, Se from 0.36 to 1.0 mg kg^{-1}, Cd from 0.024 to 0.025 mg kg^{-1}, Pb from 0.047 to 0.079 mg kg^{-1}. Rognerud et al. (2002) also found that concentrations of Hg in fish muscle from high-mountain lakes in Europe ranged from 0.021 to 0.179 mg kg^{-1}. Thus, results presented by other studies demonstrate that high-mountain lakes function as a regional contaminant convergence zone by medium and long-range atmospheric transport of contaminants. Since our results are in line with these authors, and no studies have been performed previously, we supposed that trace elements amount detected in brook trout from Balma Lake have an origin both from anthropogenic and pedogenic sources.

Metallothioneins have been widely considered as valuable biomarker reflecting the level of trace elements in aquatic environment, acting as metal-trapper against mainly Cd, Hg, Pb, Cu and Zn in fish (Bourdineaud et al., 2006; Morcillo et al., 2016). The higher levels of MTs measured in autumn were related to the increased trace elements concentration in this tissue, and a strong correlation with Cu, Hg and Pb values emerged in females of *S. fontinalis.* It may advice for an alarming scenario, as it would suggest an increased concentration of these elements in the environment. However, although chemical analyses have not been carried out on field water, the peculiar geomorphological characteristics of Balma Lake allow us to exclude an increase in such contaminants during autumn. It is well known that seasonal variations in fish metals concentrations can be related to several environmental factors such as food availability, temperature and biological cycle (Hermesz et al., 2001; Amiard et al., 2006; Dragun et al., 2009). Furthermore, also fish size may play a key role in metal uptake (Wright and Mason, 1999). In the present study, an increased weight and length was measured in *S. fontinalis* through both sampling seasons, and the frequency of occurrence of terrestrial invertebrates recorded in the diet of brook trout during the ice-free season of Balma Lake was in accordance with other studies performed on this species in other Alpine lakes (Sotiropoulos et al., 2006; Tiberti et al., 2016a). It is thus feasible that fish growth due to higher intake of food in summer could have favored the uptake of heavy metals, such as Pb and Hg.

This hypothesis is corroborated by previous study on black scabbardfish *Aphanopus carbo* (Trichiuridae), in which an exponential increase of total Hg load was found in all fish tissues in a length-dependent manner (Bebianno et al., 2007). Furthermore, previous studies showed that also fluctuations toward high pH values can play a key role in modulating metals uptake, affecting their speciation and bioavailability (Playle, 1998). This outcome may explain the strong correlation between pH and the two hydroxyl scavengers MTs and CAT in female muscle in autumn. Trace elements also affected the activity of several enzymes in different tissues in fish from autumn. In particular, a strong correlation between Cr and SOD activity in liver of *S. fontinalis* emerged, as reported in previous study in rock fish *Sebastes schlegelii* (Kim and Kang, 2016). Furthermore, a recent study showed that Cr can induce conformational changes of CAT enzyme and decrease the activity depending on its valence states and concentration (Chen et al., 2018). These findings may explain the different CAT activity measured through the seasons in female brook trout muscle. In general, numerous elements have influenced the activity of this biomarker, and As and Fe were the most involved elements in modulating enzymes activity in the investigated tissues. Arsenic is a global contaminant deriving from natural or anthropogenic sources and arouses great concern for terrestrial and aquatic ecosystems (Elia et al., 2018). At high concentration, arsenic may induce oxidative stress by interacting with antioxidants resulting in the accumulation of free radicals in cells. In particular, arsenite species can interact with sulfhydryl groups of biomolecules such as enzymes or reduced glutathione (Elia et al., 2018). On the other hand, redox active metals such as iron generate ROS or are involved in the Fenton route, leading to lipid peroxidation (Mahboob, 2013). However, despite trace elements amount has increased in autumn, the concentration of the three most harmful Pb, Hg and Cd in fish muscle considered in Regulation 1881/2006 (European Commission, 2006) is far below the established limit threshold. This fact should be taken in account and may suggest an adaptive condition of *S. fontinalis* to seasonal changes rather than to oxidative stress.

PCA analysis showed how samples from liver in summer were strongly related to oxidative biomarkers level, since this tissue is the site of multiple oxidative reactions and maximal free radical generation (Gul et al., 2004; Avci et al., 2005).

For ectothermic organisms, temperature represents a crucial abiotic factor (Hassan et al., 2017). Daily temperature fluctuations and, mainly, seasonal variations have different influences on fish (Buckley et al., 2006; Place and Hofmann, 2004; Podrabsky and Somero, 2004). Wellness and growth are optimal within a well-defined temperature range depending on the species (Godowsky and Caddell, 1991). Temperatures deviating excessively from the optimum can exert harmful effects and, as well, induce mortality (Rijnsdorp et al., 2009). In the present study, the marked drop of water temperature in autumn due to seasonality was related to changes mainly in GPx’s activity in male kidney and in female spleen of *S. fontinalis*. Moreover, it is well known that higher temperature results in increased GPx activity in other fish as the Antarctic *Pagothenia borchgrevinki* (Almroth et al., 2015). The thermal changes have also been associated with the up regulation of NRF2 transcription factor, involved in expression of antioxidants via binding to the antioxidant responsive element (ARE) (Almroth et al., 2015). Therefore, the elevated GPx’s activity during summer may indicate a strengthening of this fundamental defense line against ROS. However, the unchanged SOD and CAT activity, as well as the lower trace elements tissue concentration, and the constant levels of the main physicochemical parameters of water, except for pH and temperature, allow to exclude an oxidative pressure scenario, rather suggesting an adaptive ability of *S. fontinalis* to higher temperature. On the other hand, the increased SOD activity in both sexes muscle is linked to an abiotic factor, such as NO_3^-, in summer. Furthermore, nutrients also influenced SOD concentration in female muscle and Se-GPx activity in female kidney in summer. In the aquatic environment, it is not unusual that fish species are simultaneously challenged by different abiotic factors. Conversely, at very high concentrations ammonia can induce a range of toxicological effects in fish, such as metabolisms alteration, lack in growth and mortality (Dosdat et al., 2003; Sinha et al., 2012, 2015). Ammonia exposure can also lead to oxidative stress in fish species (Sun et al., 2012; Sinha et al., 2014). However, previous study showed that conductivity plays a crucial role in maintaining the ammonia ionization equilibrium (NH_3 and the non-toxic form NH_4^+) in aquatic environment (Sinha et al., 2015). In the present study, the unchanged conductivity and nutrients as phosphorus through the seasons suggests that changes in antioxidant parameters may be related to food intake (which indirectly promotes the metals uptake) rather than to nutrients concentration. Undoubtedly, in their habitats, high-mountain lakes included, fish are often exposed to periods of food insufficiency in response to several factors such as temperature, conductivity, and biological cycle (Pérez-Jiménez et al., 2007; Furné et al., 2009). In particular, increasing food intake during summer may also explain the fluctuation of values in biomarkers of oxidative stress.

8. CONCLUSIONS

Alpine lakes are “natural laboratories” in which to investigate global changes in water quality and biodiversity (Rogora et al., 2008; Rossetti et al., 2008) and to assess the large-scale effects of anthropogenic activities (Battarbee et al., 2009). With this PhD thesis we focused on two Alpine lakes, one located in the Western (Balma Lake) and one in the Eastern Italian Alps (Dimon Lake). In the first part, we used new and advanced cartography technologies to accurately reconstruct the complete digital terrain model of both lakes, including the surrounding out of water terrain (with drones) and the submerged part (with an equipped boat), since no topographic and bathymetric data were previously available. Our results demonstrated the potential utility of unmanned aerial vehicles for future monitoring, management, and conservation of aquatic ecosystems.

The physicochemical features of the lakes were in line with previously published literature but differed from each other in geo-lithological context: sandstone and volcanic rock in Dimon Lake, ophiolite metamorphic bedrock in Balma Lake. These features made a difference in macrobenthic community assemblage between the two lakes, where Diptera Chironomidae and Oligochaeta generally predominated. For this PhD thesis, macrobenthic invertebrates were used as trace element tracers in Alpine lakes. The taxa from the two lakes located at opposite ends of the Alps were found to differ in trace element concentration, reflecting the different levels of pollution from the plain: much higher in Dimon Lake. This study is the first to report on environmental monitoring of Alpine lakes based on a survey of trace element accumulation in macrobenthic communities. Our protocol may help environmental agencies to assess environmental contamination in Alpine lakes in the future. In such environments, because fish are often non-native, it would be more accurate to use macrobenthic invertebrates to monitor environmental contaminants in mountain lakes.

Paleolimnological analysis of Balma Lake highlighted significant differences in subfossil chironomid communities before and after fish introduction, and between modern and subfossil communities, with a significant reduction in diversity. Fish play a pivotal role in shaping the community structure of most aquatic insects in lake environments, where their role may be more prominent than either lake origin or physiography (Binckley and Resetarits, 2005; Schilling et al., 2009). Chironomids are the available prey for introduced predators: studies elsewhere have shown predation by fish to be an important driver in shaping chironomid communities (Goyke and Hershey, 1992; Milardi et al., 2016). Paleolimnological analyses are an important tool for climate reconstruction and are developed from transfer functions based on the study of biological assemblages such as chironomids and diatoms (Fritz et al., 1991; Heiri et al., 2014; Raposeiro et al., 2017). These analyses assume, however, that the main driver that triggers the oscillations observed in their assemblages is climate fluctuations and does not account for the consequences of alien fish introduction largely carried out for recreational fishing, especially during the 20th century. Based on the observed consequences on biological assemblages, such species introductions can have significant effects on climate reconstructions and their interpretation (Raposeiro et al., 2017). Our findings on fish assemblages showed that the brook trout population of Balma Lake is well structured, with individuals in 0+ to 4+ age classes sampled. Since fish sampling was performed only with gillnets, no smaller fish (< 8 cm) were caught. This fact precluded evaluation of the fish pressure on zooplankton, the main prey of the youngest brook trout (Tiberti et al., 2016a). We found a high predation pressure on terrestrial insects (Hymenoptera), however. Areas surrounding the lake include protected habitats. For example, Alpine prairies are included in annex I of the Habitat Directive 92/43/EEC (Council of the European Commission, 2000) and host endangered species, which may be negatively impacted by the direct and indirect effects of fish predation. These observations ought to be considered by local government administrations when implementing conservation actions such as fish eradication. Since the heavy metals detected in fish muscle were in line with the limit established by the European Commission, parallel strategies similar to eradication can be implemented, for example, the use of angling sessions to support eradication campaigns. Because Alpine lakes play a pivotal role as indicators of global environmental change and “early warning systems”, it is of primary importance to improve our knowledge about the impact of fish introduction in such lotic environments and provide correct evaluations.

In Dimon Lake, brook trout is no longer present; instead, we recorded the bullhead and observed liver alterations in this species. The causes may might be related to adaptation to the high-altitude lake, unsuitable environment for this species, accumulating lipids in the liver to tackle the adverse season that typically lasts 6 months of the year. Further investigations are ongoing on other bullhead populations that inhabit alpine and subalpine freshwater watercourses of Friuli-Venezia Giulia to better explore this phenomenon.

Results from this PhD allowed to highlight the influence of human activities on two Alpine lakes, which clearly cannot be generalized to all lakes located in Alps, however.

By applying an integrated, multidisciplinary approach to implement the objectives of this PhD thesis, we obtained new and interesting data that could be useful for environmental agencies and local government administrators. The study data may be used to inform preparatory actions and to develop projects funded under the LIFE programme.

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APPENDIX I

Average density (ind m^{-2}) and functional feeding guilds (FFG) of taxa recorded in littoral sites (Lit_1-5) of Balma Lake.

		Balma Lake - Summer					**Balma Lake - Autumn**				
Taxon	**FFG***	**Lit_1**	**Lit_2**	**Lit_3**	**Lit_4**	**Lit_5**	**Lit_1**	**Lit_2**	**Lit_3**	**Lit_4**	**Lit_5**
BIVALVIA											
Pisidium Pfeiffer, 1821	FC	16.7	20.0	26.7	6.7	66.7	46.7	13.3	110.0	10.0	83.3
OLIGOCHAETA											
Naididae											
Naidinae											
Nais alpina Sperber, 1948	GC										
Nais bretscheri Michaelsen, 1899	GC										
Nais barbata Müller, 1774	GC										
Tubificinae											
Potamothrix Vejdovský & Mrázek, 1903	GC			13.3		40.0	23.3	16.7			220.0
Limnodrilus Claparède, 1862	GC										
Spirosperma ferox Eisen, 1879	GC	20.0	90.0	1066.7	123.3	3.3		213.3	373.3	76.7	390.0
Tubifex Lamarck, 1816	GC		23.3								
Tubifex tubifex Müller, 1774	GC			3.3	6.7	56.7					
Lumbriculidae											
Lumbriculus variegatus Müller, 1774	GC	60.0	130.0	33.3	123.3	90.0	30.0	180.0	26.7	70.0	180.0
Stylodrilus heringianus Claparède, 1862	GC										
Enchytreidae											
Cognettia Nielsen & Christensen, 1959	GC	146.7			36.7	23.3					
Henlea Michaelsen, 1889	GC	10.0				13.3					
Mesenchytraeus Eisen, 1878	GC					3.3					
HIRUDINEA											
Glossiphoniidae											
Helobdella stagnalis Linnaeus, 1758	P	3.3									3.3
HEXAPODA											
Coleoptera											
Dytiscidae											
Hydroporus memnonius Nicolai, 1822 (l)	P		10.0		40.0	10.0	3.3	46.7		40.0	10.0
Hydroporus memnonius Nicolai, 1822 (a)	P				16.7						
Diptera											
Chironomidae											
Tanypodinae											
Macropelopia Thienemann, 1916	P			3.3	3.3	153.3	50.0	13.3	13.3		66.7
Thienemannimyia Fittkau, 1957 (series)	P										
Zavrelimyia Fittkau, 1962	P		150.0	510.0	186.7	183.3	183.3	413.3	173.3	260.0	210.0
Chironominae											
Chironomini											
Endochironomus Kieffer, 1918	FC										
Tanytarsini											
Micropsectra Kieffer, 1908	GC	33.3					123.3				
Paratanytarsus Thienemann & Bause, 1913	GC	150.0	250.0	153.3	106.7	286.7	123.3	573.3	226.7	430.0	73.3
Diamesinae											
Pseudodiamesa Goetghebuer, 1939	P	50.0				26.7					
Prodiamesinae											
Prodiamesa olivacea Meigen, 1818	GC	3.3									

		Balma Lake - Summer					**Balma Lake - Autumn**				
Taxon	**FFG**	**Lit_1**	**Lit_2**	**Lit_3**	**Litt_4**	**Lit_5**	**Lit_1**	**Lit_2**	**Lit_3**	**Lit_4**	**Lit_5**
Orthocladiinae		13.3	6.7								
Chaetocladius Kieffer, 1911	GC	3.3									
Cricotopus (Isocladius) Kieffer, 1909	GC										
Cricotopus/Orthocladius van der Wulp, 1874	GC	26.7						186.7	66.7	116.7	
Hydrobaenus Fries, 1830	GC	280.0	16.7				16.7				
Metriocnemus van der Wulp, 1874	GC	3.3									
Parorthocladius Thienemann, 1935	GC						86.7				
Psectrocladius Kieffer, 1906	GC						20.0				
Psectrocladius psilopterus group	GC	76.7	133.3	120.0	350.0	126.7	403.3	586.7	593.3	956.7	300.0
Ceratopogonidae											
Atrichopogon Kieffer, 1906	P										
Leptoconops Skuse, 1889	P					6.7					
Ephemeroptera											
Heptageniidae											
Ecdyonurus Eaton, 1868	GC	56.7					13.3				
Megaloptera											
Sialidae											
Sialis Latreille, 1802	P			20.0		3.3		10.0	16.7		26.7
Odonata											
Aeshnidae											
Aeshna juncea Linnaeus, 1758	P										
Plecoptera											
Nemuridae											
Nemoura cinerea Retzius, 1783	SH										
Perlodidae											
Perlodes Banks, 1903	P	6.7									
Trichoptera											
Limnephilidae											
Limnephilus Leach, 1815	SH	20.0					13.3				
Polycentropodidae											
Plectrocnemia conspersa Curtis, 1834	P	10.0		20.0			3.3				

*Functional Feeding Guilds (FFG) are assigned for each taxon in agreement with Merritt and Cummins (2006), Moller Pillot (2009a, 2009b, 2009c), Timm (2009). FFG are indicated as follows: FC=Filtering Collectors, GC=Gathering Collectors, SC=Scrapers, SH=Shredders, P=Predators.

APPENDIX II

Average density (ind m^{-2}) and functional feeding guilds (FFG) of taxa recorded in littoral sites (Lit_1-5) of Dimon Lake.

		Dimon Lake - Summer					**Dimon Lake - Autumn**				
Taxon	**FFG***	**Lit_1**	**Lit_2**	**Lit_3**	**Lit_4**	**Lit_5**	**Lit_1**	**Lit_2**	**Lit_3**	**Lit_4**	**Lit_5**
BIVALVIA											
Pisidium Pfeiffer, 1821	FC										
OLIGOCHAETA											
Naididae											
Naidinae											3.3
Nais alpina Sperber, 1948	GC			3.3							
Nais bretscheri Michaelsen, 1899	GC						3.3				
Nais barbata Müller, 1774	GC						16.7				
Tubificinae											
Potamothrix Vejdovský & Mrázek, 1903	GC										
Limnodrilus Claparède, 1862	GC			120.0	33.3		80.0	13.3	103.3		6.7
Spirosperma ferox Eisen, 1879	GC										
Tubifex Lamarck, 1816	GC			53.3	13.3	93.3	100.0	30.0	333.3	93.3	50.0
Tubifex tubifex Müller, 1774	GC										
Lumbriculidae											
Lumbriculus variegatus Müller, 1774	GC	66.7	73.3	6.7	453.3	166.7	70.0	346.7	383.3	530.0	663.3
Stylodrilus heringianus Claparède, 1862	GC		73.3	13.3							
Enchytreidae											
Cognettia Nielsen & Christensen, 1959	GC										6.7
Henlea Michaelsen, 1889	GC										
Mesenchytraeus Eisen, 1878	GC										
HIRUDINEA											
Glossiphoniidae											
Helobdella stagnalis Linnaeus, 1758	P	103.3	10.0	523.3	603.3	483.3	236.7	103.3	270.0	313.3	136.7
HEXAPODA											
Coleoptera											
Dytiscidae											
Hydroporus memnonius Nicolai, 1822 (l)	P										
Hydroporus memnonius Nicolai, 1822 (a)	P										
Diptera											
Chironomidae											
Tanypodinae											
Macropelopia Thienemann, 1916	P	173.3	130.0		10.0	20.0		6.7			
Thienemannimyia Fittkau, 1957 (series)	P	133.3	18.1	3.3	3.3	6.7					
Zavrelimyia Fittkau, 1962	P	69.5	30.0	149.1	16.7	6.7	470.0	10.0	213.3		6.7
Chironominae											
Chironomini											
Endochironomus Kieffer, 1918	FC	SH	23.3								
Tanytarsini											
Micropsectra Kieffer, 1908	GC										
Paratanytarsus Thienemann & Bause, 1913	GC	1120.0	766.7	626.7	403.3	426.7	336.7	13.3	13.3	16.7	23.3
Diamesinae											
Pseudodiamesa Goetghebuer, 1939	P										
Prodiamesinae											
Prodiamesa olivacea Meigen, 1818	GC	1263.3	903.3	170.0	333.3	390.0	90.0	16.7	63.3		33.3

		Dimon Lake - Summer					**Dimon Lake - Autumn**				
Taxon	**FFG**	**Lit_1**	**Lit_2**	**Lit_3**	**Lit_4**	**Lit_5**	**Lit_1**	**Lit_2**	**Lit_3**	**Lit_4**	**Lit_5**
Orthocladiinae				3.3							
Chaetocladius Kieffer, 1911	GC										
Cricotopus (Isocladius) Kieffer, 1909	GC	330.0	186.7	353.3	613.3	266.7	320.0	126.7	676.7	840.0	356.7
Cricotopus/Orthocladius van der Wulp, 1874	GC										
Hydrobaenus Fries, 1830	GC										
Metriocnemus van der Wulp, 1874	GC										
Parorthocladius Thienemann, 1935	GC										
Psectrocladius Kieffer, 1906	GC										
Psectrocladius psilopterus group	GC										
Ceratopogonidae											
Atrichopogon Kieffer, 1906	P		3.3	3.3							
Leptoconops Skuse, 1889	P										
Ephemeroptera											
Heptageniidae											
Ecdyonurus Eaton, 1868	GC										
Megaloptera											
Sialidae											
Sialis Latreille, 1802	P										
Odonata											
Aeshnidae											
Aeshna juncea Linnaeus, 1758	P						3.3				
Plecoptera											
Nemuridae											
Nemoura cinerea Retzius, 1783	SH						40.0	40.0	40.0		53.3
Perlodidae											
Perlodes Banks, 1903	P										
Trichoptera											
Limnephilidae											
Limnephilus Leach, 1815	SH	3.3		13.3			16.7	13.3	30.0		26.7
Polycentropodidae											
Plectrocnemia conspersa Curtis, 1834	P								3.3		

*Functional Feeding Guilds (FFG) are assigned for each taxon in agreement with Merritt and Cummins (2006), Moller Pillot (2009a, 2009b, 2009c), Timm (2009). FFG are indicated as follows: FC=Filtering Collectors, GC=Gathering Collectors, SC=Scrapers, SH=Shredders, P=Predators; St=site.