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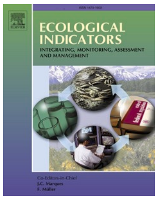

Combined effect of temperature and a reference toxicant (KCl) on *Daphnia middendorffiana* (Crustacea, Daphniidae) in a high-mountain lake

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ABSTRACT

Climate change has direct effects on aquatic systems where increased water temperature leads to range shifts and changes in the distribution of aquatic organisms. The effects of climate change on aquatic ecosystems are expected in all biomes, and in Alpine environments in particular. Anthropogenic pressure (e.g., chemical pollution) besides climate change impact on the state and quality of aquatic systems in which climate change and environmental contaminants can interact. To better understand the effect of increases in temperature and environmental pollution on high-mountain lakes, we performed an ecotoxicological assay on *Daphnia middendorffiana* collected during summer 2021 in a high-mountain lake (Upper Balma Lake, Cottian Alps, 2212 m a.s.L.). Samples were exposed to two temperature values (15 °C and 20 °C), potassium chloride (KCl) as the reference toxicant, and *D. magna* as the model organism for comparison. Findings showed immobilization after exposure to KCl in both species, but exposure to non-optimal water temperature (20 °C and 15 °C for *D. middendorffiana* and *D. magna*, respectively) enhanced this effect. The mean half-maximal effective concentration (EC_{50}; 24 h) for *D. middendorffiana* was significantly lower than that recorded for *D. magna* exposed to 20 °C (KCl) (46.9 mg/L *vs* 255 mg/L). A significantly higher EC_{50} (273.4 mg/L; 24 h) was recorded for *D. middendorffiana* exposed to 15 °C (KCl) compared to *D. magna* (EC_{50} 50.6 mg/L; 24 h). Our findings suggest that the combined effects of temperature and chemical pollution may severely affect the occurrence of *D. middendorffiana*, which occupies a central position in the food webs of high-mountain lakes in the Alps.

1. Introduction

Mountain regions cover about 20–24 % of the Earth's surface (Zhang et al., 2011). While species diversity richness decreases with altitude, mountain environments are characterized by different types of climate and ecosystems. As such, they provide a habitat for diverse species globally (Antonelli et al., 2018; Perrigo et al., 2020). The Alps are of particular interest for their endemic species (Menchetti et al., 2021) and for biodiversity conservation in Europe (Sergio and Pedrini, 2007). However, mountain regions, and the Alps in particular, are vulnerable to climate change and anthropogenic pressure (Elsasser and Bürki, 2002; Willibald et al., 2021). The temperature is rising at about double the rate of the global average recorded for the last century (Gobiet et al., 2014; Gobiet and Kotlarski, 2020). This constant rise impacts on Alpine ecosystems characterized by low temperatures. The highly specialized organisms living near the limit of physiological tolerance (Dagnino et al., 2020; Pastorino et al., 2020a) show the effects of climate change before and more pronounced than those inhabiting lowland ecosystems (IPCC, 2022).

Climate change affect the mountain biota in different ways: directly owing to the increase in temperature and the variation of precipitation regimes and indirectly owing to the variation in abiotic components of the environment (e.g., glacial retreat) (Hoorn et al., 2018; Tito et al., 2020). Ecological response to such pressure depends on the

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characteristics of a species. The response of individual species can lead to changes in the interaction between species at the same or different trophic levels and to a more general variation in ecological communities (Rogora et al., 2018). The recorded and the expected effects of global warming on fauna are largely reflected in changes in physiology, phenology, and spatial distribution (Diez et al., 2020). Species inhabiting mountain environments are particularly sensitive to climate change (Dagnino et al., 2020) and may face increased risk of extinction (Schwager and Berg, 2019).

This is particularly true for the harsh environmental conditions characteristic of high-mountains lakes that support highly specific aquatic communities (Catalan et al., 2006), which are studied as indicators of global environmental change and "early warning systems'' for the mountain environment (Moser et al., 2019). Due to their small surface area, depth, and catchment area, high-altitude lakes are extremely sensitive to the effects of global, regional, and local environmental impacts (Perilli et al., 2020; Cantonati et al., 2021). The aquatic organisms inhabiting them are adapted to the severe conditions of persistent low temperature, exposure to UV radiation, and common ultraoligo- or oligotrophic status (Pastorino et al., 2020a). The ice-free season lasts for a few months, generally from mid-June to late October. During this brief period of ideal conditions, aquatic organisms can complete their life cycle before the snow covers the lakes again. They react very sensitively to even slight changes in the environment (Pastorino and Prearo, 2020). Anthropogenic activity (e.g., chemical pollution) besides direct climate change impact on the state and quality of high-mountain lakes (Catalán et al., 2009): tourism, grazing, and long-distance airborne transport of pollutants from the lowland. For example, trace elements, persistent organic pollutants (e.g., organochloride compounds), and microplastics are a major important source of pollution in these remote ecosystems where climate change and environmental contaminants interact (Pastorino et al., 2022a, Pastorino et al., 2022b).

Because of their short life cycle, zooplankton species are particularly sensitive to climate change; they are also vulnerable to a climate-induced mismatch between food resource demand and availability (Preston and Rusak, 2010). Because temperature effects are species specific, zooplankton species, rather than functional groups, may reflect the influence of climatic warming on ecosystem changes (Carter et al., 2017). For example, the genus *Daphnia* contains over 100 species of freshwater plankton found in the world; it constitutes an important group of herbivores in freshwater ecosystems (Kotov, 2015). *Daphnia* spp. are particularly useful for answering questions about the effects of climatic warming on plankton communities and entire ecosystems because they are a major link in the energy flow between primary producers and secondary consumers in food webs; as such, they reflect both bottom-up and top-down influences (Altermatt et al., 2008; Dziuba et al., 2020). Within the genus *Daphnia*, *D. middendorffiana* has an arctic, circumpolar distribution, with some isolated southern populations confined to mountainous areas (Tiberti, 2011). Inhabiting cold, oligotrophic waters in the Alps, it is a vital species in high-mountain lakes for high energy transfer efficiency from pico- and nanoplankton to higher trophic levels (Yurista and O'brien, 2001).

In this study, our hypothesis was that an increase of 5 °C in water temperature, as expected in the years ahead due to climate change, in combination with pollutants may have a negative effect on *D. middendorffiana* in high-mountain lakes. Indeed, compared to the early years of the twenty-first century, the temperature in the Alps is expected to increase between 2 and 5 °C by 2100 (CREA Mont-Blanc, 2022). For this study, an ecotoxicological assay was performed on *D. middendorffiana* collected during summer 2021 in a high-mountain lake (Upper Balma Lake, Cottian Alps), using *D. magna* for comparison, a widely used model organism in aquatic toxicology (Olkova et al., 2018).

2. Material and methods

2.1. Study area

Upper Balma Lake is a high-mountain lake *sensu* Catalan et al. (2006) located above the tree line in the Cottian Alps (Municipality of Coazze, Piedmont, northwestern Italy) at 2212 m a. sl. (Fig. 1). The lake is of glacial origin and included in the Special Area of Conservation (SAC) and Special Protection Area (SPA) IT1110006 "Orsiera Rocciavrè". The ice cover generally lasts from late October to late May/early June. Upper Balma Lake is S-shaped, with two subbasins separated by a shallow midsection (774 m in perimeter, 1.82 m^2 in surface area, 2.77 m in maximum depth). Being shallow, the lake lacks thermal stratification. The catchment core is composed of ophiolite metamorphic bedrock and the landscape is dominated by rocky outcrops, ridges, and mountain walls. A small meadow is located at the southern end of the lake, where a small inlet flows. Although the lake was originally fishless, brook trout (*Salvelinus fontinalis*) has been introduced for recreational fishing (Pastorino et al., 2020b).

2.2. Zooplankton sampling and determination of water physicochemical parameters

A zooplankton sampling campaign was performed on 20 August 2021. For the sampling, an Apstein net (mesh size 45 μm) with a circular opening (0.30 m in diameter, 0.90 m in length) hauled by an electric rubber dinghy at low velocity (3 km/h) was used. The Apstein net was towed vertically from the lake bottom to the water surface. Random transects were placed to cover the entire lake perimeter. The bottle at the end of the net was emptied in a 2 L glass bottle, placed in a cooling box, and transported by helicopter to the lowland within a few minutes. The box was transferred to the laboratory within 1 h, where the bottle was placed in a chilled thermostat (+15 °C; Upper Balma Lake water temperature). During sampling, physicochemical water measurement were recorded at three sampling sites (Fig. 1) to replace the environmental parameters during the ecotoxicological assays. To do this, water temperature (°C), dissolved oxygen (mg/L), pH (unit), and conductivity (μS/cm) were recorded with a field probe (HI 98194; Hanna Instruments Inc., Woonsocket, RI, USA) at the water surface and at the bottom of each sampling site.

2.3. Selection of *D. middendorffiana* and ecotoxicological assays

The zooplankton sample was sorted in the laboratory a few hours after collection and live individuals belonging to *D. middendorffiana* according to morphology (distinct brown pigmentation extending to the dorsal exoskeleton and often to the proximal segments of the antennae) as described by Tiberti (2011) were selected. Species identification was confirmed by PCR amplification of COI gene region (Özdemir et al., 2017).

The populations of *D. magna* (obtained from dormant stage ephippia; MicroBioTest Inc., Daphtoxkit FTM magna 1996) and *D. middendorffiana* (obtained by parthenogenesis) were acclimated for three generations following the protocol reported by Guilhermino et al. (2021).

Daphnia middendorffiana was reared and maintained in ISO 6341 freshwater (ISO, 2013) at 15 °C, 6000 lx (photoperiod of 16:8h of light /darkness) and pH 7.5 (environmental condition of Upper Balma Lake; see Section 3) and fed with *Spirulina* spp. Five juvenile individuals per each replicate (<24 h old) of *D. magna* and *D. middendorffiana* were exposed to different concentrations (19.5, 39.1, 78.1, 156.3, 312.5, 625, 1250, 2500 mg/L) of potassium chloride-KCl for 24 h and 48 h at 15 °C (temperature of Upper Balma Lake) and 20 °C (temperature recommended by UNI EN ISO 6341:2013 guideline for the *D. magna* assay) and a photoperiod of 16:8h of light /darkness without feeding. The endpoint was immobilization after 24 h and 48 h of exposure. Potassium chloride was chosen as a reference contaminant based on interlaboratory

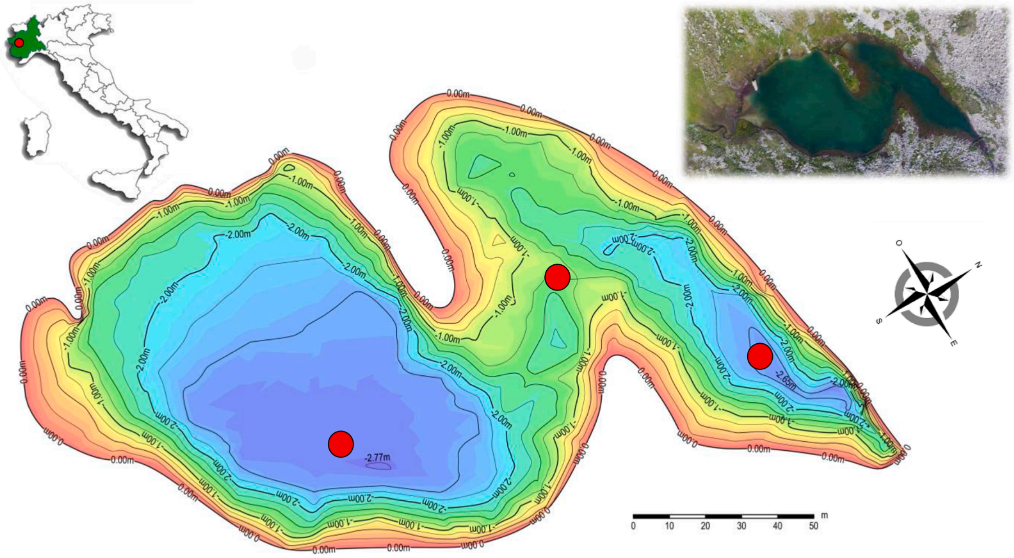

Fig. 1. Upper Balma Lake (insert). Bathymetric map showing water sampling sites (red dots). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

comparison (as indicated by the UNI EN ISO/IEC 17043:2010 standard that specifies the general requirements for the competence of interlaboratory assessment test organizers) and the published literature (Knapik and Ramsdorf, 2020). Exposure concentrations were determined by pilot testing on *D. magna* to define the correct range of dilution and based on interlaboratory comparison (ISPRA, 2017). Four replicates of each assay were performed. To meet test validity criteria, the criteria in the UNI EN ISO 6341:2013 guideline (ISO, 2013) were followed. For each batch of analyses, tests were performed under standardized conditions with negative (standard ISO freshwater; percentage of immobility, max 10 %) and positive ($K_2Cr_2O_7$; EC_{50} 24 h *D. magna*: 0.84–1.65 mg/L; EC_{50} 24 h *D. middendorffiana*: 0.62–1.52 mg/L) controls; all results were within the range of acceptability reported by the specific testing method.

2.4. Statistical analysis

Normality and homogeneity of variance were tested using the Shapiro–Wilk test and Levene's test, respectively. Since the null hypothesis for the homogeneity of variance and/or for normal distribution could not be rejected, differences in concentration of the physicochemical measurements between the three sampling sites were checked using the Kruskal-Wallis test, whereas the Wilcoxon test was used to check for differences in physicochemical measurements between the surface and the bottom of the lake. The concentrations that induced the endpoint in 50 % (EC_{50}), 20 % (EC_{20}), 10 % (EC_{10}), and 5 % (EC_5) of exposed *D. magna* and *D. middendorffiana* were calculated using the U.S. EPA Toxicity Relationship Analysis Program (TRAP version 1.30), with a Gaussian distribution and logarithmic transformation of exposure variables sized for ecotoxicological tests and expressed as mean and range. Wilcoxon test was used to check for differences in effective concentration (EC_x: EC_{50}, EC_{20}, EC_{10}, EC_5) values. Principal component analysis (PCA) was applied to check for trends in ecotoxicological response (EC_{50}, EC_{20}, EC_{10}, EC_5; response at 24 h and at 48 h were merged) between *D. magna* and *D. middendorffiana* exposed to KCl at 15 °C and at 20 °C. PCA was also used to differentiate the EC_{50} (standard indicator of chemical potency in the ecotoxicological test) between *D. magna* and *D. middendorffiana* exposed to KCl at 15 °C and at 20 °C. Eigenvalues were used to evaluate the interpretation of the principal components (PCs) (only PCs with an eigenvalue > 1 were retained). The significance of the results was set at $p < 0.05$. Statistical analysis was performed using the free open-source data analysis software RStudio® (RStudio, Inc.).

3. Results and discussion

Concerns about the impact of climate change on ecosystems and on human society (Malhi et al., 2020; Hooper et al., 2013) have prompted research into its global effects on ecosystems. For this study, we performed an ecotoxicological assay on *Daphnia middendorffiana* collected during summer 2021 at Upper Balma Lake. Physicochemical water parameters were recorded in the field to better reproduce local environmental conditions. No statistically significant differences were noted in water temperature (mean ± standard deviation, 14.93 ± 0.15 °C), conductivity (11.67 ± 0.58 μS/cm), pH (7.55 ± 0.13), and dissolved oxygen (6.51 ± 0.53 mg/L) measured at the three-sampling site (Kruskal-Wallis test; $p > 0.05$) and at the surface and the bottom of the lake (Wilcoxon test; $p > 0.05$). Generally, the water physicochemical parameters matched those reported by Tiberti et al. (2010) for twelve Alpine lakes in Gran Paradiso National Park (Western Alps, Italy). In the present study area, Upper Balma Lake rests on granite bedrock, which is why it has a lower pH than lakes on sandstone bedrock. The pH was consistent with previous data for high-altitude lakes. As expected, the oxygenation level was high. The low conductivity was consistent with previous data (Füreder et al., 2006; Fjellheim et al., 2009).

For the present assay, 15 °C and 20 °C were set as temperature of exposure, potassium chloride (KCl) as the reference toxicant, and *D. magna* as the model organism for comparison. Upper Balma Lake has a water temperature of 15 °C; 20 °C was selected for this study since an increase of 5 °C in temperature is expected for the Alps in the near future (CREA Mont-Blanc, 2022). On this path, it is important to point out that certain high-mountain lakes are shallow-water environments, and the water temperature is very close to that of the air. Furthermore, 20 °C is an optimal temperature for a *D. magna* ecotoxicological assay. Table 1 presents the ecotoxicological response of *D. middendorffiana*. Greater effect (100 % immobilization) was observed after exposure to higher KCl concentrations (625–2500 mg/L) after 24 h and 48 h at both water temperatures. A decrease in the percentage of effects (%E) was observed at lower KCl concentrations but %E was greater at 20 °C compared to those recorded at 15 °C. Exposure to KCl had a negative effect on *D. middendorffiana,* but the higher temperature (20 °C) seems to have exacerbated the effect. For example, at lower KCl concentration (19.5 mg/L) the percentage of the endpoint (immobilization) at 24 h was 6.7 %, whereas the %E at 20 °C was 20 % (33.5 % higher). EC_x for KCl were

Table 1
Ecotoxicological response of *Daphnia middendorffiana* exposed to KCl (19.5–2500 mg/L) at 15 °C (optimal condition) and 20 °C (non-optimal condition). Association between the percentage of effect (%E) after exposure for 24 h and 48 h and effective concentration values (EC_5, EC_{10}, EC_{20}, EC_{50}; mean and range; data are expressed as mg of substance per liter of solution; mg/L).

		%E (15 °C)				**%E (20 °C)**			
		24 h		**48 h**		**24 h**		**48 h**	
	KCl (mg/L)	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Daphnia middendorffiana	2500	100	0	100	0	100	0	100	0
	1250	100	0	100	0	100	0	100	0
	625	100	0	100	0	100	0	100	0
	312.5	53.3	11.5	80	0	93.3	11.5	93.3	11.5
	156.3	46.7	11.5	53.3	11.5	86.7	11.5	93.3	11.5
	78.1	33.3	11.5	46.7	11.5	66.7	11.5	73.3	11.5
	39.1	26.7	11.5	33.3	11.5	46.7	11.5	53.3	11.5
	19.5	6.7	11.5	13.3	11.5	20	20	33.3	11.5
EC_x (mg/L)	$\mathbf{EC_{50}}$	273.4	(234.6–318.7)	141.8	(110.84–181.46)	46.9	(22.78–96.70)	40.7	(14.96–110.88)
	$\mathbf{EC_{20}}$	172.2	(135.59–218.58)	67.1	(45.55–98.75)	18.5	(6.35–53.99)	15.7	(3.62–67.65)
	$\mathbf{EC_{10}}$	135.2	(100.25–182.26)	45.3	(28.16–73.01)	11.4	(3.13–41.45)	9.5	(1.60–56.43)
	$\mathbf{EC_5}$	110.7	(77.362–157.89)	32.8	(18.79–57.31)	7.6	(1.69–34.38)	6.3	(0.76–51.84)

consistently higher at 20 °C after 24 h and 48 h of exposure. EC_{50} (24 h) was significantly higher for organisms exposed to 15 °C (mean values: 273.4 mg/L *vs* 46.9 mg/L at 15 °C and 20 °C, respectively; Wilcoxon test; $p < 0.05$). The same trend was also observed after exposure for 24 h for EC_{20} (172.2 mg/L *vs* 18.5 mg/L at 15 °C and 20 °C, respectively), EC_{10} (135.2 mg/L *vs* 11.4 mg/L at 15 °C and 20 °C, respectively), and EC_5 (110.7 mg/L *vs* 7.6 mg/L at 15 °C and 20 °C, respectively; Wilcoxon test; $p < 0.05$). To our best knowledge there are no comparative toxicity studies on *D. middendorffiana*; ours is the first study to assess acute toxicity of KCl in this endemic species.

Our findings suggest that the expected 5 °C increase in water temperature could have a huge impact on the life processes of organisms whose metabolism is dependent on environmental temperature (poikilotherms). This thermal input, combined with a reference pollutant (KCl), is more stressful to aquatic poikilotherms at lower organizational levels such as zooplankton. An increase in water temperature can increase organism metabolism, decrease oxygen concentration (organisms require more oxygen to support their temperature-enhanced metabolic activity), and increase contaminant concentration due to water evaporation (Ikeda et al., 2001; Bonachea, 2021). All such factors are a cause of osmotic and metabolic stress in poikilotherms, making them more vulnerable to other environmental threats such as chemical pollutants. Khan and Khan (2008) provided preliminary data on the effects of increased temperature on *D. magna*, suggesting that as the temperature rises above 6 °C, the daphnids increase their breathing rate and heartbeat. This behavior favors the uptake of contaminants from the water, explaining the immobilization at low concentrations of KCl we noted.

High temperature tolerance is largely dependent on the ability of oxygen transport mechanisms to meet the increasing demand for oxygen due to an increase in metabolic rate caused by a rise in temperature (Audzijonyte et al., 2019). For example, hemoglobin (Hb) is a central component of the oxygen transport system and is critical for thermal tolerance (Zeis, 2020). The increase in oxygen demand parallel to an increase in temperature, combined with less oxygen available due to decreased solubility, drives the need for intense Hb synthesis (Zeis, 2020). Because Hb takes time to adjust, a sudden rise in temperature causes a short-term mismatch between oxygen transport and energy demand, with negative effects on the physiological function of daphnids (Pirow, 2003; Zeis, 2020).

Findings from previous studies suggest that K^+ from KCl is responsible for cladoceran (*Ceriodaphinia dubia*) toxicity (Mount et al., 2016). Romano and Zeng (2007) provided evidence that exposure of juvenile blue swimmer crabs (*Portunus pelagicus*) to high KCl concentrations (>450 mg/L) caused an increase in K^+ in the hemolymph due to active transport from the external aquatic environment. Hyperkalemia is known to be highly lethal via disrupted cardiac function and/or depolarization of cell membranes (Cripps et al., 2013), and a similar situation could explain the death of *D. middendorffiana* following KCl treatment. About other chemicals, Heugens et al. (2003) assessed the effects of temperature on cadmium toxicity in *D. magna* and found that higher temperature lowered the internal threshold concentrations and increased the mortality rate and the rate of metal uptake.

For this study, *Daphnia magna* was used as a model organism for comparison. Table 2 presents the ecotoxicological response of *D. magna*. Greater effects (100 % immobilization) were observed at higher KCl concentrations (625–2500 mg/L) after exposure for 24 h and 48 h at 15 °C. Furthermore, 100 % immobilization was also observed at a higher KCl concentration (2500 mg/L) after exposure for 24 h and 48 h at 20 °C. The %E was less at lower KCl concentrations but higher at exposure to 15 °C compared to 20 °C. EC_x were always higher at 20 °C after exposure for both 24 h and 48 h. EC_{50} mean (24 h) was significantly higher after exposure to 20 °C (mean values: 255 mg/L *vs* 50.6 mg/L at 20 °C and 15 °C, respectively; Wilcoxon test; $p < 0.05$). The same effect was observed for EC_{20} (100.6 mg/L *vs* 21.3 mg/L at 20 °C and 15 °C, respectively), EC_{10} (61.9 mg/L *vs* 13.6 mg/L at 20 °C and 15 °C, respectively), and EC_5 (41.4 mg/L *vs* 9.4 mg/L at 20 °C and 15 °C, respectively) (Wilcoxon test; $p < 0.05$). Overall, *D. magna* performed as expected based on the published EC_{50} range for KCl (250–880 mg/L; 48 h) (Mount et al., 1997; Mohammed, 2007; Struewing et al., 2015).

Here, as observed for *D. middendorffiana*, exposure to KCl resulted in immobilization of *D. magna,* but the lower temperature (15 °C) seemed to increase the effect. The optimal temperature recommended by the ISO guideline (ISO, 2013) for ecotoxicological assays is 20 ± 2 °C. Because of its increased solubility in water, oxygen demand is reduced in colder water (15 °C). Daphnids decrease ventilation and muscular activity according to oxygen supply. The resulting decrease in partial pressure of oxygen levels in the blood and activation of anaerobic metabolism depletes the organism's physiological stores. Müller at al. (2018) showed that swimming activity is greatest and lactate production (anaerobic metabolism) is lowest in *D. magna* individuals near their acclimation temperature, indicating that *D. magna* vitality depends on the aerobic provision of energy and is therefore maximized within an optimal temperature range. The same was observed for the filtration rate (Müller et al., 2018). In the present study, the non-optimal temperature of 15 °C probably had a much greater effect on *D. magna* immobilization compared to exposure to KCl, due to the drop in filtration capacity, i.e., the drop in KCl adsorption from the aquatic medium.

Principal component analysis clearly discriminated the ecotoxicological response of the two daphnid species (Fig. 2). Eigenvalues revealed that the first two principal components (PC1 and PC2) accounted for a significant portion of total variance (98.2 %), while the two other components (PC3 and PC4) accounted for a much smaller

Table 2

Ecotoxicological response of *Daphnia magna* exposed to KCl (19.5–2500 mg/L) at 15 °C (non-optimal condition) and 20 °C (optimal condition). Association between the percentage of effect (%E) after exposure for 24 h and 48 h and effective concentration (EC_5, EC_{10}, $EC_{20,}$ EC_{50}; mean and range; data are expressed as mg of substance per liter of solution; mg/L).

		%E (15 °C)				**%E (20 °C)**			
		24 h		**48 h**		**24 h**		**48 h**	
	KCl (mg/L)	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Daphnia magna	2500	100	0	100	0	100	0	100	0
	1250	100	0	100	0	93.3	11.5	80.0	0
	625	100	0	10	0	73.3	11.5	53.3	11.5
	312.5	93.3	11.5	86.7	11.5	60	0	60	0
	156.3	86.7	11.5	93.3	11.5	46.7	11.5	33.3	11.5
	78.1	66.7	11.5	73.3	11.5	13.3	11.5	13.3	11.5
	39.1	46.7	11.5	60.0	0	13.3	11.5	0	0
	19.5	13.3	11.5	26.7	11.5	6.7	11.5	0	0
EC_x (mg/L)	$\mathbf{EC_{50}}$	50.6	(28.88–88.79)	35.2	(9.39–131.8)	255.0	(207.7–313.2)	340.9	(290.3–400.4)
	$\mathbf{EC_{20}}$	21.3	(9.17–49.64)	11.7	(1.75–77.12)	100.6	(72.4–139.85)	122.9	(95.59–158.02)
	$\mathbf{EC_{10}}$	13.6	(4.89–37.71)	6.5	(0.66–64.65)	61.9	(41.05–93.28)	72.1	(52.35–99.3)
	$\mathbf{EC_5}$	9.4	(2.85–30.69)	4.0	(0.27–61.09)	41.4	(25.55–67.13)	346.4	(31.66–68.03)

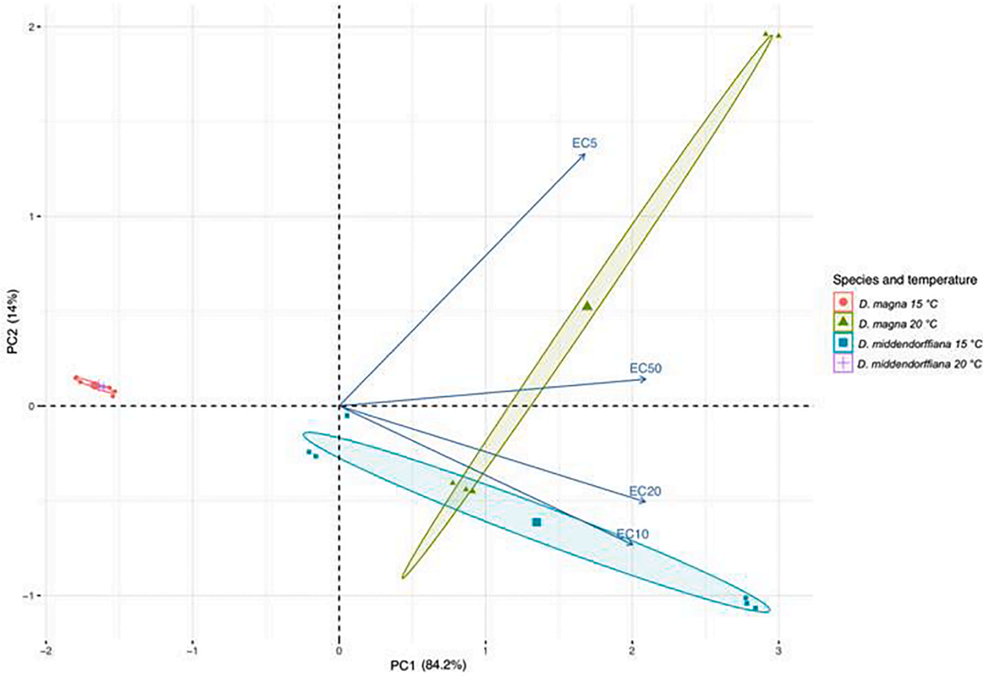

Fig. 2. Biplot of loadings (variables) and scores (observations) in the principal component analysis. The scores of each species (*D. middendorffiana* and *D. magna*) exposed to 15 °C and 20 °C are denoted by a symbol (largest symbol denotes average). Confidence ellipses (95 %) plot values of each species.

portion of variance (1.8 %).

The biplot of loadings (EC_x) and scores (*D. middendorffiana* and *D. magna*) revealed which species exposed to which temperature (15 °C or 20 °C) were similar to each other (Fig. 2). Each score has a different symbol and color (largest symbol denotes average value). Almost all variables moved to PC1 because they were more closely related. *D. middendorffiana* and *D. magna* exposed to KCl at non-optimal temperature (20 °C and 15 °C, respectively) are located in the left part of the plot in relation to lower EC_x (more sensitive). In contrast, *D. middendorffiana* and *D. magna* exposed to KCl at optimal temperature (15 °C and 20 °C, respectively) are located in the right quadrant in relation to higher EC_x (less sensitive). *D. magna* exposed to KCl at 20 °C is located in the upper right quadrant in relation to higher EC_5 and EC_{50}. *D. middendorffiana* exposed to KCl at 15 °C is located in the lower right quadrant in relation to higher EC_{10} and EC_{20}. There is a higher variability (wide confidence ellipses) in the EC_x for individuals exposed to KCl at optimal temperature, while very little variability (small confidence ellipses) is observed for individuals exposed to non-optimal temperature, confirming the displacement of species from their viable environmental temperature condition.

Finally, Fig. 3 presents the PCA scores of the two species in relation to EC_{50}. The plot shows an increase in KCl EC_{50} from left to right where optimal temperatures of the species are met.

4. Conclusions

Our findings show immobilization in both species after exposure to KCl, the reference toxicant, and an enhanced effect at non-optimal water temperature. This response is particularly true for stenotherm organisms like *D. middendorffiana* inhabiting the extreme and remote environment of high-mountain lakes where they are adapted to living at low temperature. Extinction of this species or a decrease in their number could have serious repercussions on the food web, in which zooplankton are a key component of the aquatic ecosystem.

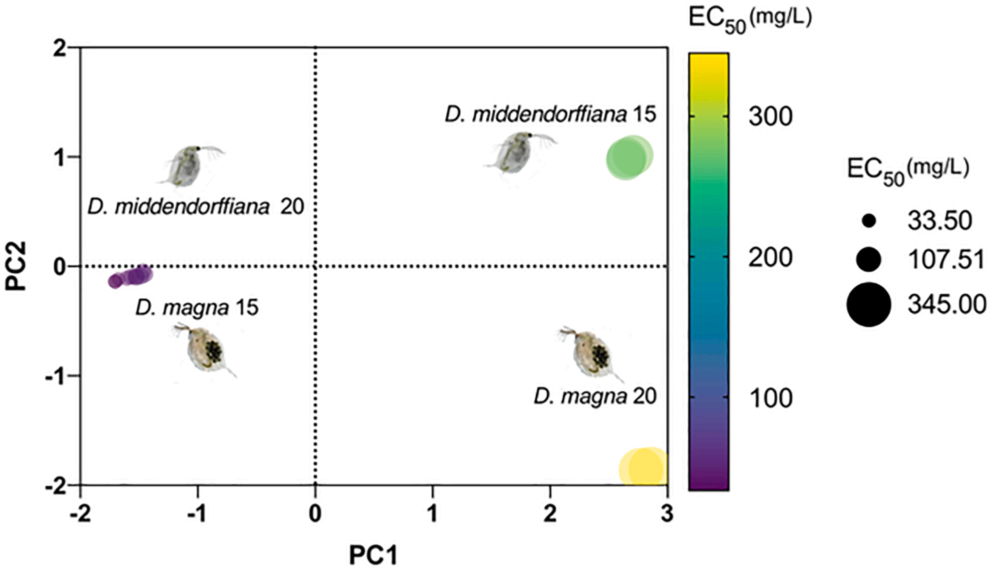

Fig. 3. Scores (*D. middendorffiana* and *D. magna*) in the principal component analysis. Data point clusters are grouped by color (violet for low, yellow for high EC values) and circle size (small for low, large for high values). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Global warming has been associated with an increase in the occurrence of extreme temperature events. Adaptation to changes in temperature will mark the behavior and survival rate of organisms. Further studies are needed to better understand temperature-driven response reversibility and short-term acclimation of *D. middendorffiana.*

CRediT authorship contribution statement

Paolo Pastorino: Investigation, Conceptualization, Methodology, Data curation, Funding acquisition, Project administration, Writing - original draft. **Marino Prearo:** Investigation, Conceptualization, Methodology, Data curation, Writing – review & editing. **Serena Anselmi:** Investigation, Methodology, Writing – review & editing. **Tecla Bentivoglio:** Investigation, Methodology, Writing – review & editing. **Giuseppe Esposito:** Investigation, Methodology, Conceptualization, Writing – review & editing. **Marco Bertoli:** Investigation, Methodology, Conceptualization, Writing – review & editing. **Elisabetta Pizzul:** Investigation, Methodology, Conceptualization, Writing – review & editing. **Damià Barceló:** Investigation, Methodology, Conceptualization, Writing – review & editing. **Antonia Concetta Elia:** Investigation, Methodology, Conceptualization, Data curation, Writing – review & editing. **Monia Renzi:** Investigation, Methodology, Conceptualization, Supervision, Data curation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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