Online Resource 3: Recovery indicators: sampling and analytical methods

Article title: Recovery of high mountain Alpine lakes after the eradication of introduced brook trout *Salvelinus fontinalis* using non-chemical methods

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Trophic state: nutrients, water transparency, chlorophyll-a concentration.

259 water samples (1000 mL) for chemical analysis were collected from 18 lakes, while a subset of 88 samples was analyzed also for Chlorophyll-a concentration. Samples were averaged over depth with a horizontal Van Dorn bottle. The samples were stored in the dark at 4 °C and analyzed at the laboratory of CNR ISE in Verbania Pallanza, Italy. Details on the analytical methods and the quality controls adopted in the laboratory can be found at: http://www.idrolab.ise.cnr.it/. Water samples were analyzed for total phosphorus (TP, UV-VIS spectrophotometry, spectrophotometer SAFAS UV mc2; Valderrama, 1981), total nitrogen (TN), and total organic carbon (TOC) (catalytic combustion with a Skalar Formacs series TOC/TN Analyzer; APHA, 2005). Some additional chemical variables (pH, conductivity, alkalinity; acidimetric titration, Gran's method) were measured to better characterize the study lakes and to quantify their buffer capacity against the risk of acidification, which could influence some of the biological indicators, independently by fish presence.
Chlorophyll-a concentration was measured on all the samples collected in 2013 (23 samples from 4 fish removal lakes and 5 control lakes) and on all the samples collected in 2014-2017 in the fish-removal lakes. Chlorophyll-a measurements were made with a spectrofluorimetric method on the same samples collected for chemical analysis at the laboratory of CNR ISE in Verbania Pallanza, Italy, using a laboratory probe (FluoroProbe^{TM}, bbe-Moldaenke).
143 vertical profiles of light intensity from 14 lakes were measured *in situ* throughout the water column at 1 or 0.5 m intervals (0.5 m in very shallow lakes with < 4 m maximum depth), using a light meter (LI-COR LI-250) with a submersible sensor for Photosynthetically Active Radiation (PAR, 400–700 nm), from one centimeter below the water surface to the bottom. The vertical extinction coefficient (*k*) was estimated for each lake by fitting an exponential curve of the percent PAR reaching each sampling depth, assuming the mean of the surface measurements to be 100%. The PAR profiles were measured in constant lighting conditions (full sunlight or persistent cloud cover without bright spells or drifting cloud patches).

Zooplankton

291 zooplankton samples from 18 lakes were collected during the ice-free season between 2006 and 2017. Zooplankton samples were collected up to eight times per lake each year during the ice-free period, but, usually, at least twice: one at the beginning (Jun-Jul) and one at the end (Aug-Sep) of summer to observe both the early and the mature stages of the highly dynamic zooplankton communities inhabiting mountain lakes (Tiberti et al., 2013). Sampling frequency was increased (3-8 samples per year) between 2013 and 2017 in the "eradication" lakes to describe in more detail the dynamics of the recovering zooplankton communities.
Zooplankton samples were collected at the deepest point of each lake by taking vertical tows from the bottom to the surface with a conical plankton net (40 cm diameter, 50 μm mesh). Sample fixation in 70% ethanol was carried out in the field as early as possible to avoid damages to animal tissues by bacterial action and autolysis. Zooplankton samples were analyzed following Edmondson and Winberg (1971): zooplankton samples were concentrated in a known volume and 3–5 subsamples (volume from 0.1 to 1 ml) were removed with a wide-bore pipette; zooplankton in each subsample were identified and counted using a closed counting chamber under a binocular dissecting microscope (Olympus CH-BI45-3) at 40× in order to obtain density data (number of individuals per m^{-3}). When some taxa were much more abundant than others (usually some common rotifer

species), the high-density and low-density taxa were counted at different dilutions. Crustaceans and rotifers were identified to the species level following Alonso (1996), Stella (1984), Krajíček et al. (2016), and Braioni and Gelmini (1983). Coarser taxonomic levels indicating a group of species (gr.) or the genus, have been used instead of the species name for those organisms with an uncertain taxonomy (e.g. *Keratella* gr. *quadrata*) or when the specific identification was not possible because of morphological deformations due to the conservative medium (e.g. *Synchaeta* gr. *stylata-pectinata*, *Ascomorpha* sp.).
We described the size distribution of each taxon by measuring the length of zooplankton with a calibrated eye-piece micrometer. The number of measured individuals per taxon varied depending on its abundance in the sample (mean ± SD = 52.5 ± 66.6 individuals per sample).
Based on their maximum length, zooplankton taxa were divided into small (total max. length < 1.25 mm) and large (total max length ≥ 1.25 mm) taxa. The threshold at 1.25 mm is based on the results by Tiberti et al. (2014a), who analyzed the stomach contents of zooplanktivorous brook trout and report that size selective predation on zooplankton is exerted by large (>20 cm) brook trout and affects zooplankton individuals larger than 1.25 mm.
The dry mass of measured crustaceans and of some rotifers with high size variability (i.e. *Synchaeta* sp., *Hexarthra bulgarica* Wiszniewski 1933, *Euchlanis* gr. *dilatata-parva*) was determined from length measurements using the length-biomass regression equation (Table OR3.1). For rotifer taxa with limited size variability (*Keratella cochlearis* Gosso, 1851, *Polyarthra* gr. *vulgaris-dolichoptera*, *Notholca squamula* Müller, 1786, *Notholca labis* Gosse, 1887, *Lecane* gr. *lunaris*, *Lecane* gr. *luna*, *Ascomorpha* sp., *Cephalobdella* sp.) we used the standard individual wet weights proposed by Ejsmont-Karabin (1998). Wet biomass was converted to dry mass assuming a dry:wet ratio of 0.1 (Pace and Orcutt, 1981).
The average dry mass of each zooplankton taxa was finally multiplied by the corresponding density to obtain the specific biomass per cubic meter.

Table OR3.1 Length-dry biomass (W in µg) equations and Standard Individual W (SIW) used to calculate zooplankton biomasses. L_1: individual length in µm; L_2: individual length in mm; References: [1]: Dumont et al. (1975); [2]: Ejsmont-Karabin (1998); [3]: Ruttner-Kolisko, (1977).

Taxon	Equation	SIW	Reference taxon from literature	Ref.
Crustacea				
Acropaerus harpae	$W = 9.05 \times 10^{-3} \times (L_1)^{0.85}$		*Acropaerus harpae*	[1]
Alona sp.	$W = 15.92 \times (L_2)^{3.84}$		*Alona* sp.	[1]
Arctodiaptomus alpinus	$W = 7.70 \times (L_2)^{2.33}$		*Arctodiaptomus* sp.	[1]
Chydorus sp.	$W = 89.43 \times (L_2)^{4.08}$		*Chydorus sphaericus*	[1]
Cyclops abyssorum and *Eucyclops serrulatus*	$W = 2.20 \times 10^{-8} \times (L_1)^{2.82}$		Cycopoids pooled equation	[1]
Daphnia longispina and *Daphnia pulicaria*	$W = 1.50 \times 10^{-8} \times (L_1)^{2.84}$		*Daphnia* sp. pooled equation	[1]
All copepod nauplii	$W = 1.10 \times 10^{-5} \times (L_1)^{1.89}$		Nauplii pooled equation	[1]
Scapholeberis mucronata	$W = 8.9 \times 10^{-8} \times (L_1)^{2.7}$		*Scapholeberis mucronata*	[1]
Rotatoria				
Ascomorpha sp.		W = 0.0393	*Ascomorpha ecaudis*	[2]
Cephalobdella sp.		W = 0.0250	*Cephalobdella catellina*	[2]
Euchlanis sp.	$W = 0.1 \times (L_2^3) \times 50$		*Euchlanis* gr. *dilatata-parva*	[3]
Hexarthra bulgarica	$W = 0.13 \times 10^2 \times (L_2)^3$		*Hexarthra* sp.	[3]
Kertella gr. *quadrata*		W = 0.0444	*Keratella hyemalis*	[2]
Keratella cochlearis		W = 0.0155	*Keratella cochlearis*	[2]
Lecane gr. *luna*		W = 0.0515	*Lecane luna*	[2]
Lecane gr. *lunaris*		W = 0.0254	*Lecane lunaris*	[2]
Notholca labis		W = 0.0339	*Notholca labis*	[2]
Notholca squamula		W = 0.0303	*Notholca squamula*	[2]
Polyarthra gr. *vulgaris-dolichoptera*		W = 0.0421	*Polyarthra dolichoptera*	[2]
Synchaeta sp.	$W = 0.10 \times 10^2 \times (L_2)^3$		*Synchaeta* sp.	[3]

Macroinvertebrates

168 macroinvertebrate samples from 17 lakes were collected during the ice-free season between 2006 and 2017. During the same surveys described above, all the shore-accessible habitats were sampled for semiquantitative macroinvertebrates estimates. Three littoral habitats were chosen according to clast diameter: sand shores (clast diameter < 2 mm), gravel shores (2 mm ≤ clast < 64 mm), and stony shores (clast ≥ 64 mm).

In each of these habitats, ten sweeps, each about 1 m long, were conducted with a standard d-frame net (mouth, 25 9 20 cm; mesh 0.5 mm) following Knapp et al (2001). If some microhabitats were not accessible, the missing sweeps were equally divided between the remaining microhabitats. Benthic macroinvertebrates were separated from detritus and sediments in the field and preserved in 70% ethanol. Macroinvertebrates were identified to a family or genus level following Campaioli et al., (1994), and they were grouped into three ecological categories: swimmers, clingers, and burrowers (see Knapp et al., 2001; Tiberti et al., 2014b).

Amphibian monitoring

R. temporaria is the only amphibian species inhabiting the high-altitude lakes of GPNP. The presence of egg masses, tadpoles or adult individuals of *R. temporaria* was recorded by Visual Encounter Surveys (VES) (Crump and Scott, 1994) walking along the perimeter of each lake. The presence of adult *R. temporaria* in high altitude lakes is usually restricted to the breeding period, at the beginning of the ice-free season (Lanza et al., 1999). However, tadpoles and some adult frogs can be found during the larval development and later in the season. Therefore, the lakes have been usually surveyed twice during the summer, once during the breeding season (June- July) and once late in the season (August-September) to detect the presence of surviving tadpoles and fresh metamorphs. Between June 2006 and Sepetember 2017, 249 surveys from 18 lakes were performed. In addition, between 2013 and 2017, the population trends of *R. temporaria* were monitored through annual egg-mass counting (Crouch and Paton, 2000) in the four fish-removal lakes. Accurate counts of *R. temporaria* egg-masses are possible because their clutch size is large (up to 4500 eggs), they are easily visible in shallow transparent water, and laid simultaneously by numerous frogs in a confined area (Bernini and Razzetti, 2006). Eggs-masses were counted walking along the entire edge of each lake just after the putative breeding season during several (2-3) surveys repeated at 5-7 days intervals. The date of the first survey was decided based on the weather conditions (e.g. persistence of the snow cover) and on the lakes' features (e.g. altitude and exposure) during the usual breeding period in the study area, which was highly predictable (ice-break in June-July depending on the lakes). The position of the egg-masses was recorded at each survey as well as their developmental stage to recognize freshly laid egg-masses and avoid re-counts or count underestimates. Of 20 potential egg mass counts (4 sites for 5 years), only one was not achieved (Lake DJO in 2015) because the first survey was done after the eggs were already hatched.

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