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Methods

Methodological design to study the effects of heatwaves on natural plankton communities from Mediterranean vulnerable ecosystems

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Abstract

The Mediterranean Region is considered one of the areas most exposed to climate warming, with artificial lakes and coastal lagoons representing particularly vulnerable ecosystems, that provide essential goods and services. Amongst the extreme events linked to warming, heatwaves are of growing concern, yet their ecological effects on the functioning of Mediterranean aquatic systems remain poorly investigated. We present a methodological framework designed by the Project “*a warmer Future world: effects on plankton commUnities and paThogens in Mediterranean vUlneRable Ecosystems (FUTURE)*“ to study how natural plankton communities respond to abrupt and sustained thermal stress. The approach targets on entire plankton communities, from bacteria to zooplankton, integrates laboratory experiments and field monitoring activities and combines classical techniques with molecular tools to capture changes in biodiversity, food web size-structure and the occurrence of potentially pathogenic and antibiotic-resistant bacteria. We selected two diverse aquatic ecosystems in the western

Mediterranean as case studies: an artificial lake, an important source for drinking water and a coastal lagoon vital for fishery, both of high ecological and economic importance. By applying controlled experimental simulations with ecological relevance, the framework provides a replicable approach to investigate plankton community-level responses to heatwaves. This methodological contribution provides a comparative framework for vulnerable Mediterranean ecosystems and promotes standardised approaches to assess the impacts of extreme climate-driven events. The scalable and reproducible *protocol* presented here fills a critical regional knowledge gap and will support the effective management of climate-sensitive aquatic ecosystems.

Keywords

heatwaves, plankton community size structure, microcosms, artificial lake, coastal lagoon, Mediterranean, traditional taxonomy/molecular tools, climate change

Introduction

Global warming is increasingly disrupting aquatic ecosystems, altering plankton community structure, biodiversity and trophic interactions in both marine and freshwater environments (Jeppesen et al. 2021, Meunier et al. 2025). The Mediterranean Basin is amongst the most sensitive regions to climate change, with artificial lakes and coastal lagoons being particularly vulnerable due to their shallow depth, limited water exchange and their exposure to strong anthropogenic pressure. These ecosystems deliver essential goods and services, including drinking water, irrigation, fisheries and recreation, yet their ecological responses to thermal stress remain underexplored and insufficiently understood. Extreme climate events, such as heatwaves, are becoming more frequent, intense and prolonged, posing major threats to aquatic organisms (Garrabou et al. 2022, Marullo et al. 2023, Woolway et al. 2025). Plankton plays a foundational role in aquatic food webs and biogeochemical cycles and is highly sensitive to warming (Calbet et al. 2022, Kim et al. 2024). Rising temperatures are expected to reduce plankton diversity and to shift food web efficiency in favour of smaller-sized organisms and pathogenic bacteria (Garzke et al. 2016, Brehm et al. 2021, Courboulès et al. 2022, Zhan et al. 2024). These shifts may reduce energy transfer to higher trophic levels, thereby weakening food web resilience and diminishing ecosystem productivity. In turn, such changes threaten the ecosystem services on which both natural systems and humans depend (Bongaarts 2019). Additionally, very little is known on how the pathobiome (total content of potentially pathogenic bacteria) and its associated antibiotic-resistome (total content of antibiotic resistance genes, ARGs) may respond to climate change, mainly manifested by increasing temperature. Nowadays, it is well known that the aquatic ecosystem constitutes a reservoir of faecal bacteria potentially antibiotic-resistant and pathogenic for humans (Di Cesare et al. 2013). An increase in surface water temperature may enhance the survival of faecal bacteria, including human pathogens, as already reported for autochthonous aquatic pathogens (Vezzulli et al. 2016). Thus, it is crucial to investigate the effects of global warming on the abundance and diversity of antibiotic-resistant and pathogenic bacteria, as they pose a serious threat to human, animal and environmental health (Intergovernmental Panel on Climate Change (IPCC) 2023).

Despite these urgent concerns, standardised experimental approaches for studying heatwave effects on natural plankton communities in Mediterranean aquatic environments are still limited. Comprehensive studies on the entire plankton assemblages, from bacteria to mesozooplankton, under warming scenarios remain limited (Vidussi et al. 2010, Soulié et al. 2022, Soulié et al. 2023). Most experimental studies on plankton responses to warming have been conducted in the Baltic Sea (e.g. Müren et al. (2005), Lewandowska and Sommer (2010), Lewandowska et al. (2014), Sommer et al. (2015)) and in cold, northern lakes (e.g. Liboriussen et al. (2005), Kratina et al. (2012), Özen et al. (2012), Jeppesen et al. (2021) and references therein). Some studies employed both traditional microscopy-based identification and DNA metabarcoding (18S rRNA and cytochrome oxidase I (COI) markers) to assess shifts in phytoplankton, ciliates and mesozooplankton, but not including pathobiome and picoplankton (Hall et al. 2025). The response of phytoplankton from Cabras Lagoon (Italy) to experimental warming, assessed through laboratory incubations of a plankton community excluding mesozooplankton, was also investigated (Pulina et al. 2020). Further, in situ mesocosm experiments were conducted in Thau Lagoon (Mediterranean Sea, south France) to study heatwave effects on plankton food web components (Vidussi et al. 2010, Soulié et al. 2022, Soulié et al. 2023, Eglaine et al. 2025). These studies are restricted to coastal lagoons and there is still a lack of studies investigating plankton responses to warming in Mediterranean artificial lakes. However, existing evidence underscores the urgent need for targeted research in the Mediterranean Region.

In this context, the objective of the Project “*a warmer Future world: effects on plankton commUnities and paThogens in Mediterranean vUlneRable Ecosystems (FUTURE)*“ was to investigate the effects of climate warming on natural Mediterranean plankton communities (lagoon and artificial lake), from bacteria to zooplankton, using experimental and field approaches combined with classical analysis and molecular tools. Specifically, we aimed to assess changes in diversity, size-structure of the plankton food web and the abundance of potentially pathogenic and antibiotic-resistant bacteria during an experimental heatwave. To achieve this goal, we developed a methodological framework that integrates laboratory-based heatwave simulations, monthly field monitoring and high-throughput sequencing (Next-Generation Sequencing). This framework is designed to disentangle the direct effects of warming from indirect effects mediated by predator–prey interactions in plankton communities under controlled conditions.

In summary, the originality of this methodological design lies in its provision of a robust and adaptable protocol for investigating the effects of heatwaves on plankton communities, from bacteria (including pathobiomes and ARGs) to zooplankton, in vulnerable ecosystems. The protocol integrates field monitoring, traditional taxonomic and counting methods, heatwave simulation experiments and cutting-edge molecular techniques (amplicon sequencing of the 16S and 18S rRNA genes, shotgun metagenomics, Sanger sequencing and COI barcoding) to synergistically assess the impacts of heatwaves on plankton communities. The protocol was developed and applied in two sensitive Mediterranean ecosystems: a coastal lagoon and an artificial lake.

Design of the methodological framework

The methodological framework was developed within the Project FUTURE (https://www.lifewatchitaly.eu/en/pg-related-projects/future-about) funded by the Italian Ministry for University and Research (PRIN 2022 Programme). This Project was realised by a multidisciplinary team of experts in aquatic ecology, microbiology and data management through collaboration between two Italian interdisciplinary research units: University of Sassari (UNISS) and the National Research Council of Italy (CNR) represented by two institutes, Water Research Institute (CNR-IRSA) and the Research Institute on Terrestrial Ecosystems (CNR-IRET).

To understand the consequences of heatwaves for Mediterranean plankton communities, we developed and applied the framework in two *case studies* (an artificial lake and a coastal lagoon) in Sardinia (Italy, western Mediterranean) (Table 1, Fig. 1): Cabras Lagoon and Lake Bidighinzu. The main use of Lake Bidighinzu is the storage of drinking water, whereas Cabras Lagoon has a high economic rating due to extensive fishery activities. These sites were selected because ecological studies, especially those on phytoplankton ecology, began a few decades ago, leading to the collection of a considerable amount of information (e.g., Luglié and Sechi (1993), Padedda et al. (2010), Padedda et al. (2012), Pulina et al. (2012), Mariani et al. (2015a), Mariani et al. (2015b), Pulina et al. (2019), Pulina et al. (2022), Pulina et al. (2023)). Both sites are part of the Long-Term Ecological Research network LTER- Italy (details at https://deims.org/3707cf71-7e04-41e3-8afc-518b293f6c07, https://deims.org/d5071d21-9c8f-47cc-b534-1b1162a5e09c).

Direct assessment of marine heatwaves by application of metrics, based on the 90th percentile of a 30-year climatology (Hobday et al. 2016, Hobday et al. 2018) was not possible because long-term daily water temperature records were unavailable in the selected ecosystems. To address this limitation, we evaluated the occurrence of atmospheric heatwaves in the ecosystems using available air temperature climatology, noting that these metrics to define the heatwaves are also applicable to freshwater systems (Woolway et al. 2025). Data from January 1990 to December 2023 for Cabras Lagoon (weather station 2 km from the lagoon) and from January 1960 to December 2023 for Bidighinzu Lake (reservoir weather station) were analysed with the rerddap (Chamberlain 2015) and heatwaveR (Schlegel and Smit 2018) packages in R software (R, Core Team 2021), detecting several atmospheric heatwaves in both ecosystems. These events occurred mainly in summer, with peaks of air temperature anomalies exceeding +8°C (Fig. 2, Fig. 3, Tables 2, 3).

Table 1.

Main physical and environmental features of the study sites.

Parameter	Site	
	Lake Bidighinzu	***Cabras Lagoon***
Latitude	40°33'22"N	39°56'37"N
Longitude	8°39'41"E	8°28'43"E
Catchment area (km^2)	52.18	430.00
Waterbody area (km^2)	1.70	23.80
Mean depth (m)	10	1.6
Maximum depth (m)	39.50	3.00
Theoretical volume (m^3)	12.2×10^6	38.1×10^6
Trophic state	Eutrophic	Eutrophic

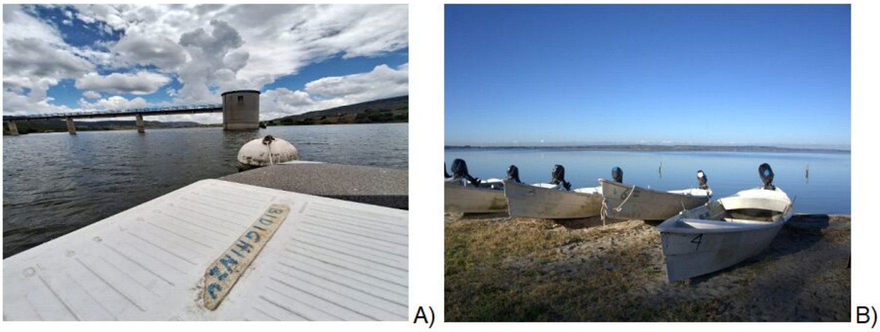

Figure 1.

Study sites in Sardinia, Italy, western Mediterranean: A) Lake Bidighinzu; B) Cabras Lagoon.

Table 2.

The five most intense atmospheric heatwaves detected in Lake Bidighinzu.

heatwaves	duration (days)	start dd/mm/yyyy	date peak dd/mm/yyyy	max intensity (°C)	cumulative intensity (°C x day)
1	5	05/04/1961	06/04/1961	10.1443	34.8457
2	6	14/10/1988	16/10/1988	9.8979	40.0282
3	5	07/08/1999	10/08/1999	9.4762	29.5485
4	8	18/02/1978	24/02/1978	9.2315	49.6100
5	5	08/10/1970	09/10/1970	8.9251	30.1159

Table 3.

The five most intense atmospheric heatwaves detected in Cabras Lagoon.

heatwaves	duration (days)	start dd/mm/yyyy	date peak dd/mm/yyyy	max intensity (°C)	cumulative intensity (°C x day)
1	6	18/09/1993	20/09/1993	9.6588	36.2900
2	5	21/05/2009	24/05/2009	9.2923	35.0736
3	5	02/07/1993	05/07/1993	9.1785	30.2898
4	10	10/10/1990	12/10/1990	8.6305	51.8533
5	6	15/09/2023	17/09/2023	8.6271	34.2165

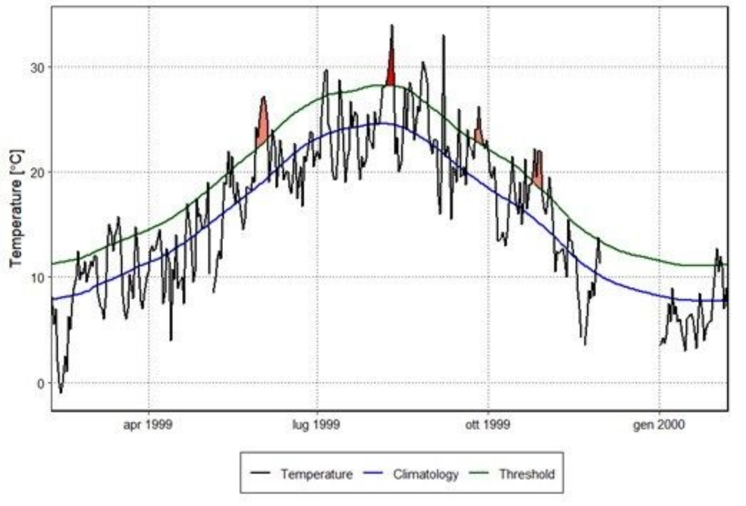

Figure 2.

The most relevant heatwave events (in red) detected in Lake Bidighinzu.

Methodological approaches

The methodological framework is designed as an integration of three complementary approaches. By applying this framework across ecosystems with contrasting characteristics (e.g. depth, salinity gradients, nutrient availability and hydrological dynamics), we gain a broader understanding that enhances the framework applicability and enables the collection of data on the ecological consequences of heatwaves for plankton communities of various ecosystems. The three approaches are:

1. Field-based one-year monthly monitoring to characterise the natural seasonal dynamics of plankton communities across temperature gradients (*status quo*);
2. Laboratory simulation of a 14-day summer heatwave to investigate effects on natural communities;

3. Advanced molecular tools for screening biodiversity, particularly for microbial components including potentially pathogenic bacteria.

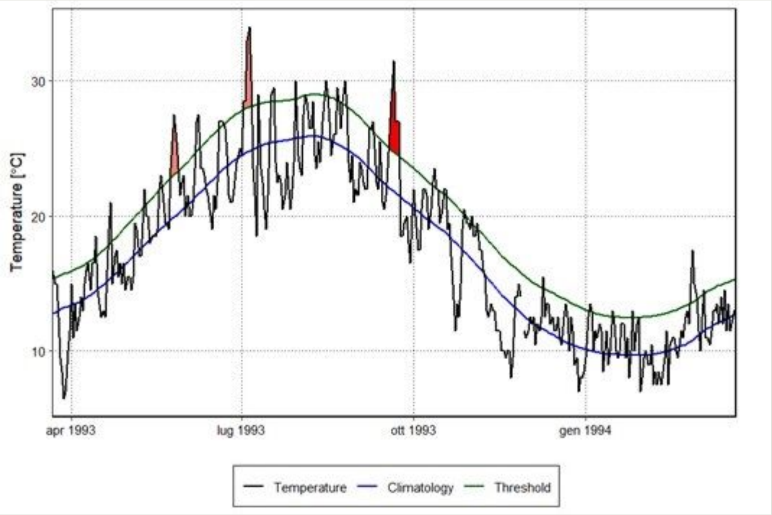

Figure 3.

The most relevant heatwave events (in red) detected in Cabras Lagoon.

1.Field-based one-year monthly monitoring

The sampling strategy differed between the two ecosystems due to their physical and environmental features. Monthly sampling was conducted at the deepest point in the lake (approx. 40 m), while in the lagoon, sampling was performed at three points (1: 39°56'01.1"N 8°30'48.8"E; 2: 39°57'08.5"N 8°29'06.9"E; 3: 39°58'53.0"N 8°30'26.6"E) along the salinity gradient from the sea inlet to the main freshwater inflow (Fig. 4).

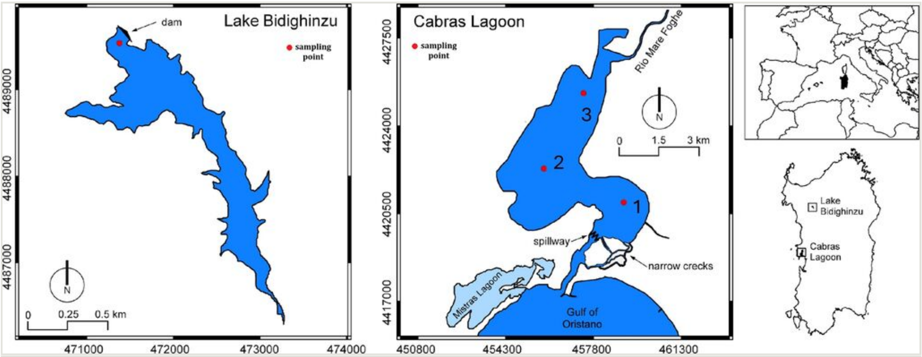

Figure 4.

Sampling points at Lake Bidighinzu and Cabras Lagoon (UTM zone 32 datum WGS84), western Mediterranean.

Sampling was conducted during the second ten days of each month from January to December 2024, with timing adjusted for weather conditions. Parameters measured monthly were:

- water temperature, pH, dissolved oxygen, conductivity/ salinity/ turbidity and Chlorophyll *a* were measured at each site (lake and lagoon) and at each sampling point by multiparameter probe (HL7, OTT-Hydrolab), equipped with various sensors. Measurements were taken along the entire water column profile until reaching 1 m from the bottom in each site. The probe was checked and, if necessary, recalibrated before each use. Certified standards were employed for verification and calibration. Specifically, for pH at 7 and 10 units, for conductivity at 10 m/S, for oxygen in water saturated at 100% and in water at 0% and for temperature using a precision thermometer;
- water transparency measured with a Secchi disc;
- concentration of algal nutrients (nitrate $N\text{-}NO_3$, nitrite $N\text{-}NO_2$, ammonium $N\text{-}NH_4$, orthophosphate $P\text{-}PO_4$ and silicate $Si\text{-}SiO_4$) carried out spectrophotometrically following Strickland and Parsons (1969) and American Public Health Association, American Water Works Association, Water Environment Federation (2023). Dissolved Inorganic Nitrogen (DIN) was obtained by summing up $N\text{-}NO_3 + N\text{-}NO_2 + N\text{-}NH_4$. These nutrients were measured in water samples collected at the sampling point of the Lake, using a Niskin bottle at 0.30, 1, 2.5, 5, 7.5, 10 and 15 m depth and subsequently at intervals of 10 m until reaching 1 m from the bottom. Water samples in the lagoon were collected at 0.30 m below the surface at the three sampling points (see Fig. 4);
- water samples for planktonic components (size fractions) were collected in the Lake from the surface to 10 m depth. Lagoon samples were collected 0.30 m below the surface at three sampling points. All samples were analysed in laboratory for: autotrophic and heterotrophic *picoplankton* (cell size 0.2-2 μm); autotrophic and heterotrophic *nanoflagellates* (cell size 2-20 μm); *phytoplankton* (cell size > 3 μm); and *microzooplankton* (body size 20-200 μm);
- *Mesozooplankton* (body size > 200 μm) was collected with a plankton net (80 μm mesh size, 30 cm diameter) by vertical hauls in the layer 10-0 m in the Lake. In the Lagoon, samples were collected at each sampling point using horizontal net hauls at a depth of 0.30–0.50 m below the surface. Haul speed and duration were recorded to calculate the filtered volume, given the Lagoon's shallow maximum depth (3 m). We used 80 μm mesh size net for capturing also juvenile stages of certain organisms (e.g. copepods, ctenophores) and larger microzooplankton species (e.g. rotifers). From that point onwards, we refer to this component as 'mesozooplankton' or 'larger zooplankton' to distinguishing it from the microzooplankton fraction.

All samples were immediately transported to the laboratory (within 5 h) and analysed with *traditional techniques* (optical microscopy and flow cytometry) for each size fraction. Seasonally, an additional aliquot of water was collected from the central sampling point of Cabras Lagoon and from the sampling point of Lake Bidighinzu for identification of eukaryotic and bacterial community composition with advanced technological approach (e.g. Next-Generation Sequencing), enabling taxonomic and functional insights, particularly for microbial components and potentially pathogenic and antibiotic-resistant bacteria.

Optical microscopy and flow cytometry

Picoplankton (0.2-2 µm) was fixed by adding 200 µl of a sterile filtered solution, containing 10% formaldehyde and 0.5% glutaraldehyde, to a total volume of 2 ml of sampled water and stored in dark condition at -80°C until further analysis. Samples were thawed at room temperature and processed by a flow cytometer. Picocyanobacteria were quantified using the CytoFlex flow cytometer (Beckman Coulter), integrating signals derived from light scattering (forward and side light scatter named FSC-H and SSC-H, respectively), fluorescence of phycoerythrin (PE channel = 585/40 nm), fluorescence of allophycocyanin (APC channel = 660/10 nm) and fluorescence of Chlorophyll-*α* (PC5.5 channel = 690/50); larger autofluorescent cells (mainly eukaryotic) were enumerated counting the cells that form a distinct cloud in the cytograms with higher PC5.5 signal. Total bacterial cells were quantified, after staining with SYBR Green I Nucleic Acid Gel Stain (Invitrogen), combining signals from FSC-H and SSC-H and fluorescence on FITC channel (525/40 nm). Flow cytometry data were analysed with CytExpert software 2.4.0, provided with the flow cytometer. Manual gating was applied for all samples to allow optimal detection as well as distinction amongst populations (Di Cesare et al. 2020).

Nanoflagellates (autotrophic and heterotrophic with cell size 2-20 µm) were analysed using epifluorescence microscopy (Zeiss, Axiovert 100). Samples were preserved with a 25% glutaraldehyde filtered solution and fixed sub-samples were filtered on to 0.8 µm black-stained polycarbonate membranes. Heterotrophic nanoflagellate samples were stained with DAPI before filtration. Duplicate slides were prepared and observed with a microscope with a blue filter, set for counting autotrophic flagellates and DAPI filter set for the heterotrophic ones. At least 30 random fields of view were counted for each slide at 1000× magnification. Cell sizes of about 30 randomly selected cells were measured on each slide.

Phytoplankton and Microzooplankton fractions were analysed according to the Utermöhl technique (Utermöhl 1958). Density was determined from sub-samples fixed with 2% acid Lugol's solution using an inverted light microscope. Counts were made at a magnification of 100× on the entire bottom of the settling chamber for the larger and more easily identifiable species and at magnifications of 200× and 400× from at least 10% of the total bottom area of the settling chamber for smaller species. For species identification, fresh samples were also observed, aiming to reach the highest certain taxonomic level. At least 20 individuals of each taxon were measured in each sample for calculating biovolume. Cell volumes were calculated approximating the shape of each taxon to a geometric shape following Hillebrand et al. (2002), Vadrucci et al. (2013) and "Atlas of Shapes" powered by LifeWatch Italy (https://www.phytovre.lifewatchitaly.eu/vre/shapes-groups). For each taxon, the cell carbon content was obtained by applying the conversion formulas to the mean cell volume suggested by Menden-Deuer and Lessard (2000).

Mesozooplankton samples were fixed with ethanol. Taxonomic identification was performed to species level when possible; when not feasible, organisms were identified to higher taxonomic levels (genus, family or order/class) using a compound microscope. Abundance was calculated by counting individuals in a HydroBios counting chamber using a subsample comprising at least 10% of the total sample volume. Morphological traits, such as the body length/width of at least 20 individuals measured for each taxon (or taxonomic group) in a sample, were measured by digital images and open-source Image J software (https://imagej.net/ij/), together with identification of geometric shapes to estimate biovolumes of the animals. The biomass was estimated by converting body length measurements to dry weight using published length–weight regressions (Downing and Rigler 1985) and biovolume to weight equations, assuming taxon-specific conversion factors. In addition, seasonal samples were collected also for molecular taxonomical identification of the species (e.g. DNA barcoding).

2. Heatwave simulation experiment

Set-up

Two independent laboratory experiments were carried out in summer 2024 to simulate plankton community responses to heatwave. Summer was selected because the strongest marine heatwaves in the Mediterranean occurred during this season (Martínez et al. 2023), which is also consistent with recent findings indicating an intensification of summer sea surface temperature trends in the region (Calbet et al. 2022, Ciappa 2022). Using natural plankton communities, the lake experiment was conducted from 1 to 15 July 2024, while the lagoon experiment from 23 July to 6 August 2024. Water samples were collected at the Lake on 1 July, while samples from the Lagoon on 23 July 2024 and immediately transferred to the laboratory. Further, the entire plankton community was incubated in semi-transparent plastic buckets and exposed in triplicate for 14 days. For each 14-days experiment, two combined treatments were applied to the incubated planktonic community:

Treatment 1) +5°C increase in water temperature compared to the control units at environmental temperature. Environmental temperature in the control units corresponded to *in situ* average water temperature of each site in July, based on long-term data. A +5°C increase in temperature represented the maximum sea surface temperature anomaly recorded in the Mediterranean (Marullo et al. 2023). It is also the maximum intensity value detected during the marine heatwaves observed in the last decade in the Mediterranean, all categorised as “severe” events (Martínez et al. 2023). Moreover, a +5°C increase in water temperature has been also used in one of the few previous studies investigating the heatwave effects on a Mediterranean coastal lagoon plankton community (Soulié et al. 2023).

Treatment 2) presence/absence of mesozooplankton: to understand whether the effect of the simulated heatwave on the plankton community was directly or indirectly mediated by predator-prey dynamics (presence and absence of the larger zooplankton).

For each laboratory experiment (Lake and Lagoon), 12 semi-transparent plastic buckets (incubation units) of 10 litres each were used, corresponding to a combination of the two applied treatments (Treatment 1 and Treatment 2) and consequently to four different experimental conditions:

- natural plankton community at *in situ* environmental (natural) temperature with mesozooplankton (Env M);
- natural plankton community at *in situ* environmental temperature without mesozooplankton (Env);
- natural plankton community at +5°C above *in situ* environmental temperature with mesozooplankton (HW M);
- natural plankton community at +5°C above *in situ* environmental temperature without mesozooplankton (HW).

Each treatment was performed in triplicate (Fig. 5).

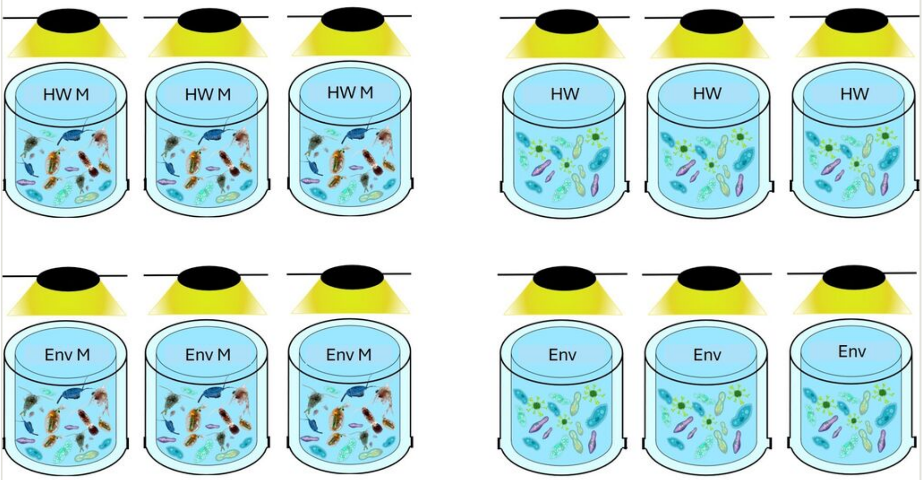

Figure 5.

Experimental design with 12 incubation units: 3 units HW M (heatwave and mesozooplankton), 3 units HW (heatwave treatment without mesozooplankton), 3 units Env M (environmental temperature and mesozooplankton), 3 units Env (environmental temperature without mesozooplankton).

A water heater connected to a thermostat was inserted in each unit. The thermostats permitted us to regulate and keep constant the temperature in all buckets throughout the experiment. For warming conditions, the target temperature was reached by gradually increasing value during the first 48 hours. In each bucket, water was constantly and gently mixed. During the experiment, the evaporated water was replenished once per experiment with pre-filtered (0.2 µm) autoclaved water collected from the site of sampling. In both experiments, light conditions simulated the summer season with a Photosynthetically Active Radiation (PAR) of 2000 µmol photons m^{-2} s^{-1} (air light intensity) in a 15:9-hour light/dark cycle.

The two experiments differed in the setup for the larger zooplankton (mesozooplankton). Although the overall goal was to design a reproducible experimental set-up, the experiments were not intended to be directly comparable between the two systems, given the intrinsic differences between the ecosystems. For this reason, the inclusion or exclusion of mesozooplankton to the experimental units was carefully designed and adapted for each study site. We considered the shallow depth and the massive presence of the non-native ctenophore *Mnemiopsis leidyi* in the Lagoon. On the other hand, the depth of approximately 40 m maximum of the Lake, allowed effectively to collect zooplankton with a vertical net. Consequently, we adopted two different mezooplankton approaches to account for ecosystem-specific characteristics, but maintaining the same experimental rationale and targeting the same ecological processes in the experiments. For the "*Lake experiment*", mesozooplankton included in the buckets was collected by two vertical net hauls from 0 to 10 m depth, corresponding to approximately 600 litres of filtered lake water. This sample was used to reproduce natural zooplankton densities (approximately 100 ind. l^{-1}), based on densities observed in the field monitoring. After collection, zooplankton samples from the two net hauls was gently mixed and transfered to the laboratory with minimal stress, and then gently mixed and equally aliquoted into the six experimental units of 10 litres in the treatments with larger zooplankton (Env M, HW M - Fig. 5). In contrast, in Cabras Lagoon, the shallow depth (approximately 3 m) and the high abundance of *M. leidyi*, already abundant in the Lagoon from May onwards, made vertical and horizontal net hauls impractical, due to both logistical constraints and frequent clogging of the net. Therefore, the community sampled from sampling point 2 (Fig. 4) was incubated "*as it is*" in the "*Lagoon experiment*" treatments with mesozooplankton (Env M, HW M) to preserve the natural community composition. A filtration through a 200 µm mesh was applied only to the control treatments to exclude larger zooplankton (Env, HW - Fig. 5).

Timing

The 14-day experiments were set up, so the *Lake experiment* was conducted from 1 to 15 July 2024, while the *Lagoon experiment* from 23 July to 6 August 2024. An aliquot was collected at the start of the incubation (T0) and regularly at different time (T1, T2, T3, T4 - Fig. 6) throughout the experiment from each unit at 30 cm below the surface for algal nutrient analyses and for the analyses of picoplankton and nanoflagellates (autotrophic and heterotrophic), phytoplankton and microzooplankton. To compensate for evaporation during the incubation period, lost water was replenished once per experiment (at T2 or at Day 7 for the Lake community and at T3 at Day 9 for the Lagoon community) with pre-filtered with 0.2 µm autoclaved water collected from the site of sampling (Fig. 6).

Over the experiments, temperature, pH, dissolved oxygen and conductivity were monitored daily directly in each unit using the multiparameter probe. PAR was also measured daily as well, with a Light Meter (Li-Cor LI-250A Light Meter). Water samples for molecular analyses of eukaryotic and bacterial community composition, as well as for assessing the richness of the antibiotic resistome and the abundance of ARGs, were collected from each unit at the start (T0), before the replenishment of lost water (at T2 of the *Lake experiment*; at T3 of the *Lagoon experiment*) and at the end of each experiment (T4). Mesozooplankton from each experimental unit was analysed at the start (T0) and at the end of the experiment (T4).

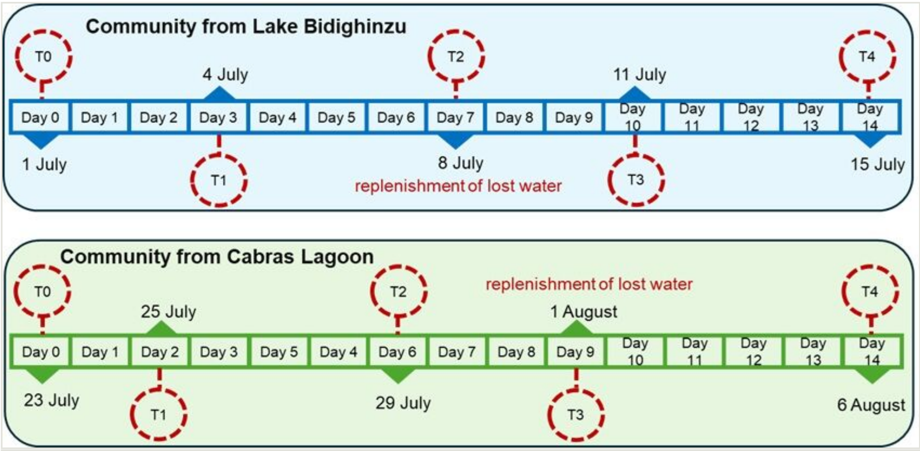

Figure 6.

Time-schedule of the 14-day heatwave simulation experiments for Lake Bidighinzu (upper panel) and Cabras Lagoon (lower panel): start-day (T0), sampling during the experiment at specific days (T2, T3) and the end (T4) of the experiment (day 14); replenishment of lost water (once per experiment with pre-filtered autoclaved water from the sampling site) was undertaken to compensate for evaporation.

3. Molecular approach

An aliquot of water samples collected from the field monitoring was passed through a series of sieves/filters (20 µm, 3 µm and 0.22 µm porosity size) and processed for amplicon sequencing. Another aliquot was directly filtered on to a 0.22 µm porosity size filter to be processed for shotgun metagenomic sequencing. Waters from the experiments were directly filtered on to a 0.22 µm porosity size filters, used for both amplicon and shotgun metagenomic sequencings. All samples were processed for DNA extraction using commercial DNeasy UltraClean Kit (QIAGEN), following the manufacturer's instructions. The quality of the obtained DNA was spectrophotometrically checked and its concentration fluorometrically measured.

Eukaryotic and bacterial community composition were characterised using 18S rRNA gene and 16S rRNA gene amplicon sequencing, respectively, whereas the antibiotic resistome in the integrated samples was assessed by shotgun sequencing. The dynamics of potentially pathogenic bacteria and their co-occurrence with ARGs were analysed to evaluate the potential risk to human health in the scenario of global warming. The DNA sequences obtained from 18S and 16S amplicon sequencing and shotgun metagenomic sequencing were processed by means of bioinformatic tools. Specifically, for 18S and 16S rRNA gene data, sequences were elaborated using the DADA2 pipeline, following the instructions of the online tutorial available at https://benjjneb.github.io/dada2/tutorial.html\h. The taxonomic assignment was done through the PR2 and Silva databases, respectively. All the data processing were performed in the R environment (R, Core Team 2021). For the shotgun metagenomic sequencing, raw reads were cleaned and trimmed. After trimming, reads were analysed for the ARG-like sequences (hereafter ARGs) with the Resistance Gene Identifier (RGI, bwt mode) and aligned against the Comprehensive Antibiotic Resistance Database (CARD), including also CARD's Resistomes & Variants data contained in the WildCARD reference database. The abundance of ARGs was expressed as gene copies per 16S rRNA gene copy. Given the implications for human health, a dataset of potentially pathogenic bacteria was prepared by selecting, after the DADA2 workflow, the genera that include clinically relevant pathogenic species, as listed by Bartlett and colleagues (Bartlett et al. 2022).

Similarly, from the total ARG dataset, genes classified in risk I and II ranks, according to what was previously described by Zhang and co-authors were taken (Zhang et al. 2021). Contigs were recovered from shotgun metagenomic sequences after the assembly of trimmed reads using metaSPAdes. To investigate contig antibiotic resistome, the prodigal-annotated proteomes were analysed with RGI (main mode) against the CARD and WildCARD reference database. After assembly, metagenome-assembled genomes (MAGs) were obtained using three different tools, for example, MaxBin2, Metabat2 and CONCOCT. Then, DASTool was used to optimise the output. Resulting bins were evaluated for their quality using CheckM. Genomes with a quality score ≥ 50 were classified as high quality MAGs and further analysed. MAGs were annotated for the taxonomy against the Genome Taxonomy Database (GTDB), allowing us to identify the bacterial hosts for ARGs.

Further, specimens from the mesozooplankton fraction collected during the field monitoring were processed for Sanger sequencing (DNA barcoding) to complement the classical taxonomic identification by optical microscopy with the molecular species profile. For this purpose, two genetic markers (18S rRNA and COI genes) were used to enable species-level identification. DNA extraction was performed using InstaGene Matrix (Bio-Rad) according to the manufacturer’s instructions, followed by PCR amplification and sequencing of each target.

Finally, a Data Management Plan (DMP) was developed to cover all data collected during the study, ensuring proper management of the data lifecycle and facilitating data accessibility for use and reuse.

Transferability, limitations and future perspectives

Our motivation for developing and documenting this methodological framework stems from the need to support scientists and environmental managers both in conducting similar studies and in relying on results that yield robust and comparable data. This " *protocol*" designed to study the effects of heatwaves on natural plankton communities from Mediterranean vulnerable ecosystems could be broadly applied and adapted to diverse ecosystems, as demonstrated by its application to an artificial lake and to a coastal lagoon. Some limitations may be encountered when performing heatwave experiments, but all can be mitigated through appropriate measures. For instance:

1. *Logistical constraints*: Temperature control in indoor microcosms may be challenging. Power supply stability and precise temperature regulation equipment can mitigate this limitation;
2. *Microcosm size and complexity*: Although microcosms and laboratory performed experiments offer controlled conditions, they might not fully replicate natural spatial and trophic complexity. Specifically, the exclusion of higher trophic levels may influence the cascading effects of warming on plankton through top-down control;
3. *Molecular resolution*: While metagenomics is reliable for characterisation of microbial communities, including microorganisms that cannot be cultivated under laboratory conditions and represent the majority in environmental samples, it provides limited resolution at a species level and does not permit identification at the strain level. Changes in abundance and biomass cannot be reliably assessed using molecular analyses alone. However, coupling traditional taxonomic identification and counting with molecular methods, as proposed here, can mitigate this limitation.

The originality and utility of the proposed methodological framework are reflected in two main aspects:

1. the combination of both, field monitoring and controlled heatwave simulation experiments;
2. the combination of traditional counting methods and advanced molecular tools.

The strength of the methodology lies in integrating indoor heatwave experiments, those capturing the direct effects of warming on natural plankton communities across multiple trophic levels (including bacteria and pathogens), with field monitoring studies that reveal plankton dynamics along natural temperature gradients. Long-term monitoring data collected over decades are invaluable for understanding ecosystem responses to diverse perturbations (Magurran et al. 2010), including heatwave events. Therefore, sustaining systematic long-term monitoring programmes is essential. Further, the integration of field/monitoring survey and traditional counting methods (e.g. optical microscopy, flow cytometry) with molecular advanced techniques, enhances methodological robustness by uniting quantitative information on abundance and biomass with high-resolution taxonomic and functional insights. The capacity of metabarcoding for estuarine plankton monitoring has been evaluated by comparing results obtained with this approach with those based on traditional taxonomic analyses (Abad et al. 2016). Some studies compared zooplankton composition identified through COI and 18S rRNA genes markers to traditional identification by microscopy, exploring the relationship between biomass and the relative abundance of sequences (Djurhuus et al. 2018). Further, the environmental DNA (eDNA) metabarcoding can represent a valuable tool for early detection of non-native species; however, it requires the combination of molecular and morphological approaches (Varrella et al. 2025), confirmed also by our preliminary and highly promising results combining optical microscopy with molecular tools.

The utility of the methodology is in providing a reproducible and adaptable "*protocol*" for assessing climate-driven changes in aquatic ecosystems across diverse environments. Our preliminary results from two case studies in the western Mediterranean, encourage the application of this framework to other ecosystems. The protocol is flexible, as it can be adjusted to available resources and research objectives. Rather than comparing ecosystems with fundamentally different characteristics, it permits assessment of the effects of heatwaves on plankton communities, regardless of ecosystem type. This approach is essential not only for advancing our understanding of plankton dynamics under climate stress, but also for providing harmonised and coherent information to authorities implementing adaptation strategies in vulnerable areas.

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Ethics and security

The authors declare no ethical issues and no security concerns related to this research and its publication.

Author contributions

S.P., L.K., B.M.P., J.T., I.R. and A.D.C. conceived the ideas and designed methodology. B.M.P. collected the data for the atmospheric heatwave statistical analyses and analysed the data. S.P., L.K., J.T. led the writing of the original manuscript. All authors (S.P., L.K., J.T., B.M.P., R.P., I.R, R.S. and A.D.C.) have contributed critically to the drafts, gave final approval for publication and agreed to be accountable for all aspects of the work.

Conflicts of interest

The authors have declared that no competing interests exist.

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