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Population Status and Ecology of the Steno-Endemic Fairy Shrimp *Chirocephalus sibyllae* Cottarelli and Mura, 1975 Inhabiting a Mountain Temporary Pond (Central Italy)

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Abstract: High-elevation ephemeral waters are sentinels of climate change, as they quickly respond to decreasing precipitation levels and increasing air temperatures. Fairy shrimps are among the most threatened invertebrates in ephemeral waters, as they are extremely vulnerable to habitat loss. *Chirocephalus sibyllae* is a fairy shrimp endemic to the Palazzo Borghese temporary pond, located within Sibillini Mountains National Park (Central Italy). The aims of the present study were to: (i) evaluate the physicochemical characteristics of *C. sibyllae* habitat, with special reference to climate changes over twenty years; (ii) document the life history, size, and abundance of *C. sibyllae*; and (iii) document the coexisting zooplankton fauna in Palazzo Borghese pond. The zooplankton community was monitored fortnightly, during the filling phases of the pond, from April 2019 to June 2021, using an 80 μm mesh net, within transects of known length. On each sampling occasion, 13 physicochemical parameters were measured, and water-level fluctuations and pond surface area were recorded. Compared to what was reported in the literature, in the last two years the wet phase of the Palazzo Borghese pond was shorter, and the pool dried up much earlier than in the past. The water quality was good and reflected the typical characteristics of high-mountain oligotrophic ponds. Orthophosphates seem to play a key role in zooplankton population abundance, increasing over time during the filling period. According to the extreme unpredictability of environmental features, the zooplankton community was composed of a very limited number of species, adapted to face drought conditions for most of the year. *C. sibyllae* life-history traits showed a high number of cysts in the broodpouch of ovigerous females (mean ± SD = 66.0 ± 38.9), and a higher mean total length of adults (1.72 cm for females and 1.76 cm for males), in comparison to data reported in the literature. The year 2019 was configured as the season with the most favorable conditions for the development of *C. sibyllae*; in 2020, the short duration of the pond did not allow the species to complete its life cycle. Climate change seems to pose the main threat to the species, considering that the progressive increase in air temperatures and the decrease in snowfall will, likely, lead to increasingly shorter filling phases of its habitat.

Keywords: fairy shrimp; steno-endemic species; high-elevation pond; ephemeral waters; climate changes; biodiversity conservation

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1. Introduction

Climate change is one of the most severe threats to inland waters, including the rich biodiversity they host and the ecosystem services they provide [1–4]. The reason for this vulnerability is that inland waters are isolated, often fragmented, heavily exploited, and are, already, afflicted by multiple anthropogenic stressors [5,6]. This is particularly true for the Mediterranean area, where an increase in the intensity and frequency of hot temperature extremes and a greater severity of drought events is expected in the near future [7].

In particular, high-elevation ephemeral waters are among the most sensitive sentinels of climate change [1,8], as they are affected more quickly than other ecosystems, by the effects of decreasing precipitation levels and increasing air temperatures [3]. In fact, their hydrological cycle is driven, mainly, by the duration and timing of snow cover and melting; moreover, air temperature is a key parameter, affecting many aspects of the ecology of aquatic invertebrates [9,10]. In temporary alpine ponds, water temperature could be unsuitable for optimal growth and the development of aquatic invertebrates, especially during the final stages of drying [9].

Therefore, analyzing the response of these vulnerable ecosystems to global warming's effects could be extremely useful in understanding the future impacts of global climate changes on inland waters biodiversity and predicting how they will influence the ecophysiology and development of the cold-adapted stenotherm species inhabiting high-elevation ponds [9]. Often, these ecosystems fall within national parks or protected areas, so it is easier to analyze cause–effect relationships than in more altered environments, where outcomes could be affected by other stressors. The ability to predict future scenarios is playing an increasingly important role, in supporting the development of mitigation strategies, capable of reducing the impacts of climate change on freshwater biodiversity [11,12].

Fairy shrimps (Anostraca, Branchiopoda, Crustacea) are among the most threatened invertebrates in ephemeral waters [13]. They are extremely vulnerable to habitat loss because many species are endemic, and many populations inhabit small temporary basins. The dormant stage in fairy shrimps is represented by resting eggs that can live for decades, while the active individuals appear during the filling phase of the pond [13]. They often exhibit plasticity in their life cycle, in response to seasonal changes in temperature and to the duration of the wet phase [9], but it is not clear to what extent they will be able to cope with the effects of climate change, without incurring local extinction phenomena. This is a crucial point in the evaluation of their extinction risk, which should be recognized (i.e., inclusion in national and international red lists of threatened species), based on the analysis of long-term environmental and biological data series [14]. For instance, out of a total of 53 species belonging to the genus *Chirocephalus* Prévost, 1803 [15], only 3 species are included in the red lists of the International Union for Conservation of Nature (i.e., *Chirocephalus croaticus* (Steuer, 1899), *C. reiseri* Marcus, 1913 and *C. pelagonicus* Petkovski, 1986) and listed among the vulnerable species [16].

Chirocephalus sibyllae Cottarelli and Mura, 1975 is a fairy shrimp endemic to the Palazzo Borghese pond, a small ephemeral pool located within Sibillini Mountains National Park (Central Italy) [17,18]. From a phylogenetic point of view, its origin is relatively recent, likely dating to the last Pleistocene glaciation event [19,20]. It is a species of considerable conservation interest, represented by a single population with a range limited to a single, extremely unstable, biotope, whose existence is closely linked to climatic conditions. Additionally, the restricted thermal tolerance and the reduced dispersal ability contribute to the vulnerability of the species [17]. The only possibility for the species to colonize new environments is, exclusively, linked to the production of resting eggs that can survive drought and be passively transported, for example, by water birds, grazing animals, and wind [15,21]. However, this hypothesis is considered unlikely, given that the presence of the species has never been detected in another temporary basin, occurring a short distance away in the same mountain massif, Pilato Lake, where another steno-endemic species (i.e., species known only from a single locality, *sensu* Ruffo and Vesentini [19]) of fairy shrimp, *Chirocephalus marchesonii* Ruffo e Vesentini, 1957 occurs; more likely, both *Chirocephalus* could be considered relict species occupying their own evolutionary refuge area [17].

C. sibyllae was described by Cottarelli and Mura in 1975. Since then, the information reported in the literature, regarding the biology and ecology of the species in nature, has been scarce. Only one paper on the life cycle of the species in nature is available [22]. Information on the characteristics of the Palazzo Borghese pond, and on the composition of the zooplankton community that it hosts, is, also, limited. Currently, the main threats to *C. sibyllae* seem to be represented by the vulnerability of the habitat to global climate change and by tourism. Low winter snowfall observed in recent years, combined with increasing summer temperature, has led to increasingly frequent episodes of the early drying up of the Palazzo Borghese pond, which caused concern regarding the survival of the species. For *C. sibyllae*, tourism and grazing animals can represent an additional threat to the species and to the integrity of the cysts deposited in the sediments, which, during the summer months, remain exposed to the trampling of hikers and livestock. This study, funded and promoted by Sibillini Mountains National Park, started in 2019 with the following objectives: (i) to evaluate the physicochemical characteristics of *C. sibyllae* habitat, with special reference to climate changes over twenty years; (ii) to document the life history, size, and abundance of *C. sibyllae*; and (iii) to document the coexisting zooplankton fauna in the Palazzo Borghese pond.

2. Materials and Methods

2.1. Biology and Ecology of Chirocephalus sibyllae

The life cycle of *C. sibyllae* is univoltine and is characterized by rapid growth and short duration, according to the rapid seasonal evolution of habitat conditions [22]. It coincides with the pond wet phase and, usually, lasts from April to the beginning of June. The larval stages of the species, generally, appear shortly after the creation of the basin, which is fed by the spring melting of the surrounding snow, and disappear with the drying up of the pond, which occurs after about six to eight weeks. The survival of *C. sibyllae* is ensured, by the production of desiccation-tolerant dormant eggs (cysts). Cyst hatching begins when environmental conditions become favorable and proceeds in different asynchronous stages, so that the simultaneous presence of different development stages could be observed [22]. There is no certainty about the mechanisms that induce the end of the dormant phase in the cysts of *C. sibyllae*. Some authors have formulated hypotheses for other species belonging to the genus *Chirocephalus*; according to their findings, the triggering factors could be thermal excursions [23] or the thickness of the overlying layer of water [24]. However, experiments conducted in the laboratory have not confirmed these hypotheses for *C. sibyllae*, whose cysts hatch within rather wide ranges of temperature and water depth [22].

The average total length of adults reported in the literature is equal to 16 mm [22]. In males, the color is a uniform light orange, and in females, the head and thorax are light orange, while the abdomen and the ovisac of adults take on a greenish color. Sexual dimorphism is characterized by the morphology of the second pair of male antennae, with the distal article longer than the proximal and the apophysis of the proximal article short and squat [22,25].

2.2. Study Area

The Palazzo Borghese pond (Lat 42° 52′ 11′′ N; Long 13° 14′ 33′′ E) is located in the Apennine chain (Central Italy) (Figure 1). It is a temporary pond of karst-glacial origin, located at 1702 m a.s.l., in pasture land that is enclosed in a cirque basin [17]. The Palazzo Borghese pond fills each spring with water from snowmelt, and it, generally, dries up after one to two months. At the end of the snowmelt, which, usually, coincides with the end of May, the pond reaches its maximum hydrometric level, with a depth of 2.5 m and a water volume of 5000 m^3 [22]. Based on these characteristics, it can be classified as a "predictable melt pond" *sensu* Belk [26], showing a temporary but periodic and predictable hydrological cycle, compared to temporary pools that receive water exclusively by rain [8].

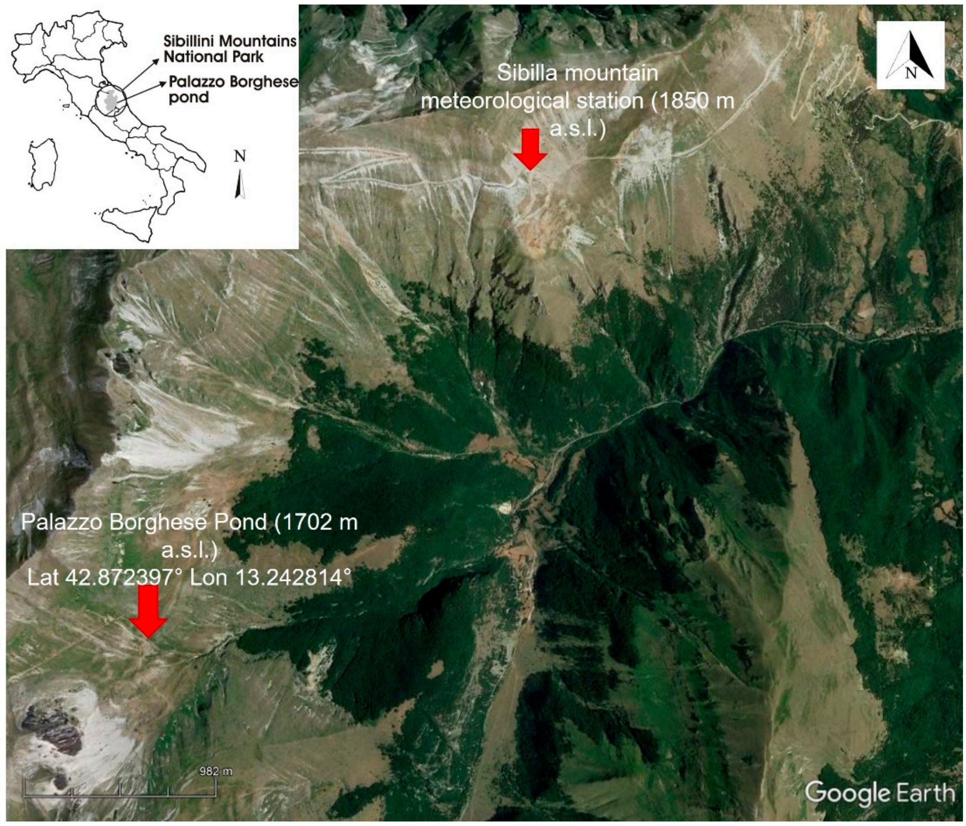

Figure 1. Study area and location of the meteorological station on Sibilla Mountain. © 2022 Google.

However, the hydrological cycle of the Palazzo Borghese pond is extremely variable and strictly connected to the climatic conditions, with special reference to the winter snow cover thickness. Additionally, the karst geology of the valley and the soil permeability contribute to increasing the speed with which the desiccation of the pond occurs.

2.3. Data Collection

1. Environmental characterization

Physicochemical, geomorphological, and biological data were collected during the filling phase of the pond, approximately every two weeks, for three sampling seasons: from 27 April to 9 June 2019, from 4 May to 14 May 2020, and from 14 May to 11 June 2021. In the other periods of the years 2019–2021, the sampling activity was prevented by the drought of the pond. On each sampling date, 13 physicochemical parameters were measured (supplementary material, Table S1). Electric conductivity (μS cm^{-1}), pH (units), water temperature (°C), and dissolved oxygen concentrations (mg L^{-1}) were measured near the pond shore, at a depth of approx. 10 cm, at the time of biological sampling (approx. 12:00 pm), using electronic meters (YSI, Yellow Springs, OH, USA; Hanna Instruments, Padova, Italy; WTW GmbH, Weilheim, Germany). Water samples were collected in 1 L polyethylene bottles, according to environmental protection agency specifications [27,28] for the analysis of chlorides (mg L^{-1}), ammonia (mg L^{-1}), nitrites (mg L^{-1}), nitrates (mg L^{-1}), orthophosphates (mg L^{-1}), BOD_5 (mg L^{-1}), and COD (mg L^{-1}) in the laboratory. To produce a bathymetric map, the surface area and perimeter of the pond were determined, using a portable GPS (Garmin GPSMAP 64S); for this purpose, on each 2019 sampling date, a walk around the pond was undertaken to georeference the shoreline. Temporal changes in the surface area were, also, characterized using aerial images collected during flights of an unmanned aerial vehicle, equipped with a camera. To investigate local trends in climatic factors, with the potential to affect the zooplankton community composition and population densities of *C. sibyllae*, we processed a twenty-year data series of mean daily air temperatures, from 2002 to 2021, from the Sibilla Mountain meteorological station, located at 1850 m a.s.l., at a short distance from the Palazzo Borghese pond (Figure 1). Air temperature data were provided by the Marche Region Meteorological-Hydrological Information System—Civil Protection Service.

2. Zooplankton community composition and *C. sibyllae* population status

To characterize zooplankton community composition, including *C. sibyllae* densities of each life stage, five quantitative samples during each sampling date were collected. During each survey, an Apstein plankton net (diameter: 30 cm; height: 90 cm; mesh size: 80 μm) was used to carry out horizontal catches, which were made at a depth of approx. 10 cm below the water surface, along 5 transects of known length (range 22–66 m), placed to explore a significant number of representative stretches of the pond (Figure 2). The choice to perform horizontal catches at a short distance from the surface is linked to the shallow depth of the pool, which did not allow vertical samplings. The volume of water filtered from the plankton net (V = A × L, in m^3) was calculated using the mouth area (A, in m^2) and the transect length (L, in m). At the end of each haul, the sample was concentrated into a 0.24 L Plexiglas collector at the end of the net and preserved using a 4% formaldehyde solution.

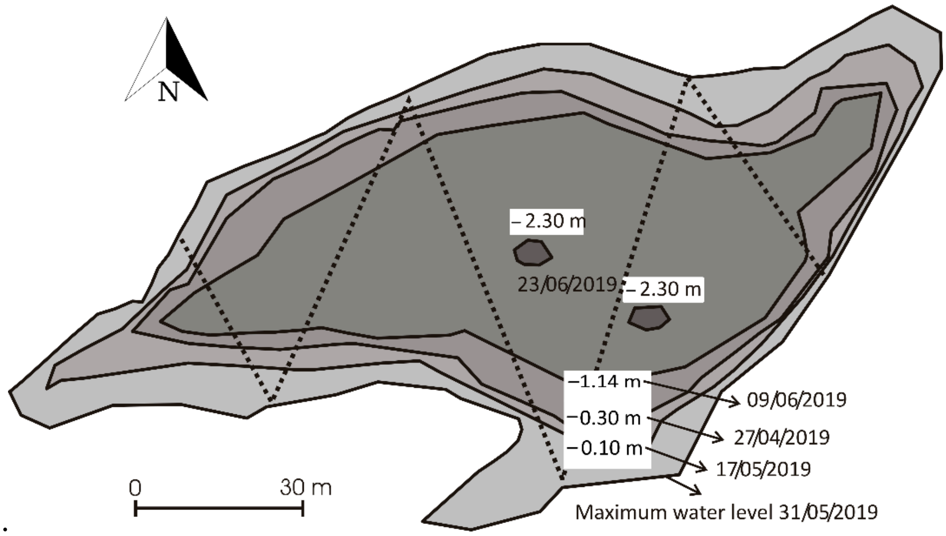

Figure 2. Pond planimetry and bathymetry. Numbers indicate meters below the maximum water level, for each sampling date in 2019. Dashed lines indicate the position of the transects.

2.4. Laboratory Analysis

Biological samples were analyzed in the laboratory, using an Olympus binocular microscope, at a magnification of 0.8× to 5.6×. All specimens were identified to the genus- or species-level and counted [29,30]. Each *C. sibyllae* specimen was classified, according to its life stage (metanauplius, juvenile, male, empty ovisac female, or ovigerous female), following Cottarelli [26]. Twelve ovigerous females were inspected under the stereomicroscope, to count the number of cysts in their broodpouch. In total, 30 females and 30 males were photographed and measured, with regard to body length. The total length of each specimen was measured, from the tip of the head to the posterior margin of the caudal furca, using ImageJ software [31].

2.5. Statistical Analysis

To evaluate temporal changes in air temperature (dependent variable), a linear regression with year (independent variable) was performed. The significance of the regression was tested, using analysis of variance (ANOVA), and the significance of the regression coefficient (i.e., the probability that the slope differs from 0) was tested using a t-test.

To investigate the relationships between environmental and biological variables, canonical correspondence analysis (CCA) [32] was conducted, using environmental variables, previously selected by a principal component analysis (PCA). The five environmental variables most strongly associated with each of the first five principal components were selected, and the final matrix used to perform CCA included five environmental variables (water volume, nitrites, water temperature, phosphates, time) and ten observations (sampling events). Time was included as an 'environmental' variable, to allow the examination of temporal changes in biological variables and was defined as the number of continuous days after the starting dates of 1 January 2019, 1 January 2020, and 1 January 2021, respectively. Biological variables included in the matrix comprised densities of each *C. sibyllae* life stage (ovigerous females, empty ovisac females, juveniles, metanauplii, males), Copepoda, and Ostracoda. Cladocera were not included in the analysis, due to the small number of specimens detected. All variables were $\log_{10}$ (N + 1)-transformed, to normalize their distributions. Significance was tested using Monte Carlo tests, with 999 permutations [33]. Pearson correlation coefficients were used, to examine relationships between environmental variables, biological variables, and CCA axes. CCA was conducted in CANOCO 4.5 (Microcomputer Power, Ithaca, NY, USA). ANOVA and linear regression were performed in Dell STATISTICA 13 (Dell Inc., Aliso Viejo, CA, USA).

3. Results

3.1. Environmental Characterization

The summary statics for the physicochemical parameters of the Palazzo Borghese pond are reported in Table 1. For water and air temperature, the observed values increased, according to the progress of the spring season (supplementary material, Table S1). For conductivity, a decreasing trend over time, within each sampling season, was observed (Table S1). The pH was always alkaline (Table 1).

Table 1. Mean, standard deviation, and range of physicochemical parameters measured in the Palazzo Borghese pond in the years 2019, 2020, and 2021.

	2019		**2020**	**2021**	
	n = 4		**n = 2**	**n = 4**	
	Range	**Mean ± SD**	**Range**	**Range**	**Mean ± SD**
Air temperature (°C)	5.0–22.5	10.4 ± 8.2	12.5–16.2	9.3–18.5	13.0 ± 4.3
Ammonia (mg L^{-1} NH_4^+)	0.06–0.10	0.07 ± 0.02	0.12–0.17	0.03–0.07	0.04 ± 0.01
BOD_5 (mg L^{-1})	0.0–1.8	0.8 ± 0.76	1.7–1.8	0.4–3.0	1.6 ± 1.15
Chlorides (mg L^{-1})	4.0–23.0	14.0 ± 7.8	11.0–12.0	14.0–34.0	26.3 ± 8.7
COD (mg L^{-1})	8.0–32.4	16.0 ± 11.2	8.0–10.7	10.7–23.1	15.3 ± 5.5
Conductivity (μS cm^{-1})	193–211	201 ± 7.8	180–238	105–161	143 ± 26.3
Dissolved oxygen (mg L^{-1})	7.2–9.8	8.3 ± 1.1	7.2–8.8	6.8–11.4	9.2 ± 1.9
Nitrate (mg L^{-1} NO_3-N)	0.30–1.10	0.58 ± 0.38	0.3–0.7	0.4–0.8	0.50 ± 0.20
Nitrite (mg L^{-1} NO_2-N)	0.05–0.07	0.06 ± 0.01	0.07–0.09	0.06–0.09	0.08 ± 0.01
pH (units)	7.4–8.6	8.0 ± 0.53	7.4–7.8	7.5–8.3	8.0 ± 0.38
Orthophosphates (mg L^{-1} PO_4-P)	0.07–0.22	0.12 ± 0.07	0.04–0.21	0.03–0.12	0.06 ± 0.04
Sulphates (mg L^{-1})	0.00–0.00	0.00 ± 0.00	0.00–5.00	0.00–0.00	0.00 ± 0.00
Water temperature (°C)	4.9–19.8	10.1 ± 6.7	8.8–18.0	13.6–6.6	13.6 ± 6.6

Pond perimeter and surface area measurements showed that the pond reached its maximum volume in May 2019 (4379.97 m^3), after which water levels gradually decreased, until the last sampling date in June, when water levels had fallen to 2.30 m above the hydrometric zero, and the pond appeared to be almost dry (Figure 2). In 2020, the pond lasted for an extremely short period; in just over 10 days, it made its appearance and, subsequently, dried up: on 4 May, the level of the pond was 5 cm below the maximum depth (2.30 m). By 14 May, the level had, already, dropped by almost a meter, compared to the previous monitoring (maximum depth = 1.35 m) (Figure 3). On the last sampling of 2020 (20 May), the pond was completely dry.

Unlike what happened in previous years, in 2021, the filling phase of the pond started in May and the pond never reached hydrometric zero; as in 2020, in this year, the permanence of the pond was short: on 18 June, the pond appeared totally dry (Figure 3).

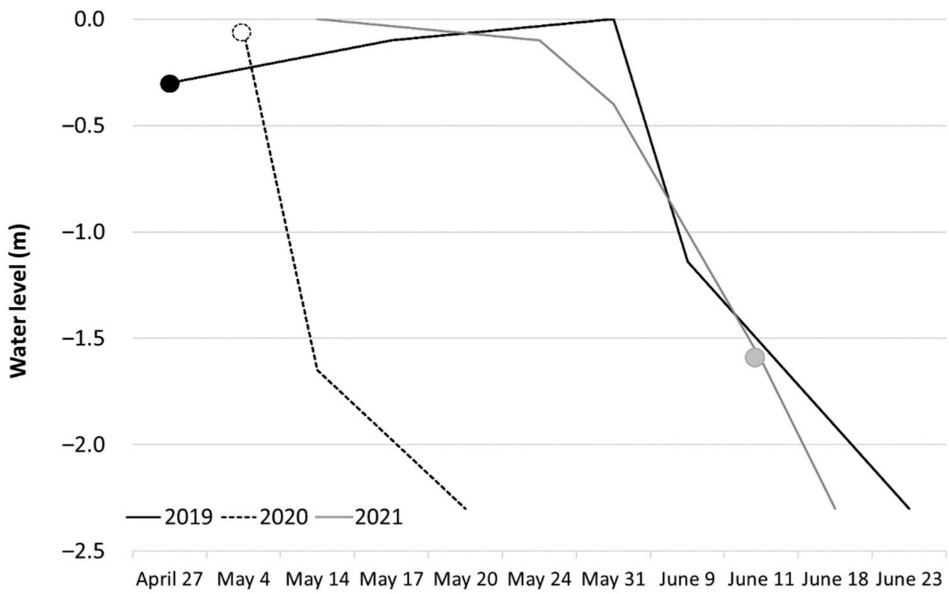

Figure 3. Trend of the hydrometric levels of the Palazzo Borghese pond, in the three years of monitoring. Black, dotted, and grey circles represent the appearance of *C. sibyllae* active stages for the years 2019, 2020, and 2021, respectively.

3.2. Climatic Conditions

Air temperature significantly increased between 2002 and 2021 (y = −3.597 + 0.0003x; $R^2 = 0.01$; ANOVA: F = 689.24; $p < 0.001$) (Figure 4), as, also, indicated by the regression coefficient (*t*-test: $t = 26.25$, $p < 0.001$). Mean monthly air temperatures in 2019–2021 were higher than during the period 2002–2018, except in April and May, while particularly marked differences in temperatures between the two periods were observed in the winter season (Figure 5). The mean air temperature increased significantly ($p < 0.001$) over a 20-year period, with a magnitude of +0.92 °C.

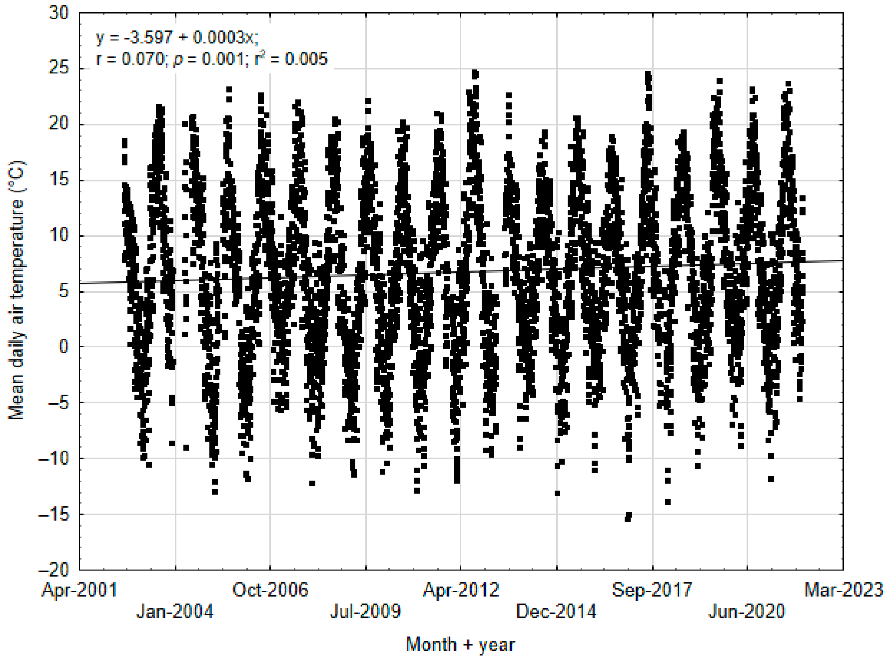

Figure 4. Trend over time of air temperature from 2002 to 2021. Data from Sibilla Mountain meteorological station.

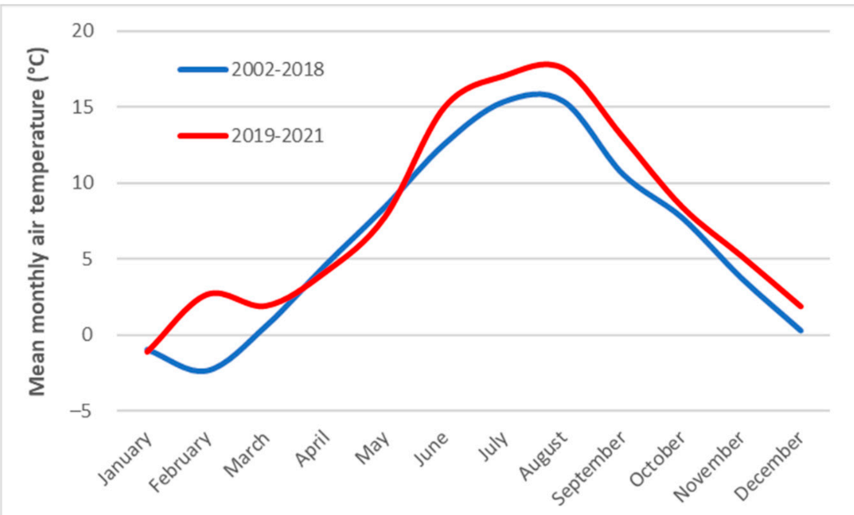

Figure 5. Mean monthly air temperature for the years 2019–2021, and comparison with the period 2002–2018. Data from Sibilla Mountain meteorological station.

3.3. Composition of the Zooplankton Community

The zooplankton community of the Palazzo Borghese pond was composed of a limited number of species. In addition to the Anostraca, represented by *C. sibyllae*, another four zooplankton taxa were identified: two Ostracoda species in two genera, *Herpetocypris reptans* and *Cyclocypris* sp., one cladoceran species, *Chydorus sphaericus* (Müller, 1776) (Chydoridae), and one copepod species, *Diacyclops bisetosus* (supplementary material: Table S2).

In 2019, Copepoda appeared in the sampling in April, together with Anostraca, and, subsequently, during the two samplings in May, they constituted most of the zooplankton community, accounting for 81% and 95% of sampled individuals of all taxa, respectively. In June, the most represented taxon was Anostraca (91%). In this year, Cladocera were totally absent. Ostracoda occurred at a very low relative abundance in May and June (Figure 6a).

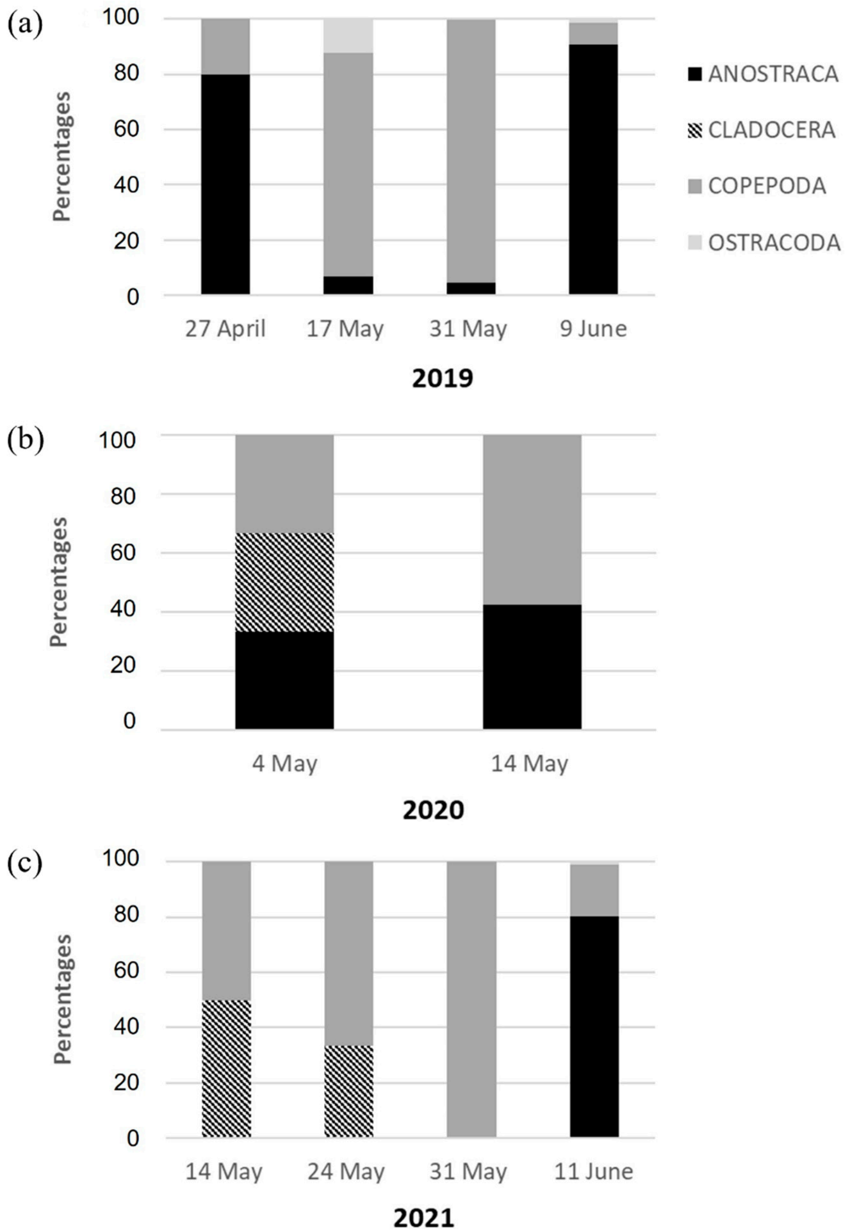

Figure 6. The relative abundance (%) of Anostraca, Cladocera, Copepoda, and Ostracoda in the Palazzo Borghese pond zooplankton community, for the years 2019 (**a**), 2020 (**b**), and 2021 (**c**).

In the first sampling of May 2020, the zooplankton community was represented equally by Anostraca, Copepoda, and Cladocera. In the second and last sampling the disappearance of the Cladocera was observed, and the community was mainly represented by Copepoda (Figure 6b).

In the first two samplings of 2021, the zooplankton community was composed of Cladocera and Copepoda; in the subsequent sampling, at the end of May, Copepoda was the only taxon present. The Anostraca appeared in June, in the final sampling, representing 80% of the total zooplankton community, while the relative abundance of Copepoda declined, up to 20% (Figure 6c).

3.4. *Chirocephalus sibyllae* Population Status, and Life History Traits

A total of 1919 *C. sibyllae* specimens were collected, of which 209 were at the larval stage, 41 were juveniles, 842 were adult females, and 827 were adult males.

Except for the first two samplings of 2019 and 2020, when the total sample was totally composed of metanauplii, in all other samplings the *C. sibyllae* population included individuals belonging to different life stages, confirming that the hatching of the cysts was not synchronous (Figure 7). In 2019, the larval stage was observed from 17 April to 31 May, and in 2020, it was observed in both sampling dates. Ovigerous females having cysts in the broodpouch, in 2019, peaked in the last sampling (9 June), while in 2021, this group represented the most abundant fraction of *C. sibyllae* females on a single date, when the presence of active fairy shrimps was detected (11 June). The presence of empty ovisac females was detected, with comparable abundances in 2019 (at the end of May and early June) and on 11 June 2021.

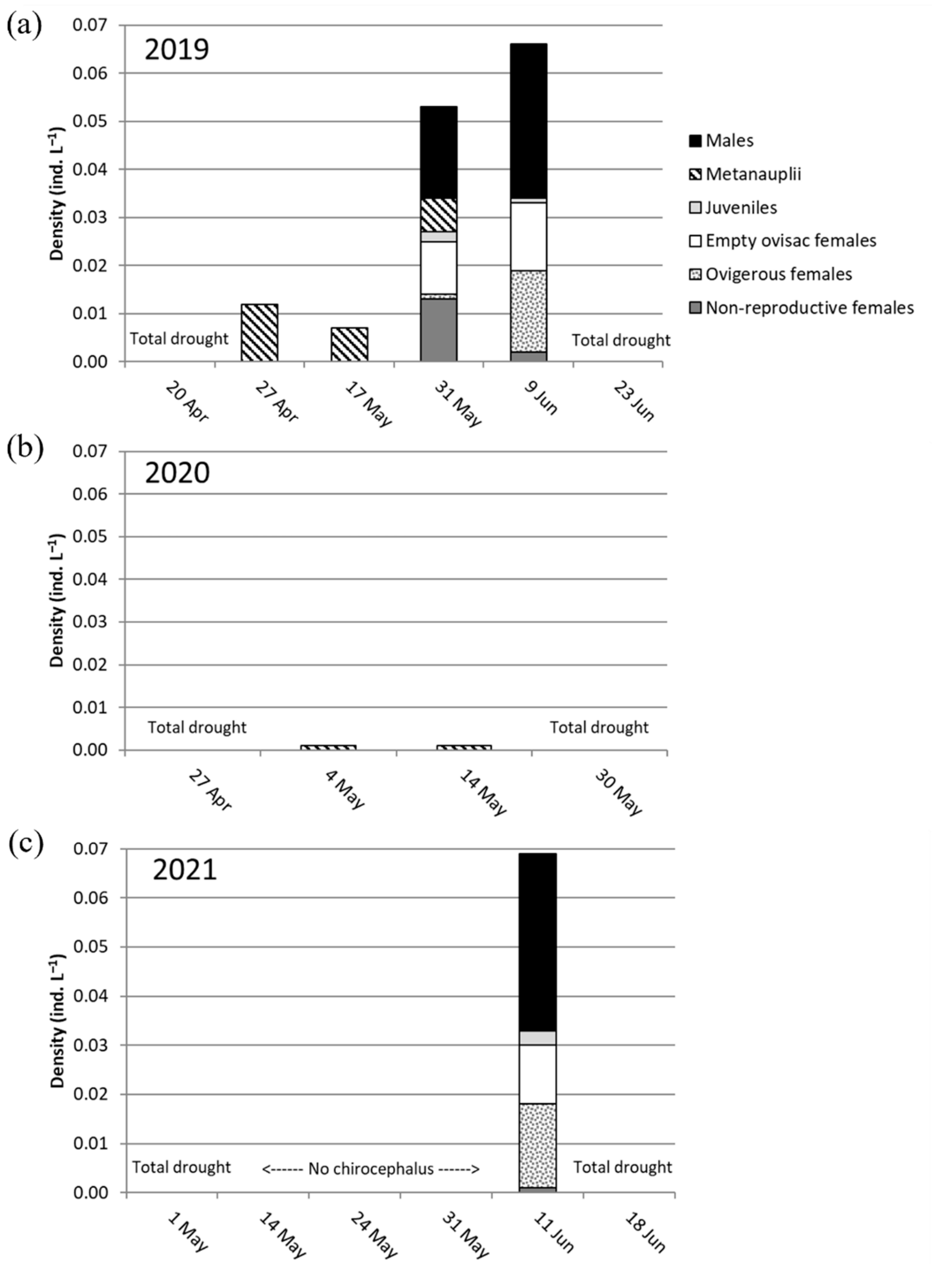

Figure 7. Mean densities (individuals [ind.] L^{-1}) of each *Chirocephalus sibyllae* life stage in the Palazzo Borghese pond: (**a**) from April to June 2019; (**b**) May 2020; (**c**) June 2021.

Regarding lifespan (days), in 2019, the *C. sibyllae* population completed its life cycle within 44 days (Figure 7a). In 2020, the mere presence of a few specimens at the larval stage showed that the species was unable to complete its life cycle in that year (Figure 7b). In 2021, active individuals were recorded only in the last sampling; thus, the species concentrated all stages of the development process in the period between 1 and 17 June (Figure 7c).

The mean body length (±SD) of the *C. sibyllae* females was 1.724 ± 0.199 (range 1.20 – 2.10 cm), whereas the mean body length of males was 1.758 ± 0.233 (range 1.24–2.23 cm); there were no statistically significant differences between the sexes in the t-test (p = 0.546). The mean number of cysts per broodpouch for clutch (±SD) was 66.0 ± 38.9 (range 11–157). Regarding sex ratio, in 2019, females represented 51% of all adults; in 2021, the percentage of females dropped to 43.7%.

3.5. Biotic Responses to Environmental Drivers

Axes 1 and 2 of CCA explained 92.7% of the total variability, and the analysis was highly statistically significant in the Monte Carlo test (F = 29.17, p = 0.001, total inertia = 1.052). The first CCA axis was most influenced by time and orthophosphates; in particular, the Pearson correlation coefficients showed that time (p = 0.008) and PO_4-P (p = 0.049) were positively related to this axis (Table 2).

Table 2. Canonical and correlation coefficients of biological and environmental variables with axis. $p < 0.05$ is in bold.

	Correlation with axis			
	AX1	***p***	**AX2**	***p***
Conductivity (µS cm^{-1})	−0.341	0.335	0.070	0.847
Nitrites (mg L^{-1} NO_2-N)	−0.365	0.299	0.271	0.449
Orthophosphates (mg L^{-1} PO_4-P)	0.621	**0.049**	−0.324	0.361
Water volume (m^3)	−0.413	0.236	0.030	0.398
Time (days)	0.777	**0.008**	−0.790	**0.007**
Non-reproductive females (ind L^{-1})	0.074	0.839	−0.281	0.431
Ovigerous females (ind L^{-1})	0.964	**0.000**	−0.284	0.426
Empty ovisac females (ind L^{-1})	0.838	**0.002**	−0.385	0.273
Juveniles (ind L^{-1})	0.670	**0.034**	−0.357	0.311
Metanauplii (ind L^{-1})	−0.356	0.313	0.368	0.295
Males (ind L^{-1})	0.904	**0.000**	−0.365	0.300
Copepoda (ind L^{-1})	−0.081	0.824	−0.269	0.452
Ostracoda (ind L^{-1})	−0.059	0.872	−0.238	0.509

The second CCA axis explained 33.1% of the total variance (Figure 8). It was most influenced by time (p = 0.007), to which it was negatively related (Table 2). *C. sibyllae* juveniles, males, ovigerous, and empty-ovisac females had a positive relationship with axis 1; this axis described temporal changes in the environmental and biological variables, indicating variability in *C. sibyllae* life-stage densities, along a gradient of increasing orthophosphate, in association with decreasing conductivity and reducing water volume (Figure 8).

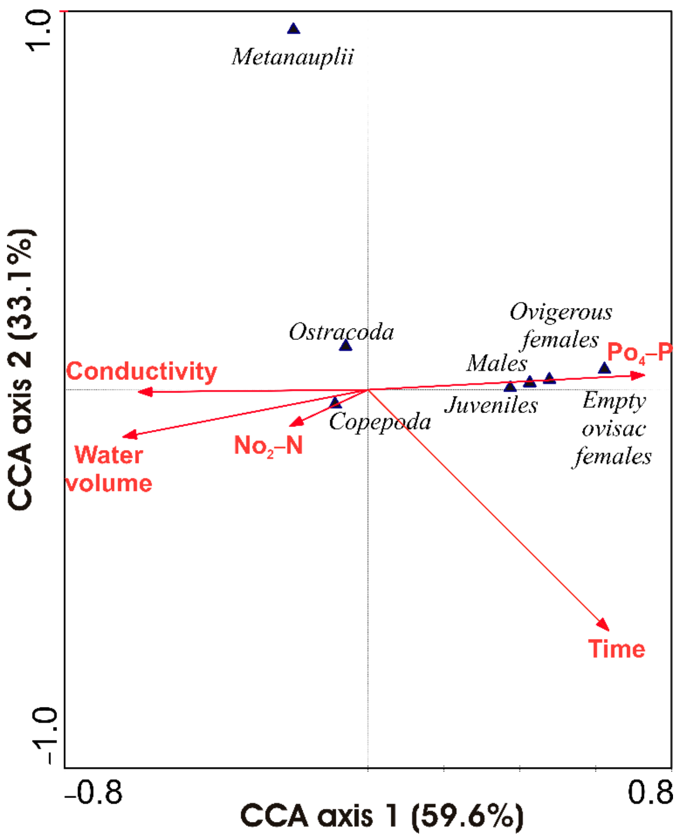

Figure 8. Canonical correspondence analysis results: biplot of biological variables (black triangles) and environmental variables (red arrows). PO_4-P and NO_2-N stand for orthophosphates and nitrites, respectively. Axes 1 and 2 explain 59.6% and 33.1% of the total variance, respectively.

4. Discussion

Our results allow us to update and integrate the little information available in the literature on the *C. sibyllae* population and its habitat. The hydrological characterization confirmed that the Palazzo Borghese pond represents a temporary, astatic, and fairly predictable reservoir, as long as it is fed by the snow melting. The permanence of the water in the pond was variable over the study period, suggesting a strict connection with the climatic conditions; since the permeability of the soil is very high, the pond tends to dry up very quickly, while the melting of the snow no longer guarantees a suitable feeding, unable to compensate for infiltration losses.

Compared to what was reported in the literature, in the last two years the wet phase of the Palazzo Borghese pond was shorter, and the pool dried up much earlier than in the past. Mura and Calzecchi Onesti [22] reported that, for the time span 1978–1982, the filling period of the pond ranged from 43 to 62 days, while the dates of desiccation fell mainly in the period between the end of June and mid-July. In the present study, in 2019, the filling phase of the pond was in line with the past (44 days), while, in the years 2020 and 2021, the pond existed for 15 days and 29 days, respectively.

Regarding water quality, all the parameters analyzed fell within the standard ranges for oligotrophic high elevation ponds. As recently reported for Pilato Lake [14], which is a high-mountain lake, located a short distance from the Palazzo Borghese pond, the pH is always alkaline, which could reflect the buffering power of the water and the calcareous nature of the substrate, while the low nutrient content and the low conductivity values reflected the low concentration of salts typical of oligotrophic high-elevation basins. The decreasing trend of conductivity, during each sampling season, confirmed that the loss of water that regulates the water volume of the pond was more influenced by infiltration rather than by evaporation processes. The low nutrient content is, also, typical of high-mountain basins.

As reported for other fairy shrimps all over the world [13,34–36], our analysis suggested that environmental alterations and the consequent habitat loss represent a serious threat to the *C. sibyllae* population. Specifically, the local air temperature trend provided some evidence of global warming effects, and higher temperatures may be a key driver of the recent shortening of the filling phase of the Palazzo Borghese pond. The significant increase in average air temperatures recorded during the study period, in the months of January and February, compared to the previous 17 years, suggested a negative impact of this phenomenon, on the snow cover of the massifs surrounding the pond. According to the findings of a previous study on the climatic conditions carried out in the same area [14], highlighting a progressive decrease over time in the mean annual cumulative precipitations, our results support the hypothesis that the poor snow cover, in particularly dry winters, can jeopardize the survival of the pond and the inhabiting species. Within certain limits, species inhabiting ephemeral waters can calibrate their reproductive strategy, based on the duration of the habitat hydrological cycle [15,37–39]. As reported in the literature [15,38–40], not all cysts of a fairy shrimp population hatch during a given reproductive season; a part of them forms an egg bank, *sensu* De Stasio [41], which serves to guarantee the survival of the species, in case, due to unfavorable environmental conditions, the population fails to reproduce. However, if the species fails to reproduce for several consecutive years, it is reasonable to hypothesize that the number of cysts could deplete until exhaustion, and the species could become extinct. Contrary to other species of fairy shrimp, such as *C. marchesonii*, in which the eggs hatch synchronously, in the case of *C. sibyllae*, the hatching of the eggs took place in a prolonged process over time, gradually increasing the population abundance. This result reflects an adaptation of *C. sibyllae* to the extreme uncertainty and shortness of the filling period of the Palazzo Borghese pond; this strategy, in fact, guarantees access to the reproduction of at least a fraction of the population, even in the most adverse environmental conditions and in particularly unfavorable years. It is known that natural selection in unstable habitats, such as ephemeral waters, pushes towards bet-hedging life-history strategies (e.g., producing offspring at different times within a reproductive season), to shield against total reproductive failure [10,42–44]. Other advantageous life-history traits, in such habitats, include high reproductive effort and a high number of young per brood [42,44], exhibiting a marked r-strategy. The high mean numbers of cysts for the broodpouch (66), observed for *C. sibyllae*, in comparison with other species belonging to the same genera inhabiting more stable environments (e.g., for *C. marchesonii*, a mean of five eggs per clutch (range 2–13) was reported by Mura [45]), was in line with the reproductive strategy adopted by *C. sibyllae*, which must face more unpredictable and, often, adverse habitat conditions. Comparing the *C. sibyllae* life-history traits observed in the present study, with the data reported in the literature [22,45], some differences could be highlighted. Regarding life span, Mura [45] reported a range of values between 35 days and 40 days; in our case, greater variability was found for this parameter; in particular, a life span equal to 44 days was observed in 2019, while in 2021, the species completed its entire life cycle in just 17 days. Mura [45], also, reported a time of 15–20 days to reach sexual maturity; in our case, the sexual maturity was reached after 35 days in 2019, and after 11 days in 2021. On this last occasion, the shortness of the life span and the quick achievement of sexual maturity could be related to the higher water temperature (18 °C ,when the active stages of *C. sibyllae* appeared in 2021). For the average total length of adults, some differences could be noted, in comparison to what was reported by Mura and Calzecchi-Onesti [22]; these authors indicated a mean total length value equal to 1.6 cm, while in the present study, the observed mean values were greater and equal to 1.72 cm for females and 1.76 cm for males.

Given the total absence of previous data on the abundance of *C. sibyllae* population, it is difficult to draw firm conclusions on the species's conservation status, from a quantitative point of view. Further monitoring and long-term data series are needed to ascertain the presence of a decreasing trend over time in population abundances.

The environmental dependence of the pond hydrological cycle, resulting in the deep abiotic factor changes occurring throughout the seasons, implies that there are significant repercussions on the adaptive strategies adopted by colonizing species [8,22]. This justifies the fact that the zooplankton community consisted of a very limited number of species. Compared to the data reported in the literature, except for the cladoceran, *Chydorus sphaericus*, which was detected for the first time during the present study, all the taxa detected during the 2019–2021 monitoring had already been reported in the past, by Mura and Calzecchi-Onesti [22], while the presence of the cladoceran, *Daphnia obtusa* Kurz, 1875, was not confirmed. The reasons for the non-detection of this species, which is suitable for living in stringent environmental conditions [44], should be investigated with further research, to verify whether the species was not collected, due to the sampling procedure applied in the present study, or whether it could reflect a suffering of the species, due to poor food availability or other environmental limitations. Copepods represent the only taxon that was always present in all samplings, dominating the zooplankton community, especially at the end of May, almost at the conclusion of the wet phase of the temporary pond.

The time and orthophosphates variables explained most of the variance in zooplankton population densities. The orthophosphates increased over time in each year, which may reflect nutrient recycling associated with sediment resuspension, driven by wind, or from trampling by livestock and hikers, which may play a crucial role in these shallow ephemeral waters [46,47]. In particular, the intensity of livestock breeding could affect pond ecosystems, through nutrient load, among other climate-related impacts. Livestock trampling on egg-laying areas could, also, affect the resuspension and hatching of cysts, which can be pushed deeper into the sediments, compared to the 5–10 mm reported in the literature [40].

5. Conclusions

The threats to *C. sibyllae* derive, above all else, from the likely and rapid changes that its habitat can undergo, as a future response to climate variations. The species has evolved in a context characterized by a wide variability of extreme environmental conditions and is, certainly, well suited to cope with them. However, the short duration of the pond in 2020 did not allow *C. sibyllae* to complete its life cycle; this event represents an alarm bell that cannot be ignored. The conservation status of the species is of particular concern, considering that the progressive increase in air temperatures and the decrease in snowfall will, likely, lead to increasingly shorter filling phases of its habitat. Possible strategies supporting the species' conservation could include: (i) the access restriction of hikers and livestock to the area occupied by the pond, even when it is totally dry, by building a fence; (ii) the creation of a cyst bank in the laboratory, to enable the reintroduction of *C. sibyllae* in the case of extinction; and (iii) the identification of suitable biotopes to host *C. sibyllae* populations, after an appropriate assessment of the risks associated with the introduction of the species into new ecosystems. Moreover, as recommended, also, by Mura [38], detailed studies on the cyst-bank dynamics and functioning (i.e., size of egg bank, survival time of cysts) could help to elucidate the potential effects of climate changes on the *C. sibyllae* population.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w14111750/s1: Table S1: Physicochemical parameters measured in the Palazzo Borghese pond in each sampling occasion over the years 2019–2021; Table S2: Mean densities (ind L^{-1}) of each zooplankton taxon collected in the Palazzo Borghese pond, during each sampling occasion, over the years 2019–2021.

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