

## Article

# Temporal Evolution of Phytoplankton Metacommunity in a Disused Mediterranean Saltwork

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**Abstract:** Saltworks are interesting sites for aquatic ecology, where marine phytoplankton plays a fundamental role in regulating ecosystem processes and sustaining large-scale biodiversity. This paper presents successional, structural, and dynamical traits of a phytoplankton metacommunity in a disused saltwork, where the industrial hydraulic management has been changed, leading to a different overlapping of natural and anthropic induced scales. We have considered the total phytoplankton biomass, size classes, and chemotaxonomical composition in monitoring the metacommunity dynamics in a series of single ponds spanning from marine to hypersaline ones between July 2008 and October 2009. Results indicate a large accumulation of biomass during summer to early autumn in hypersaline ponds, with the prevalence of small-size cells (<20 µm) in the entire system. Despite severe salinity fluctuations as a consequence of rainy events and evaporating processes, chlorophytes represented the dominant group of hypersaline ponds, while diatoms were more abundant where the influence of seawater inflow was higher. Despite the absence of ordinary maintenance, the phytoplankton metacommunity dynamic and patterns observed suggest that the existing saline gradient ensures qualitative and quantitative aspects of the primary production usually found in active saltworks. This makes the Saline di Tarquinia an important site not only for the preservation of Mediterranean biodiversity and hypersaline biotopes but also for ecological studies dealing with the trait-based responses of the phytoplankton community to large environmental fluctuations.

**Keywords:** chemotaxonomy; extreme environment; Saline di Tarquinia; size classes

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## 1. Introduction

The paramount importance of salt in the development of human societies sets saltwork systems among those providing basic ecological goods, explaining their relevant role in Mediterranean history [1,2]. Their importance in compensating for coastal habitat loss and natural wetlands, especially for aquatic bird conservation, received greater attention in last decades [3–6]. Along the human history, sectors of coastal lagoons have been transformed into the artificial systems of saltworks, thanks to the pronounced variability of the physical–chemical parameters of their waters, whose salinity values span from those of the adjacent sea to extremes, allowing salt precipitation in the most confined sectors of the system. In the Mediterranean context, saltworks are known as salinas; hence, this term will be utilized hereafter. A fundamental aspect of salinas consists of the system being kept under a stable hydraulic regime, based on strictly planned water renewal in the different ponds in order to maximize salt production. The extreme variations in salinity, along with those of temperature and other physical parameters issuing from the limited depth of the ponds, places salinas in the category of extreme environments.

In disused plants the water dynamics are largely under the influence of seminatural conditions, with human intervention acting only to preserve the persistence of the water in the system. Due to the research opportunities they offer to the study of successional dynamics, disused salinas deserve particular attention. In fact, while they keep the basic spatial–temporal characteristic of an active salina, they respond, to a large extent, to a more natural environmental forcing. The ensuing ecological characteristics of the two types of salinas are significantly different and greatly influence structural and functional aspects of their biological communities. Salinas are particularly interesting for microbial ecology because hypersaline ponds are predominantly or exclusively inhabited by microbial communities [7–9] where phytoplankton represents one of the major factors controlling the efficiency of the energy transfer toward upper trophic levels [10–12]. The typically shorter and high efficiency trophic chain triggered by phytoplankton of salinas, is the food chain phytoplankton—*Artemia salina*—fish and birds [13–15], that represent a key process sustaining the biodiversity of these systems. Phytoplankton is the base of this chain and consists of a series of photoautotrophic unicellular organisms with limited motion capability. Their inability to seek favorable conditions determines strong variations in functional traits and species composition as an effective strategy to improve the community success [10,16–23]. Studies dealing with phytoplankton in salinas systems have produced large amounts of information that are often misleading, with successional patterns depending on the scale dimension utilized and the consequent degree of connectivity in the systems [24–29].

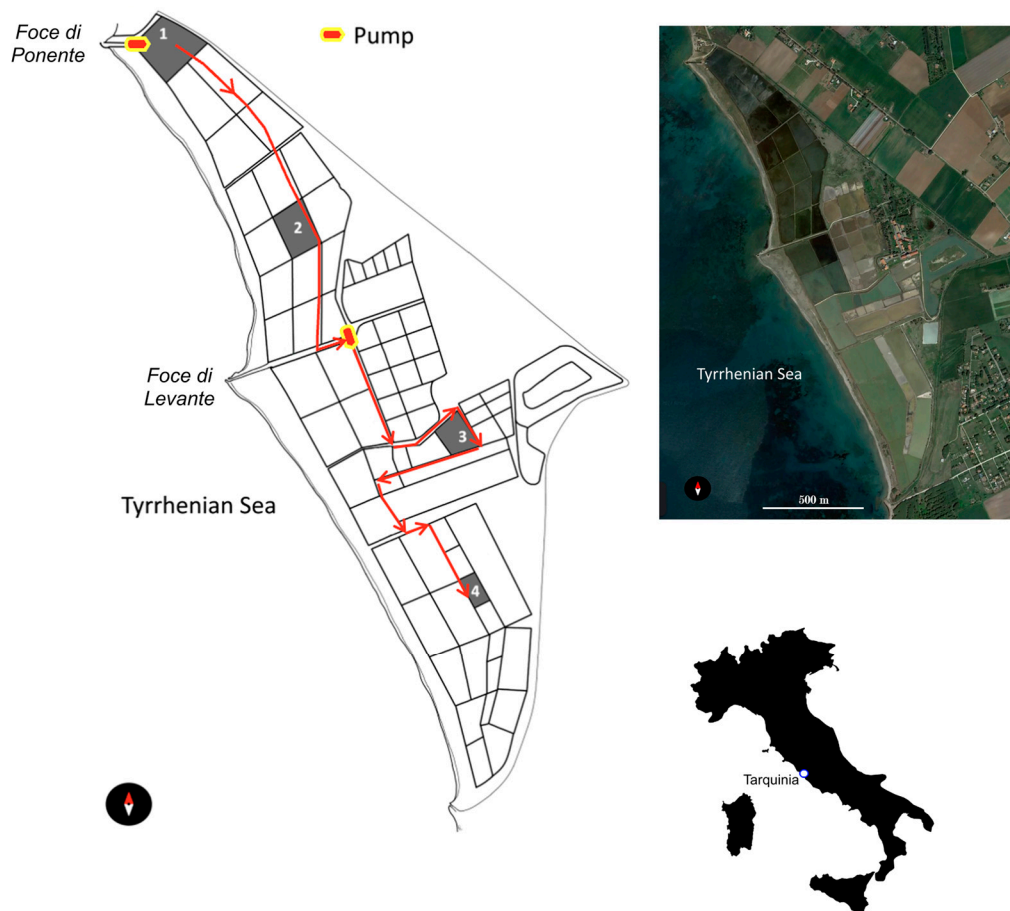
A large part of this information is derived from active salinas, where the salinity gradient is controlled and kept active by human activity to produce salt. In a kind of organization such as that of salinas, communities inhabiting ponds in both active and nonactive salinas are connected by exchanges of individuals and materials through the hydraulic net channels. Thus, from a conceptual perspective, we refer to the concept of metacommunities in studying these systems. A metacommunity is a dynamic property, identifying a set of local communities linked by the dispersal of multiple interacting species [30,31]. In recent years, researcher have deepened and applied the concept of the metacommunity to the study of plankton in different ecological systems, above all ponds, but also wetlands and limnetic and marine ecosystems, producing new insights on complex adaptative systems [31–34].

The present work aims to investigate how the phytoplankton metacommunity of Saline di Tarquinia, a disused Mediterranean salinas located on the Italian Tyrrhenian coast, respond to large environmental fluctuation because of reduced maintenance activity. The water flow of the salinas has significantly changed, with a decreased flux and an accentuated effect of rainy events leading to serious ecological consequences on the aquatic environment [35,36]. We have approached the study of the metacommunity considering the functional traits of size classes, due to its significant role in shaping the food web, and chemotaxonomical composition as reliable tools for monitoring changes along a salinity gradient in a period spanning from July 2008 to October 2009.

## 2. Materials and Methods

### 2.1. The Study Site

The Saline di Tarquinia is located on the Tyrrhenian coast of Italy, some 90 km north of Rome, covering an area of 170 hectares in a triangular shape with its longer side facing the seashore (Figure 1). Its present structure, that suffered heavy damages in 1987 after exceptional flood, was set up by the Papal State in 1805, whose administration operated the plant through to the end of the same century, when it became property of the Italian State.



**Figure 1.** Map of the Salina di Tarquinia and plant with indication of water exchange within the system—from the seawater intake to the most confined ponds—and sampling stations (ponds 1, 2, 3 and 4).

The Salina di Tarquinia consisted of about 100 ponds with a rectangular plan bordered by embankments in local limestone or wood. The production of salt took place forcing the circulation of water between various ponds (distinguished by evaporation, servants, and salters), and the water flux was guaranteed by wooden locks and a series of channels necessary for the transfer of the water between ponds, concentrating solutes with salinity values ranging from that of the sea (35 psu) to the point of precipitation of NaCl (250 psu). The external perimeter is encircled by a channel preventing fresh water from the surrounding fields from reaching the salina, while marine water seepage is prevented by another channel running parallel to the seashore. In 1987, ponds of the plant were flooded by a huge amount of water and mud, causing even the surrounding channel to overflow. About 16 ha of the saline were completely buried and never restored, and some ponds were excluded from the cycle [35].

The whole system is currently made up of 40 rectangular ponds, separated by stone or wood embankments. The seawater is brought into the plant via a hydraulic pump located in the Foce di Ponente channel, from which it proceeds via natural flow up to the basins adjacent to a channel named Foce di Levante. Here, a second hydraulic pump allows the water to overcome the altimetric difference existing between the northern and eastern side of the Foce di Levante, proceeding via natural flow toward saltern ponds. The current water management of dismissed salinas is the opposite of what was performed

during the active phase, since the main object is to keep the water level constant in different ponds, ranging from about 100 cm in northern ponds to 10 cm in hypersaline ones where salinity reaches about 190 psu. The marine water flow circulation and increasing salinity from the northern inflow channel towards the southern crystallization ponds, influences both the abiotic parameters and the biological community [36].

Considering its natural value, in 1980 Saline di Tarquinia became a natural reserve, successively designated as a SIC (Site of Community Interest) and a ZPS (Zone of Special Protection) on the bases of the European habitat and birds directives (Site Code IT6010025, IT6010026). As it is well known, both active and dismissed salinas represent systems of high ecological value as stopover points and feeding grounds for migrating birds [37–39]. While many studies have investigated the animal and vegetal communities of the Saline di Tarquinia [36,40–43], information regarding planktonic communities and related production is still poor.

## 2.2. Field Activities

Water samples have been collected from four different ponds in Foce di Levante (pond 1) to the southern sector of the plant (pond 4), following an increasing salinity gradient (Figure 1, Table 1). Each sample was hand-collected from the shore of the pond to avoid sediment resuspension and analyzed shortly after collection in an adjacent field laboratory belonging to a local administration. For the analysis of total phytoplankton biomass, 100 mL of sea water was filtered on 0.45  $\mu\text{m}$  GF/F filter (Whatman-Maidstone, UK, 25 mm diameter). For the determination of size classes, micro- (200–20  $\mu\text{m}$  size cells), nano- (20–2  $\mu\text{m}$  size cells), and pico- (<2  $\mu\text{m}$  size cells), a series of fractionated filtrations were made. A total of 100 mL of saltwork water was prefiltered through 20  $\mu\text{m}$  net obtaining two fractions, cells and colonies larger than 20  $\mu\text{m}$  on the net, and a water phase containing all the particles < 20  $\mu\text{m}$ . This last fraction was filtered on 0.45  $\mu\text{m}$  GF/F filter (Whatman-Maidstone, UK, 25 mm diameter). The same procedure was repeated by pre-filtering a 100 mL saltwork water through 2  $\mu\text{m}$  net, obtaining a water phase filtered on 0.45  $\mu\text{m}$  GF/F filter (Whatman-Maidstone, UK, 25 mm diameter). All GF/F filters obtained were immediately stored in liquid nitrogen until the analyses in the laboratory. For the estimation of the contribution of each class, the formula is reported in the section laboratory analyses.

**Table 1.** Geographical coordinates (latitude and longitude) and depth of the investigated ponds. The symbol ~ indicate the mean depth of the evaporating ponds. Sampling period: 1 and 29 July 2008; 16 September 2008; 12 November 2008; 9 January 2009; 12 February 2009; 12 March 2009; 30 April 2009; 4 June 2009; 28 September 2009; 27 October 2009; and 11 November 2009.

|        | Lat. N    | Lon. E   | Depth (cm) |
|--------|-----------|----------|------------|
| Pond 1 | 42.210502 | 11.70997 | 100        |
| Pond 2 | 42.205383 | 11.71502 | 80         |
| Pond 3 | 42.199698 | 11.72096 | ~30        |
| Pond 4 | 42.195687 | 11.72161 | ~15        |

For the determination of functional groups using chemotaxonomical criteria, 500 mL of seawater was filtered onto 0.45  $\mu\text{m}$  GF/F filters. All GF/F filters were preserved frozen at  $-80\text{ }^{\circ}\text{C}$  for successive analyses back at the university laboratory. For each sample, salinity and temperature values were determined using a YSI probe (YSI Incorporated|1700/1725 Brannum Lane|Yellow Springs, OH 45387, USA—mod. 556 MPS). The sampling period is represented by 8 months, from July 2008 to April 2009. The missing months to formally complete an annual cycle are due to logistic difficulties on the site.

### 2.3. Laboratory Analyses

The contribution of total phytoplankton biomass (total Chl a) and size classes micro-, nano-, and pico- was evaluated through the analyses of chlorophyll a (Chl a) and phaeopigments (Phaeo) according to [44], using a FluoroMax spectrofluorometer (HORIBA Instruments Inc., 20 Knightsbridge Road, Piscataway, NJ, USA) The estimation of each class was calculated as follow:

microsize: (total Chl a) – (<20  $\mu\text{m}$  Chl a);

nanosize: (<20  $\mu\text{m}$  Chl a) – (<2  $\mu\text{m}$  Chl a);

picosize: <2  $\mu\text{m}$  Chl a.

The ratio of phaeopigments (Phaeo): chlorophyll a (Chl a) has been utilized as the grazing index [44]. For the determination of the pigment content, GF/F filters were homogenized and resuspended in 100% methanol and analyzed using High-Performance Liquid Chromatography (HPLC) (Hewlett Packard, Hewlett Packard Enterprise, Palo Alto, CA, USA- 1100 Series) in a reverse phase (C8 column 3  $\mu\text{m}$  Hyperloop MOS) [45]. For the determination of chlorophylls and carotenoids, a spectrophotometer with a diode array detector was set at 440 nm, making it possible to determine the absorption spectrum of the 350–750 nm interval for each peak to check the purity of single pigments. The column was calibrated using different pigment standards (chlorophyll a, chlorophyll b, chlorophyll c1 + c2, alloxanthin, prasinoxanthin, 19'-butanoyloxyfucoxanthin, fucoxanthin, 19'-hexanoyloxyfucoxanthin, peridinin, zeaxanthin, neoxanthin, violaxanthin, and  $\beta$ -carotene). Standards were provided by the International Agency for 14 C Determination, VKI Water Quality Institute, Copenhagen, Denmark. Quantification was based on the absorbance at 440 nm and the factor response value (peak area/pigment concentration) for each pigment [46]. The contribution of main phytoplankton groups to the total Chl a was estimated using CHEMTAX 1.95 software [47], using an iterative process to find the optimal pigment:Chl a ratios and generating the fraction of the total Chl a pool belonging to each pigment-determined group. Considering the high variability of cellular pigmentary pool in relation to environmental conditions, a specific ratio matrix has been used for each pond. The chemotaxonomical groups identified are cyanophytes (Cyano\_1), chlorophytes (Chloro), prasinophytes (Pras\_3), cryptophytes (Crypto), diatoms (Diat\_1 and Diato\_2), haptophytes (Hapto\_6), dinoflagellates (Dino), and chlorophytes (Chloro\_2).

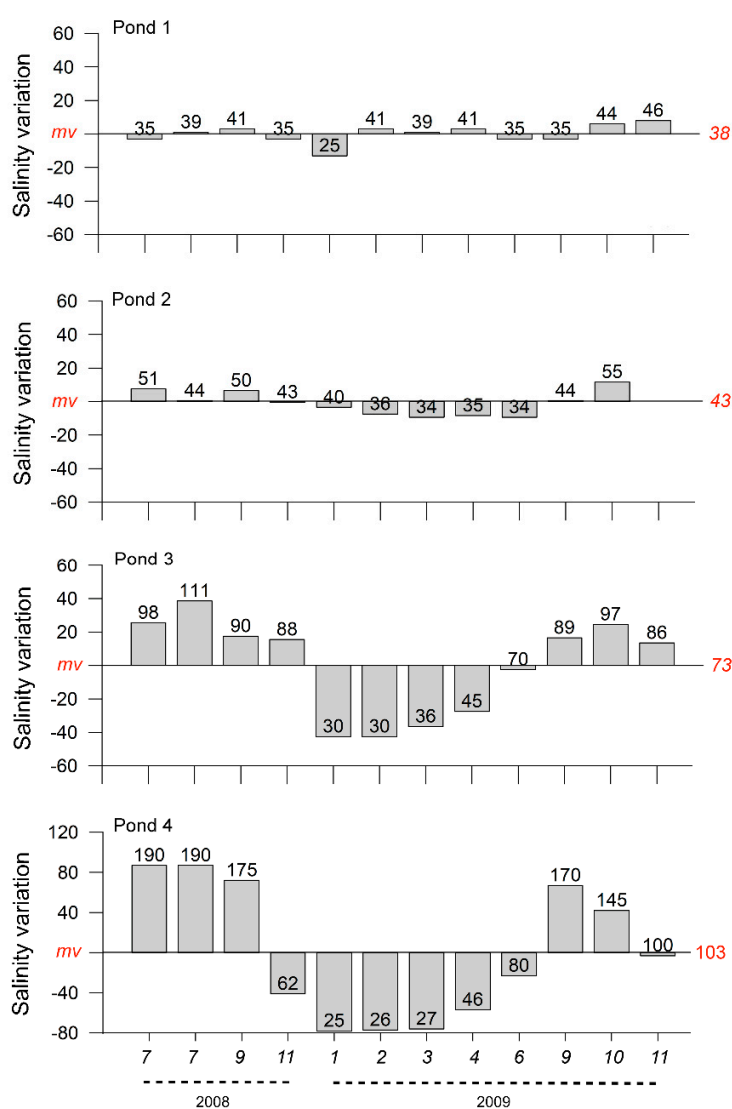
### 2.4. Statistical Analyses

Metacommunities can be analyzed using different statistical procedures; within the pertinent literature we have utilized the hierarchical cluster analysis to compare the chemotaxonomical composition of different ponds using the neighbor joining clustering analyses, based on the Bray–Curtis similarity index [48]. To assess which chemotaxonomical functional group and size class was primarily responsible for difference between ponds, a multigroup SIMPER test was performed using the Bray–Curtis similarity measure [49]. The overall significance of the difference was assessed using ANOSIM (analyses of similarity). The overall average dissimilarity was computed using the considered functional traits (e.g., size class or chemotaxonomical functional groups), while the functional-trait-specific dissimilarities were computed for each trait individually. Finally, the relationship between biological and environmental variables was calculated through a principal component analysis (PCA) based on a Pearson's correlation matrix, treating missing data with pairwise deletion and highlighting temporal and spatial dynamic grouping data for months (red numbers) and ponds (colored dots). Results were presented using projecting variables on the factor-plane described by the 1st, 2nd, and 3th principal factors. All statistical analyses were performed using PAST 4.03 software.

### 3. Results

#### 3.1. Salinity

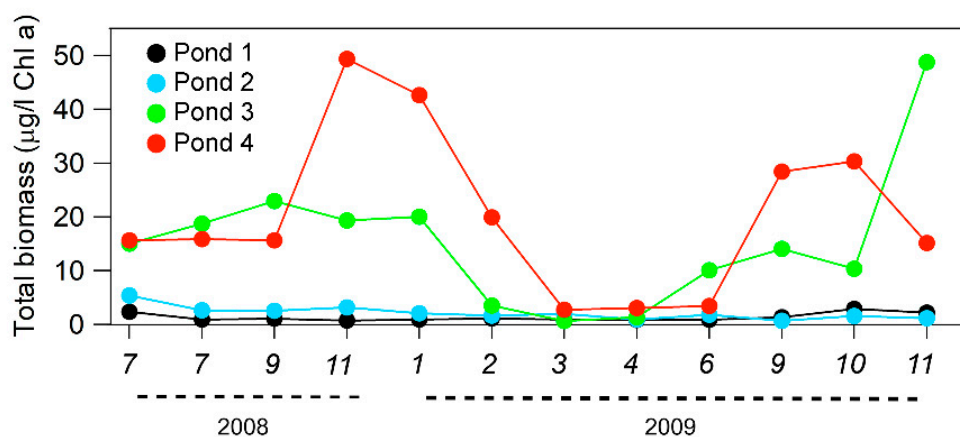
The annual variations in salinity in the different ponds are shown in Figure 2. Pond 1, affected by seawater inflow, shows a mean of 38 psu with values ranging from 25 psu (January 2009) to 46 psu (November 2009). Pond 2 shows a mean of 43 psu, ranging from a minimum of 36 psu (February 2009) to a maximum of 55 psu (October 2009). Pond 3 shows a mean of 73 psu, with values ranging between 30 psu (January–February, 2009) and 111 psu (July 2008). Pond 4 shows a mean of 103 psu, with values ranging between 25 psu (January 2009) and 190 psu (July 2008). The extreme variations observed in ponds 3 and 4 with salinity reaching values lower than seawater are related to heavy rains as reported in Table S1—SIARL-ARSIAL (<https://www.arsial.it/siarl-servizio-integrato-agrometeorologico-della-regione-lazio/>, accessed on 4 November 2022).



**Figure 2.** Histograms showing salinity variations (psu) during the year in ponds 1, 2, 3, and 4, with indication of point values on the top. The horizontal line indicates the annual mean value (*mv*, in red). Sampling months and year are reported along the X axes.

### 3.2. Total Phytoplankton Biomass and Size Classes

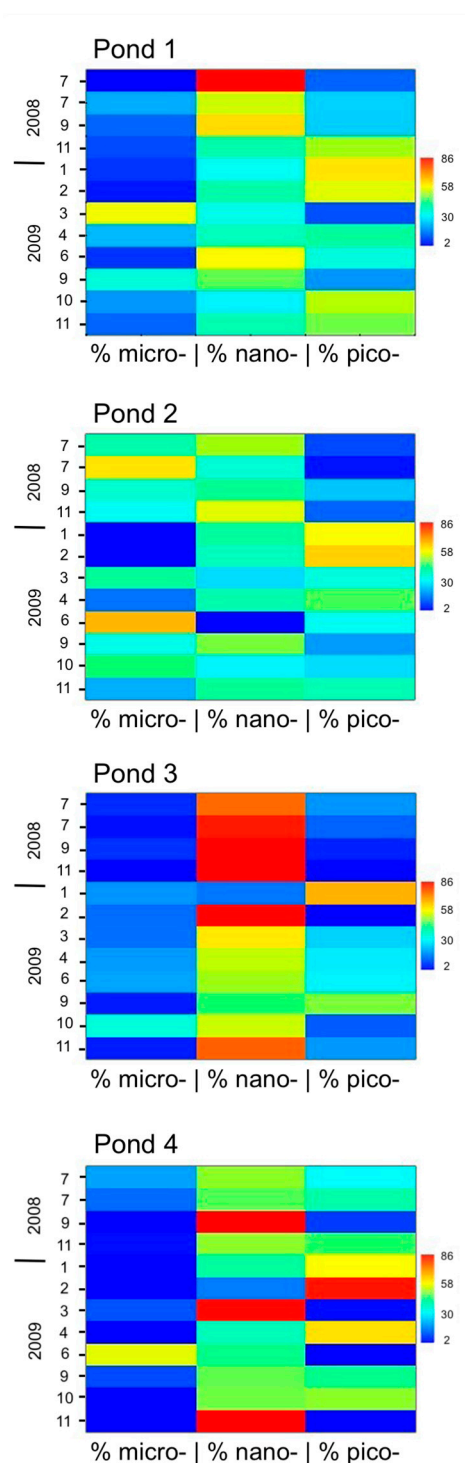
The temporal dynamic of total phytoplankton biomass (Chl a) was different in ponds 1 and 2 compared to ponds 3 and 4, where values were one order of magnitude higher in autumn (Figure 3). In particular, in pond 1 Chl a varied between 0.66  $\mu\text{g/L}$  (November 2008) and 2.87  $\mu\text{g/L}$  (October 2009); in pond 2 between 5.34  $\mu\text{g/L}$  (July 2008) and 0.64  $\mu\text{g/L}$  (28 September 2009); in pond 3 between 49.34  $\mu\text{g/L}$  (11 November 2008) and 2.72  $\mu\text{g/L}$  (12 March 2009); and in pond 4 between 48.75  $\mu\text{g/L}$  (26 November 2009) and 0.6  $\mu\text{g/L}$  (3 December 2009).



**Figure 3.** Temporal variations in total phytoplankton biomass ( $\mu\text{g/L}$  Chl a) in pond 1 (black), 2 (red), 3 (blue), and 4 (green). Sampling months and year are reported along the X axes.

The metacommunity-size structure showed different characteristic among ponds (Figure 4). In pond 1, nanophytoplankton dominated between July and September 2008 and June 2009, reaching a maximum percentage of 85% in July 2008. Picofraction dominated between November 2008 and February 2009, and October–November 2009, with a maximum of 57% on February 2009. Microphytoplankton dominated only in March 2009 (57%) representing the less abundant fraction in the overall duration of sampling activities. In pond 2, micro- and nanophytoplankton were more abundant between July and November 2008, reaching percentages up to 60% (February 2008) and 56% (November 2009), respectively; microphytoplankton reached the maximum percentage (66%) in June 2009. Picophytoplankton dominated between January and February 2009 exceeding 60%. In pond 3, nanophytoplankton was the main group, exceeding 80% between July and November 2008, in February 2009 and November 2009. Pico-phytoplankton dominated in January and September 2009, while microphytoplankton was poorly represented. In pond 4, nanofraction dominated between July and November 2008, March 2009, and November 2009, exceeding 80% in September, March, and November. Picophytoplankton dominated in January–February 2009, exceeding 80% in February and April 2009. Microphytoplankton was poorly represented showing a mean percentage of 32% and becoming dominant only in June 2009 (60%).





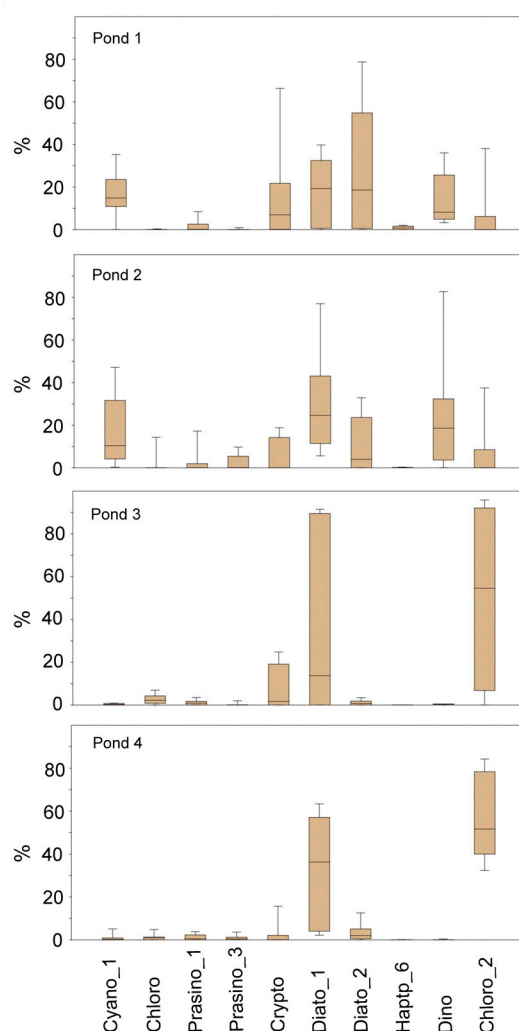
**Figure 4.** Contribution of size classes micro- (>20 μm), nano- (2–20 μm), and picophytoplankton (<2 μm) expressed as percentage contribution to total biomass, in ponds 1, 2, 3, and 4). Sampling months and year are reported along the Y axes.

### 3.3. Chemotaxonomical Composition of Phytoplankton

The chemotaxonomical composition of phytoplankton communities at different ponds is reported in Figure 5. In pond 1, diatoms (Diato\_1 and Diato\_2) represent the most abundant group, with a mean percentage of 18% (Diato\_1) and 28% (Diato\_2), followed by cyanophytes (Cyano\_1) with 16% and dinoflagellates (Dino) with 15%. In pond 2,



values show a similar trend to that observed in pond 1, with diatoms still representing the dominant groups, although with a major contribution of Diato\_1 (30%) that never reached percentages lower than 8%. Dinoflagellates represent the second most abundant group, showing high variability during the overall sampling period (highlighted by the stretching of the box and whiskers) with a mean of 24%, followed by cyanophytes (Cyano\_1) with 17%. In pond 3 chlorophytes (Chloro\_2) strongly dominated the community with a mean percentage of 49%, reaching values up to 95% in September 2008. Diato\_1 represents the second most abundant group, with a mean of 36% and a maximum of 95% reached in February 2009. Other groups were poorly represented, with cryptophytes (Crypto) showing a mean of 8% and reaching the maximum of 25% in November 2008. In pond 4, chlorophytes (Chloro\_2) dominated with a mean of 58% and never reached values lower than 33%, representing the dominant group for almost the overall duration of the sampling period. Diato\_1 showed a mean of 31%, becoming dominant only in February and March 2009 and with percentages of 57% and 63%, respectively. Other groups were less represented.



**Figure 5.** Box plots showing the chemotaxonomical composition (percentage contribution) of different ponds for the entire sampling period.

### 3.4. Similarity Tests and Neighbor Joining Clustering Analyses

The analysis of similarity between ponds for the considered functional traits is reported in Table 2. With regard to the body size, the ANOSIM test shows a weak, although significant, dissimilarity between ponds ( $R = 0.119$ ;  $p = 0.0051$ ). The multigroup

comparison indicates significant differences ( $p < 0.05$ , values in bold) among ponds 1–3, and between pond 2 with ponds 3–4. The size classes primarily responsible for the observed differences are nano- (contribution 36.28%) and picophytoplankton (contribution 35.21%), per the SIMPER test. With regard to the chemotaxonomical functional groups, ANOSIM tests indicate a significant dissimilarity between ponds ( $R = 0.3507$ ;  $p = 0.001$ ) with multigroup comparisons highlighting significant differences of ponds 1–2 with ponds 3–4 ( $p < 0.05$ , values in bold). The SIMPER test suggests that functional groups primarily responsible for the observed difference are mainly Chloro\_2 (contribution 29%) and Diato\_1 (contribution 21.53%).

**Table 2.** ANOSIM and SIMPER tests for the considered functional traits in relation with ponds.

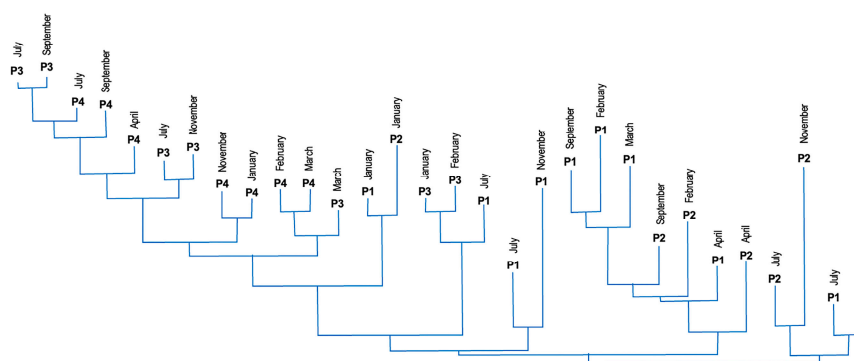
| Anosim Test—Functional Groups |             |             |          | <i>p</i> -Values. Uncorrected Significance |        |        |        |
|-------------------------------|-------------|-------------|----------|--|--------|--------|--------|
| Permutation N:                | 9999        |             |          | Pond 1                                     | Pond 2 | Pond 3 | Pond 4 |
| Mean rank within:             | 158.9       |             |          | Pond 1                                     | 0.4272 | 0.0017 | 0.0004 |
| Mean rank between:            | 235.2       |             |          | Pond 2                                     | 0.4272 | 0.0055 | 0.0009 |
| R:                            | 0.3507      |             |          | Pond 3                                     | 0.0017 | 0.0055 | 0.4403 |
| <i>p</i> (same):              | 0.0001      |             |          | Pond 4                                     | 0.0004 | 0.0009 | 0.4403 |
| Simper Test—Functional groups |             |             |          |  |        |        |        |
| Taxon                         | Av. Dissim  | Contrib. %  | Cumul. % | Pond 1                                     | Pond 2 | Pond 3 | Pond 4 |
| Chloro2                       | 19.79       | 29          | 29       | 5.91                                       | 6.1    | 49.5   | 58     |
| Diato1                        | 14.69       | 21.53       | 50.53    | 18   | 30.1   | 36.4   | 31.5   |
| Diato2                        | 9           | 13.24       | 63.77    | 27.8                                       | 10.6   | 0.973  | 3.53   |
| Dino                          | 8.34        | 12.22       | 75.99    | 14.4                                       | 24.1   | 0.281  | 0.0814 |
| Cyano_1                       | 7           | 10.15       | 86.14    | 16.3                                       | 17.3   | 0.367  | 0.98   |
| Crypto                        | 6           | 9           | 94.82    | 14.8                                       | 5.22   | 8.34   | 2.68   |
| Prasyno1                      | 1           | 2           | 96.7     | 1.73                                       | 2.48   | 1.17   | 1.11   |
| Chloro                        | 1           | 2           | 98.47    | 0.0613                                     | 1.79   | 2.66   | 1.21   |
| Prasyno3                      | 0.8032      | 1           | 99.64    | 0.121                                      | 2.18   | 0.303  | 0.957  |
| Hapto6                        | 0.2436      | 0.357       | 100      | 0.859                                      | 0.104  | 0.0243 | 0.0214 |
| Anosim Test—Size classes      |             |             |          | <i>p</i> -values. Uncorrected significance |        |        |        |
| Permutation N:                | 9999        |             |          | Pond 1                                     | Pond 2 | Pond 3 | Pond 4 |
| Mean rank within:             | 513.1       |             |          | Pond 1                                     | 0.1742 | 0.0283 | 0.3073 |
| Mean rank between:            | 580.2       |             |          | Pond 2                                     | 0.1742 | 0.0002 | 0.0161 |
| R:                            | 0.119       |             |          | Pond 3                                     | 0.0283 | 0.0002 | 0.1401 |
| <i>p</i> (same):              | 0.0051      |             |          | Pond 4                                     | 0.3073 | 0.0161 | 0.1401 |
| Simper Test—Size classes      |             |             |          |  |        |        |        |
| Taxon                         | Av. Dis-sim | Con-trib. % | Cumul. % | Pond 1                                     | Pond 2 | Pond 3 | Pond 4 |
| % nano-                       | 12.35       | 36.28       | 36.28    | 46   | 37.2   | 64.7   | 53.9   |
| % pico-                       | 11.98       | 35.21       | 71.49    | 36.2                                       | 30.6   | 22.6   | 35.5   |
| % micro-                      | 10          | 28.51       | 100      | 17.8                                       | 32.2   | 12.7   | 10.5   |

On the other hand, considering how much the metacommunity changes over the time period, with regard to the functional groups, no significant differences between months are observed ( $p = 0.6919$ ), with the negative value of  $R$  ( $-0.04656$ ) indicating a similarity between the sampling periods, suggesting, therefore, the different ponds analyzed tend to maintain their characteristics (Table 3). As concerns the body size, the metacommunity does not show marked differences ( $R = 0.09093$ ,  $p = 0.0621$ ) between months, except for summer (Month 7–9) and winter (Month 1–2) months regarding the pico- and nano- sizes as evidenced by the SIMPER test values.

**Table 3.** ANOSIM and SIMPER tests for the considered functional traits in relation with months.

| Anosim Test—Functional Groups |            | <i>p</i> -Values. Uncorrected Significance |          |        |        |        |        |        |        |        |        |      |
|-------------------------------|------------|--|----------|--------|--------|--------|--------|--------|--------|--------|--------|------|
| Permutation N:                | 9999       |  | July     | Sept.  | Nov.   | Jan.   | Feb.   | Mar.   | April  |        |        |      |
| Mean rank within:             | 211.8      |  | July     | 0.4221 | 0.7028 | 0.6576 | 0.3562 | 0.7466 | 0.7646 |        |        |      |
| Mean rank between:            | 202.3      |  | Sept.    | 0.4221 | 0.4054 | 0.1731 | 0.46   | 0.4007 | 0.3377 |        |        |      |
| R:                            | −0.0466    |  | Nov.     | 0.7028 | 0.4054 | 0.2559 | 0.1997 | 0.3736 | 0.5565 |        |        |      |
| <i>p</i> (same):              | 0.6919     |  | Jan.     | 0.6576 | 0.1731 | 0.2559 | 0.5484 | 0.4283 | 0.5446 |        |        |      |
|                               |            |  | Fabr.    | 0.3562 | 0.46   | 0.1997 | 0.5484 | 0.9705 | 1      |        |        |      |
|                               |            |  | Mar.     | 0.7466 | 0.4007 | 0.3736 | 0.4283 | 0.9705 | 0.7257 |        |        |      |
|                               |            |  | April    | 0.7646 | 0.3377 | 0.5565 | 0.5446 | 1      | 0.7257 |        |        |      |
| SIMPER TEST—Functional groups |            |  |          |        |        |        |        |        |        |        |        |      |
| Taxon                         | Av. dissim | Contrib. %                                 | Cumul. % | July   | Sept.  | Nov.   | Jan.   | Feb.   | Mar.   | April  |        |      |
| Chloro2                       | 17.49      | 26.95                                      | 26.95    | 34.3   | 43.6   | 27.8   | 32.6   | 10     | 20.3   | 23.7   |        |      |
| Diato1                        | 14.66      | 22.59                                      | 49.54    | 30     | 5.71   | 21.3   | 37.1   | 40.4   | 38.4   | 26.1   |        |      |
| Diato2                        | 9          | 13.68                                      | 63.22    | 4.2    | 22.7   | 1.28   | 3.88   | 26.9   | 13.2   | 12.6   |        |      |
| Dino                          | 8          | 11.95                                      | 75.17    | 8.88   | 8.15   | 22.5   | 1.42   | 6.11   | 13.8   | 13.7   |        |      |
| Cyano_1                       | 6          | 10.01                                      | 85.17    | 7.85   | 4.8    | 0.192  | 20.6   | 12     | 6.9    | 15     |        |      |
| Crypto                        | 6          | 9  | 94.56    | 11.1   | 11.3   | 24.5   | 0.427  | 0      | 3.54   | 0.217  |        |      |
| Prasyno1                      | 1.35       | 2  | 96.64    | 1.44   | 2.67   | 0.47   | 0.733  | 0.645  | 0.075  | 7.03   |        |      |
| Chloro                        | 1          | 2  | 98.41    | 1.48   | 0.865  | 1.47   | 0      | 1.41   | 3.87   | 0.35   |        |      |
| Prasyno3                      | 0.8088     | 1  | 99.65    | 0.474  | 0.208  | 0      | 2.71   | 2.11   | 0.005  | 1.27   |        |      |
| Hapto6                        | 0.225      | 0.3467                                     | 100      | 0.287  | 0.055  | 0.515  | 0.42   | 0.47   | 0      | 0.0567 |        |      |
| Anosim Test—Size classes      |            |  |          |        |        |        |        |        |        |        |        |      |
| Permutation N:                | 9999       |  | July     | Sept.  | Nov.   | Jan.   | Feb.   | Mar.   | April  | June   | Oct.   |      |
| Mean rank within:             | 518.7      | July                                       |          | 0.9241 | 0.4553 | 0.0165 | 0.0235 | 0.44   | 0.1514 | 0.2835 | 0.1347 |      |
| Mean rank between:            | 570        | Sept.                                      | 0.9241   |        | 0.5909 | 0.0417 | 0.0351 | 0.39   | 0.2693 | 0.2103 | 0.165  |      |
| R:                            | 0.09093    | Nov.                                       | 0.4553   | 0.5909 |        | 0.1803 | 0.2247 | 0.28   | 0.5754 | 0.2139 | 0.3316 |      |
| <i>p</i> (same):              | 0.0621     | Jan.                                       | 0.0165   | 0.0417 | 0.1803 |        | 0.6617 | 0.06   | 0.4831 | 0.0734 | 0.827  |      |
|                               |            | Feb.                                       | 0.0235   | 0.0351 | 0.2247 | 0.6617 |        | 0.22   | 0.2259 | 0.1178 | 0.5948 |      |
|                               |            | Mar.                                       | 0.4402   | 0.3908 | 0.2898 | 0.0624 | 0.2248 | 0.08   | 0.0851 | 1      | 0.4567 |      |
|                               |            | Apr.                                       | 0.1514   | 0.2693 | 0.5754 | 0.4831 | 0.2259 | 0.45   | 0.51   | 0.2524 | 0.5281 |      |
|                               |            | June                                       | 0.2835   | 0.2103 | 0.2139 | 0.0734 | 0.1178 | 0.45   | 1      | 0.2524 | 0.6075 |      |
|                               |            | Oct  | 0.1347   | 0.165  | 0.3316 | 0.827  | 0.5948 | 0.45   | 67     | 0.5281 | 0.6075 |      |
| Simper Test—Size classes      |            |  |          |        |        |        |        |        |        |        |        |      |
| Taxon                         | Av. diss.  | Con. %                                     | Cum. %   | July   | Sept.  | Nov.   | Jan.   | Feb.   | Mar.   | April  | June   | Oct. |
| % pico-                       | 12.21      | 36.4                                       | 36.4     | 19.2   | 24     | 27.6   | 51.1   | 50.6   | 18.4   | 44     | 23.9   | 42.9 |
| % nano-                       | 11.97      | 35.67                                      | 72.07    | 59.8   | 58.3   | 61.2   | 36.1   | 44.2   | 50.8   | 41.5   | 38.6   | 35.5 |
| % micro-                      | 9.37       | 27.93                                      | 100      | 21     | 17.6   | 11.1   | 12.9   | 5.17   | 30.8   | 14.5   | 37.5   | 21.6 |

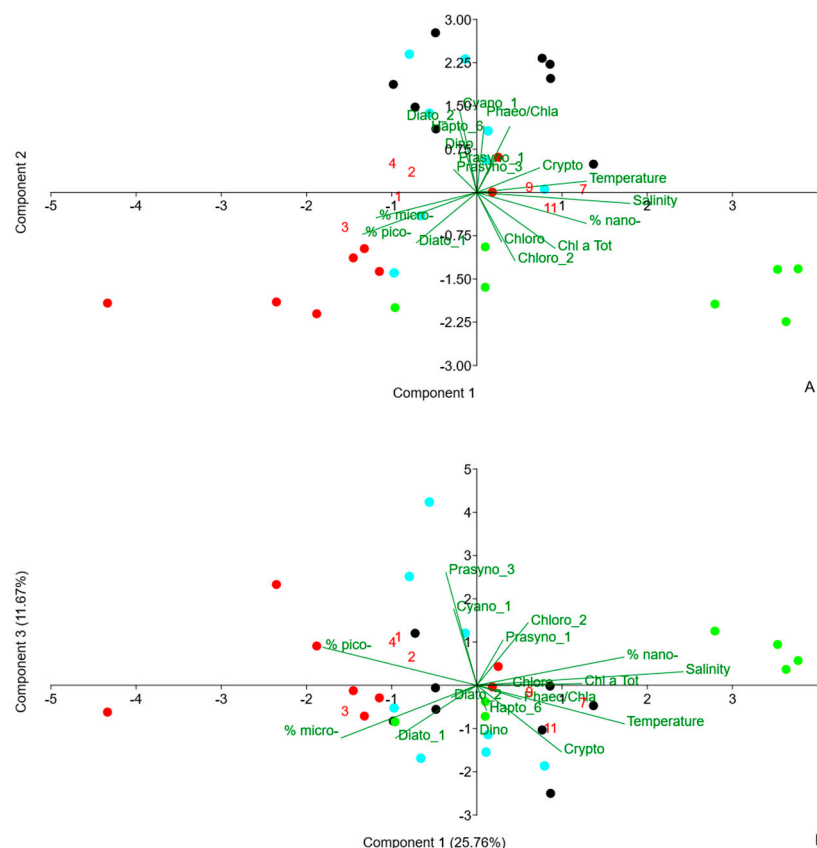
Based on what is reported above, a neighbor joining clustering analyses was performed to show the distance between ponds in terms of chemotaxonomical composition over the time period (Figure 6). The general picture shows that ponds tend to be relatively closer during winter and spring time, and maintain their typical community structure.



**Figure 6.** Neighbor joining clustering analyses based on the Bray–Curtis index, with ponds indicated with P (pond1, P1; pond 2, P2; pond 3, P3; and pond 4, P4) and related sampling months.

### 3.5. PCA

The overall picture emerging from our data is reported in PCAs, explaining 51% of total variance (Figure 7). Communities were distributed differently: pond 1 (black point) in the second quadrant, pond 2 (blue point) in the first, pond 3 (green point) in the third, and pond 4 (red) in the fourth. Temperature and salinity were the main environmental variables correlating along the positive component 1 (loading factors 0.52 and 0.37, respectively), where summer months grouped (7, 9, 11). Nanofraction (%nano-), total biomass (Chl a), and chlorophytes (Chloro\_2) showed a similar correlation with component 1 (loading factors 0.36, 0.26, and 0.13, respectively), indicating the presence of highest concentrations of biomass dominated by nanofraction and chlorophytes in most confined ponds. Pico- and micro- fractions were inversely correlated to the component 1 (loading factors 0.38 and  $-0.33$ , respectively), becoming more abundant in winter months (1, 2, and 4) when salinity was lower. Other functional groups grouped along positive component 2, as the ratio Phaeo/Chl a that was inversely correlated to diatoms (Diat\_1). Additionally, considering component 3, the distribution of variables showed a similar trend.



**Figure 7.** Principal component analyses (PCAs). Components 1 and 2 (A) explain 25.76% and 14.54% of total variance, respectively, and component 3 (B) explains 11.67% of total variance. Sampled months are reported in red numbers (1: January; 2: February; 3: March; 4: April; 5: May; 6: June; 7: July; 9: September; 10: October; 11: November; and 12: December). Chemotaxonomical groups are reported as follow: cyanophytes (Cyano\_1), chlorophytes (Chloro), prasinophytes (Pras\_3), cryptophytes (Crypto), diatoms 1 (Diato\_1), diatoms 2 (Diato\_2), haptophytes (Hapto\_6), dinoflagellates (Dino), and chlorophytes 2 (Chloro\_2). Colored dots indicate different ponds: pond 1 (black), pond 2 (red), pond 3 (blue), and pond 4 (green). %micro-, %nano-, and %pico- indicate the percentage contribution of size classes micro- (>20  $\mu\text{m}$ ), nano- (2–20  $\mu\text{m}$ ), and picophytoplankton (>2  $\mu\text{m}$ ); grazing index (Phaeo/Chl a), salinity, and temperature.

#### 4. Discussion

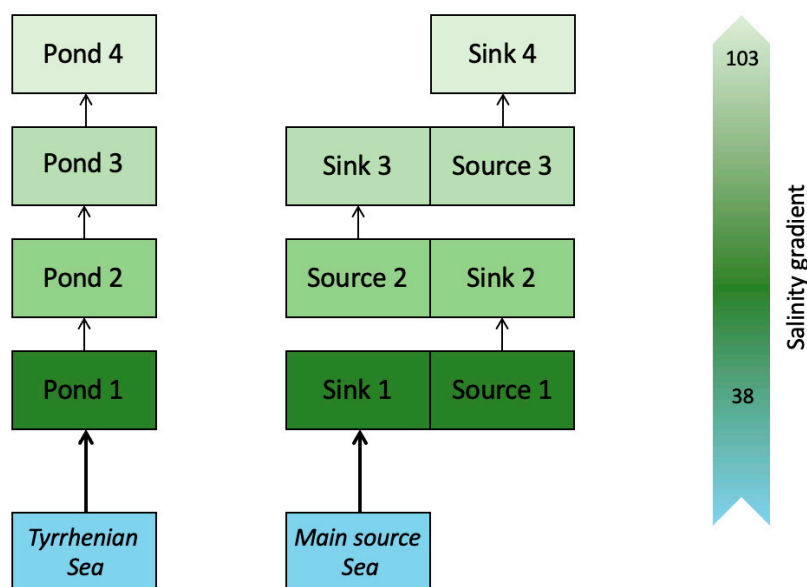
Since the turn of the eightieth century, salinas have attracted the attention of biologists, physiologists, and ecologists interested at investigating adaptive mechanisms of the organisms thriving in such demanding conditions, mostly considering single species within a physiological and an autoecological approach [13–15,28]. These studies have been largely carried out in operational plants, where environmental gradients are kept artificially constant to optimize the salt production cycle. However, the recent trend in the industrial production and international market of salt led to an increasing number of small disused salinas, many of which have resumed new life as conservation sites mainly for aquatic birds [3,50–53]. The altered hydraulic management and the underlying ecological memory of disused salinas significantly contribute to complicating the ecological dynamics of these sites, and there is a total lack of information regarding the functioning of these systems especially with regard to phytoplankton communities. In the present work we have investigated the temporal dynamics of the phytoplankton metacommunity in the disused Salina di Tarquinia, to shed light on the effects of the altered hydraulic regime on the phytoplankton communities' structure inhabiting different ponds. Our approach has

taken into account the phytoplankton functional traits with the basic assumption that these provide a reliable tool to follow successional dynamics across environmental gradients. Among traits, we have selected the size classes of phytoplankton, considering their significant role, for example, in shaping food web characteristics, and the chemotaxonomical functional groups because of their great variability in relation to environmental constraints [54–56]. This allowed us to move from a species-based autecological to a synecological perspective [57,58]. Our results indicate that the phytoplankton metacommunity in the Saline di Tarquinia points to an accumulation of biomass in summer–autumn thanks to the high productivity of hypersaline ponds, where concentrations of Chl *a* reached values up to one order of magnitude higher than ponds 1 and 2. In general, the size fraction < 20 µm was the most productive throughout the year, with the microfraction (20–200µm) relatively more abundant in ponds 2 and 1 during spring. The dominance of small size fractions has been reported by several authors working in salinas, with variable results depending on the features of the system investigated [11,24,25,59–61]. Beyond local differences, the dominance of small cells has been related to the presence of almost monospecific blooms under hypersaline conditions [28,62,63] where, thanks to the production of large amount of β-carotene, the green alga *Dunaliella* spp. represents the main and often the sole primary producer [64]. In our study, the selective pressure exerted by salt clearly emerged in the pigment: Chl *a* ratios (Supplementary Table S2), with the presence of high values of chlorophyll *c*<sub>2</sub> in ponds 1–2 and β-carotene in ponds 3–4. From a chemotaxonomically point of view, the pigmentary shift reflects in a large dominance of chlorophytes in ponds 3 and 4 throughout the sampling period, except during winter when salinity decreases as a consequence of intense rain events (Supplementary Table S1). In particular, in pond 1, near the pump station, diatoms were the most abundant group; cryptophytes, cyanophytes, and dinoflagellates showed similar percentages. Pond 2 showed a relative increase in dinoflagellates and a minor contribution of diatoms and cryptophytes.

The similarity distance between ponds in terms of chemotaxonomical composition places ponds 3 and 4 at the left side of the plots, and ponds 1 and 2 at the right ones, except during winter when the distance appears lower (Figure 6). Changes observed in the chemotaxonomy of metacommunity during the entire sampling period are also highlighted by the different Shannon indexes: 1.79 (pond 1), 1.74 (pond 2), 1.26 (pond 3), and 1.14 (pond 4).

In considering the observed changes, we must take into account that the hydraulic structure of the system determines a strong interaction between autoecological and synecological processes, with species transported from the north toward the south, from a typical ‘marine pond’ (pond 1) to a series of ponds with increasing salinity. This produces a source–sink mechanism (Figure 8) that strongly influence the productivity of the system reminding also of the concept of “second-neighbor models” of percolating systems [65,66]. At a larger scale, the entire salinas can be considered a sink, where water incoming from the sea (source site) becomes more and more salty while flowing toward the final evaporating and salt precipitation ponds (sink site). At a smaller scale, salinas can be considered a system made up by a series of source–sink sites, depending on the water flux, with a source (pond 1) and a final sink (evaporating pond) interconnected by a series of source–sink ponds. Despite the large fluctuations observed in our study, the phytoplankton metacommunity maintained basically the same structure in the overall sampling period. SIMPER and ANOSIM tests, in fact, emphasize the existence of significant differences between ponds considering both chemotaxonomical and size classes composition but none considering the sampling periods. The projections of the entire sets of variables in PCAs plots (Figure 7) show the role of salt in structuring the system, with high amounts of biomass, chlorophytes, and nanophytoplankton strongly correlated to salinity, contrary to what was observed for other groups. Although salt is undoubtedly the main driver of the observed changes, the low significance of PCAs, accounting only for 51.97% of total variance (PC1 vs. PC2 and PC1 vs. PC3), implies the existence of more complex processes contributing to the shape of the metacommunity of salinas, not least the role of successive

consumers along the trophic web, including birds whose role for similar systems is reported in several papers [3–5,7].



**Figure 8.** Conceptualization of the multiscale source–sink model proposed in this study. Blue and green colors indicate the sea and salina system, respectively; changes in the green color indicate different mean salinity values in ponds. Arrows indicate the water flux.

Based on what has been described so far, it appears evident that the stability of the metacommunity of the Saline di Tarquinia is strongly linked to the maintenance of the saline gradient between different ponds, whose presence ensures the production of high biomass values especially in the warmer months. The strong variations of salinity in hypersaline ponds, linked to heavy rains, have not led to large variations in the structure of the communities over time, despite the uncontrolled flow of water through the ponds. This indicates that, although disused, the Saline di Tarquinia represents a very important and well-structured system from an ecological point of view, that must be protected considering its role in the preservation of Mediterranean biodiversity and conservation politics [52,67]. This is particularly true in light of the observed loss of hypersaline biotopes in the Mediterranean region, partly due to the economic dismissal of many salinas along the Mediterranean coast over the past two decades, and due to the combined effects of direct anthropogenic pressure and ongoing climate change [53,68,69].

## 5. Conclusions

Our results provide additional information to the fragmentary picture arising from the available literature on salinas and complement the understanding of community dynamics influencing aspects of food webs with a conservation perspective. From a conceptual side, salinas are appropriate sites for utilizing the metacommunity concept. Their peculiar hydrographic scheme of magnifying the salinity gradient and ensuing ecological effects provide an opportunity to study community processes at proper spatial and temporal scales according to a source–sink type dynamic. This makes salinas very interesting natural laboratories for ecological studies. The high level of biomass observed, especially in warmer periods, together with the dominance of size fraction  $<20\ \mu\text{m}$  agree with other studies performed in operative reclamation projects [28]. In particular, hypersaline ponds (3 and 4), characterized by large seasonal fluctuations due to rainfall in winter and salt accumulation during summer–autumn, were dominated by chlorophytes and the size class nano-phytoplankton. Ponds 1 and 2, affected by seawater inflow, were dominated by diatoms with a relative higher contribution of micro-phytoplankton. Maintaining a



saline gradient from seawater to hypersaline levels is, therefore, a strict necessity for ensuring qualitative and quantitative aspects of the production are available for the trophic web in these complex and highly productive systems, especially for the preservation of Mediterranean hypersaline biotopes and biodiversity.

**Supplementary Materials:** The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/w15132419/s1>, Table S1: Rainfall accumulation. Daily precipitation (mm) at Portaccia Station (Tarquinia, VT, Italy) during the period 1 July 2008–1 December 2009. Table S2: Pigment ratios. Pigment: Chl a ratios for each pond and size classes <20 and <2  $\mu\text{m}$ . Chl C2 (Chlorophyll c2), Anther (Antheraxanthin), Perid (Peridinin), Prasin (Prasinolaxanthin), Violax (Violaxanthin), Zeax (Zeaxanthin), Allox (Alloxanthin), Lut (lutein), Chl b (Chlorophyll b), 19'BF (19'-hexanoyl-oxy-fucoaxanthin), Diadin (Diadinoxanthin), Diatox (Diatoxanthin), Feof (Phaeophytin),  $\beta$ car ( $\beta$ -carotene)

**Author Contributions:** Conceptualization, F.B. and O.M.; methodology, A.T.; software, F.B.; validation, F.B. and O.M.; formal analysis, A.T.; investigation, A.T.; resources, O.M.; data curation, F.B., A.T. and O.M.; writing—original draft preparation, F.B. and O.M.; writing—review and editing, F.B. and O.M.; visualization, F.B. and O.M.; supervision, F.B. and O.M.; project administration, O.M.; funding acquisition, O.M. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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