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# PHYTOPLANKTON COMMUNITY STRUCTURE IN THE GORO LAGOON ANALYZED BY MICROSCOPY AND MOLECULAR APPROACHES

Thesis in: Harmful Algal Blooms and Biotoxins

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*I am grateful to you, Lord, for everything, great and small! Thank you for showering me with far more blessings than I deserve. I'm nothing without you.*

## ABSTRACT

This study aimed at investigating the phytoplankton dynamics in a coastal lagoon with complex hydrological dynamics (Sacca di Goro, Northern Adriatic Sea, Italy) highly utilized for shellfish farming, by combining a morphological approach (microscopy) with the innovative eDNA metabarcoding, towards a more informed management of transitional areas. A monthly sampling was carried out between September 2020-2021 in 4 sites. Both the molecular and morphological method resulted valid tools for phytoplankton monitoring. Seasonal variation in phytoplankton abundances and high densities during spring dominated by diatoms (*Chaetoceros, Skeletonema*, *Pseudo-nitzschia,* and *Cyclotella* spp.) were found. Differences in taxa identification between the two methods were observed, as 147 and 158 taxa were reported using the morphological and molecular approach respectively. Although eDNA resulted efficient in detecting cryptic taxa and picophytoplankton that were not morphologically identified, limitations were reported in resolution at species level, in quantification and in identification of some groups (Cyanobacteria and Euglenophyceae), due to the lack of representative sequences in current databases. Potential HAB species were found at low densities (dinoflagellates: *Prorocentrum cordatum, Gonyaulax* sp*., Alexandrium* sp., *Heterocapsa* sp., and diatoms: *Pseudo-nitzschia delicatissima* and *seriata* complex) which could be threats to shellfish farm and human health. The study highlights the value of implementing monitoring programs using innovative tools (e.g., eDNA) to analyse the phytoplankton diversity and identify toxic species. Due to the sensitivity of transitional ecosystems, combining different approaches, such as microscopy able to quantify phytoplankton at low taxonomic level and a fast and powerful molecular tool, could be fundamental to assess the composition and ecological function of microalgal communities and facilitate a better conservation strategy in view of climate changes.



**Keywords: Transitional coastal lagoon, phytoplankton, Harmful Algal Blooms (HABs), eDNA metabarcoding, Chlorophyll-*a*.**

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| | |
|---|---|
| ASP | Amnesic Shellfish Poisoning |
| BM | Boca Mare |
| EU | Europe |
| BQEs | Biological Quality Elements |
| CNV | Copy Number Variations |
| DSH | Diarrhetic Shellfish Poisoning |
| DNA | Deoxyribonucleic Acid |
| DMS | Dimethylsulphide |
| G | Gorino |
| FV | Foce Volano |
| eDNA- | Environmental DNA |
| HTS | High-Throughput Sequence |
| HABs | Harmful Algal Blooms |
| USD | US dollars |
| MSFD | Marine Strategy Framework Directive |
| MOTU | Molecular Operational Taxonomic Unit |
| MPI | Multi-metric Phytoplankton Index |
| NGS | Next Generation Sequence |
| NSP | Neurotoxic Shellfish Poisoning |
| NGS | Next Generation Sequencing |
| OTUs | Operational Taxonomic Units |
| PCR | Polymerase Chain Reaction |
| PSP | Paralytic Shellfish Poisoning |
| PG | Porto Gorino |
| SDGs | Sustainable Development Goals |
| WFD | Water Framework Directives |
| HPLC | High Performance Liquid Chromatography |
| HPC | High performance Cluster |

## LIST OF SYMBOLS

| | |
|---|---|
| C | Carbon |
| °C | Degree Celsius |
| cells/L | Cells per Liter |
| E | East |
| g | Gram |
| mL | Milliliter |
| µg | Microgram |
| % | Percentage |
| L | Liter |
| mg | Milligram |
| mg/L | Milligram per Liter |
| N | Nitrogen |
| N | North |
| psu | Practical Salinity Unit |
| t | Tons |
| y | Year |

# CHAPTER 1: INTRODUCTION

## 1.1 Background information

Transitional waters such as estuarine coastal lagoons are highly dynamic systems with great spatiotemporal variability in physicochemical factors due to their close connection with rivers and sea (Kouadio *et al*., 2011). These ecosystems are highly productive and perform several ecological functions which contributes to the overall productivity of coastal waters providing services that support the resilience of coastal communities (Newton *et al*., 2018). Coastal lagoons are known to provide essential habitat for many aquatic organisms and are used as nursery and feeding areas by several aquatic species. They also provide economic benefits through commercial fishing and transportation of goods (Basset *et al*., 2013; Franco *et al*., 2006). Despite their ecological role, coastal lagoons are the most threatened ecosystem globally subjected to anthropogenic pressures such as climate change, habitat destruction and waste water discharge which degrades the ecosystem (Rodrigues *et al*., 2021). To reduce the impacts caused to these ecosystems, it is important to understand and quantify the response of biological communities and implement regulatory and monitoring measures that will contribute to the sustainability of coastal lagoons.

In regard to this, since 2000, European member states have been safeguarding their water resources following guidelines provided by the Water Framework Directives (WFD) (2000/60/EC) (Water Framework Directive, 2000). Under this legislation, European Member States must achieve a ‘good ecological status’ for all their water bodies by 2027 (Hering *et al*., 2018). To achieve this the WFD has employed a number of Biological Quality Elements (BQEs) including phytoplankton, benthic flora, benthic invertebrates and fish to assess the ecological status of the waters (EEA, 2018). For the implementation of this directive, the WFD has employed phytoplankton related variables such as biomass, taxonomic composition, abundance, frequency, and intensity of blooms for the definition and classification of the water quality in coastal transitional environment (Facca & Sfriso, 2009; Teresa *et al*., 2012).

Performance of aquatic ecosystems has been directly related to its Phytoplankton species diversity where it varies in the marine environment due to influence from several

parameters. Widely, Phytoplankton species diversity has been used in the monitoring of ecological change and often is used in the form of an index (Spellerberg, 2008). For this reason several biodiversity indices have been adopted and are used to assess effects of pollution and disturbance on marine ecosystems (Junshum, 2008). Diversity indices including Shannon- wiener index, evenness, and Margalef's have been used for biodiversity evaluation and indicators of ecosystem health. For instance, high diversity index is suggested to indicate a health ecosystem while a low diversity index indicates a degraded ecosystem (Ghosh *et al*., 2012). Recently a multi-metric phytoplankton index (MPI) has been adopted by Italy to accomplish the WFD requirements (Facca *et al*., 2014). The MPI index has been successfully applied in some Mediterranean lagoons such as the Venice lagoon (Aubry *et al*., 2021; Facca, *et al*., 2014), in Sardinia (Bazzoni *et al*., 2013) and currently has been employed to assess the status of the Adriatic sea coastal lagoons (Ferrari *et al*., 2021).

Although biodiversity indices allow direct comparison of communities and provide insights about a particular ecosystem in terms of pollution, it highly depends on the methods employed for taxonomic identification. A high level of expertise in taxonomic identification is required for a better characterization of the phytoplankton communities with traditional microscopy (Spatharis & Tsirtsis, 2010). In view of this, in the last decade great efforts have been made to develop alternative methods to the traditional approach for taxonomic identification of phytoplankton communities. DNA metabarcoding via Next generation sequence a newly developed method is proved to be fast and simple and enables the identification of species in environmental samples in a wide geographic location in a span of time.

The Sacca di Goro (44.78-44.83°N, 2.25-12.33°E) (Figure.1) is one of the Adriatic Sea lagoons located at the South of the Po River Delta in the province of Ferrara. The lagoon has an area of about 3700 hectares and is characterized as being shallow (an average depth of 1.5 M) with the Eastern part being the shallowest (Simeoni *et al*., 2000). To the South a sand bar named "scanno di Goro" separates the lagoon from the open sea whose waters enter the lagoon through two mouths Lido di Volano and the tip of the Scanno

about 1500m wide from each other and whose tidal dynamics (amplitude ca 80 cm) contribute to the hydrodynamic forces influencing the lagoon.

The waters are on average brackish with freshwater input generated mainly by the Po River branches, Po di Volano with a discharge of 350millioon $m^3$/y joining the lagoon at the South-Western corner, and the Po di Goro which is artificially regulated through a dam in its eastern part. The central area is influenced by the sea and canals (Giralda, Romanina, and canal Bianco) with similar flows (2.0–5.5 × $10^7$ $m^3$ /y) flowing directly into the western part of the lagoon.

The fresh water or hydraulic residence time oscillates monthly between 2.5 and 122 days with a mean value of 24.5 days, whereas the water exchange time ranges occur from 2 to 4 days. Most of the lagoon's floor is flat and the sediment consists of alluvial and mud with silty-clay contents found in the central and northern zones. In the southern shoreline the sediment is composed of sand while sandy mud sediments are found in the eastern area (Carafa *et al*., 2007). The climate of the region is Mediterranean with some continental influence (wet Mediterranean). These characteristics have been considered to contribute to the large daily and seasonal variability in the physico-chemical parameters of the lagoons' water (i.e. temperatures: 2–33°C, salinity: 6–30 psu, and pH: 7–8.8) (Corbau *et al*., 2016).

The lagoon is economically important for the residents through shellfish aquaculture Manila clam (*Tapes philippinarum*) which supports an annual production between 15000-16000 t/yr making the lagoon the leading European producers of clams. (Viaroli *et al*., 2010a). The lagoon area also represents one of the largest wetlands in the region and since 1981, the lagoon was recognized as an area under the Ramsar convection on wetlands of international importance for aquatic avifauna. Furthermore, the Eastern area of the lagoon known as Valli di Gorino together with Scanno di Goro is a State Natural Reserve.

The lagoon supports several biodiversity with submerged vegetation in the deep and brackish water of the lagoon's interior dominated by algal populations of the *Ulva* spp. and *Gracilaria* spp. Rice fields located in the lagoon watersheds and connected to the canal network draining directly into the lagoon are reported to increase the risk of pollution

from fertilizers and pesticides which consequently could affect the phytoplankton structure. (Viaroli, Giordani, *et al*., 2010b).Since 1987's after the introduction of clam farming, the lagoon experienced abnormal growth of green nitrophilous seaweeds of the Ulva complex and red macroalgae of the Gracilaria genus ( Bartoli *et al*., 2016). Clam farming deeply alters the benthic metabolism of the whole ecosystem and doubles the risk of oxygen depletion. Again, the metabolic activity of clams and their harvesting system results into water quality deterioration through release of nutrients and pollutants stored in sediments which would be assimilated in the food chain altering the productivity of the ecosystem ( Bartoli *et al*., 2003).

### 1.1.1 Phytoplankton Definition and taxonomic groups

Phytoplankton are photoautotrophic microalgae that live along the water column (planktonic) and can be solitary or colonial in form. Like other living organisms, phytoplankton is hierarchically classified from division, class, order, family, to genus level with major morphological differences and lastly to species level with smaller morphological differences within individuals. Phytoplankton can be found in a variety of forms, sizes, and structures. Based on their huge morphological variations such as cylindrical, round, oval, and fusiform cells with or without projections like flagella, cilia and thorns. Scientists have classified them into different groups: Bacillariophyceae (diatoms), Dinophyceae (dinoflagellates), Cyanophyceae (blue-green algae), Euglenophyceae, Dictyochophyceae, Chlorophyceae (green algae), Coccolithophora and Silicoflagellata (Kraberg *et al*., 2010). In addition to the taxonomic classification, phytoplankton can be classified based on organism sizes: picoplankton (0.2–2μm), nanoplankton (2–20μm), and microplankton (20–200μm) (Drews-Jr *et al*., 2013).


**Figure 1: Satellite view of Sacca di Goro, Ferrara, Emilia-Romagna**

Bacillariophyceae and Dinophyceae represents the two major groups of phytoplankton with diatoms being the most important group followed by dinoflagellates in the marine ecosystem (Maria-Teresa, 2014). Diatom is the most diverse group composed of at least 100,000 different species and contributes to about 20 % of the total primary production and 40% of the total marine primary production (Fu *et al*., 2022).

Diatoms consists of several morphological features which can be used as bases for their classification and based on valve symmetry, can be divided into two groups: Pennate (that are elongated with primarily bilateral symmetry) and centric diatoms (that are circular with radial symmetry) (Kraberg *et al*., 2010). The pennate diatoms are further classified into the raphid and araphid diatoms depending on the presence and absence of raphe. Their cell walls consist of 2 valves: a large epivalve and a smaller hypovalve. The presence of silica cover called frustule or theca and their valve morphology is mostly used for their identification (Round,1990). Blooms of some species of diatoms ( nine species of *Pseudo-nitzschia* and one species of *Nitzschia* are associated to results in Amnesic shellfish poisoning (ASP) (Hégaret *et al*., 2009).

On the other hand, Dinophyceae are unicellular organisms characterized by having two flagella: longitudinal and transversal flagella. The cells are surrounded by a complex theca and in some cases a thin additional layer, the pellicle. Dinoflagellates are divided into two: thecate dinoflagellates consisting of an outer covering theca and athecate (or naked dinoflagellates) which lack the theca. The theca, which is their cell covering structure, differentiates them from other algal groups. The theca may be smooth and simple or may have spines, pores, or grooves with various arrangement and is used to distinguish them from other algal groups. They can also be distinguished from other groups by the presence of cingulum and sulcus (Corbau *et al*., 2016; Tomas, 1996).

Dinoflagellates have been globally recognized as the causative agents of most of the HABs in the marine environments where long term spring-summer blooms results into various types of human illness through toxins production, namely paralytic shellfish poisoning (PSP), neurotoxic shellfish poisoning (NSP), diarrhetic shellfish poisoning (DSP), and ciguatera (Anderson *et al*., 2021; Yu *et al*., 2018).

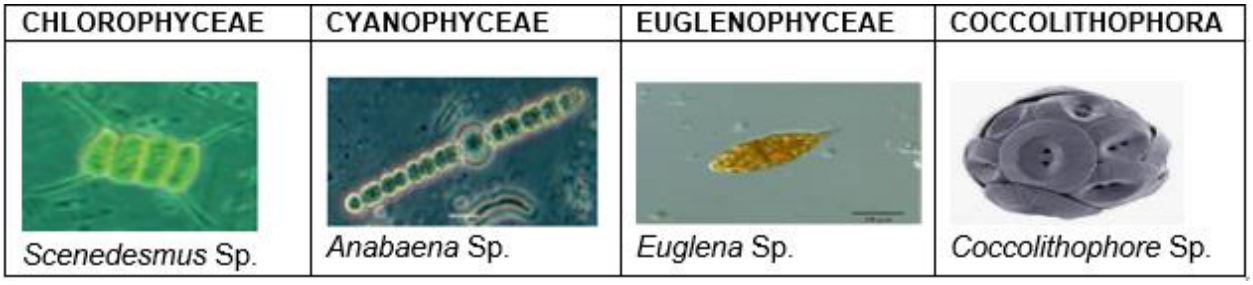



**Figure 2: Images of algal cells representative of some phytoplankton groups: Chlorophyceae, Cyanophyceae, Euglenophyceae and Coccolithophore** (ICRAM, 2006).

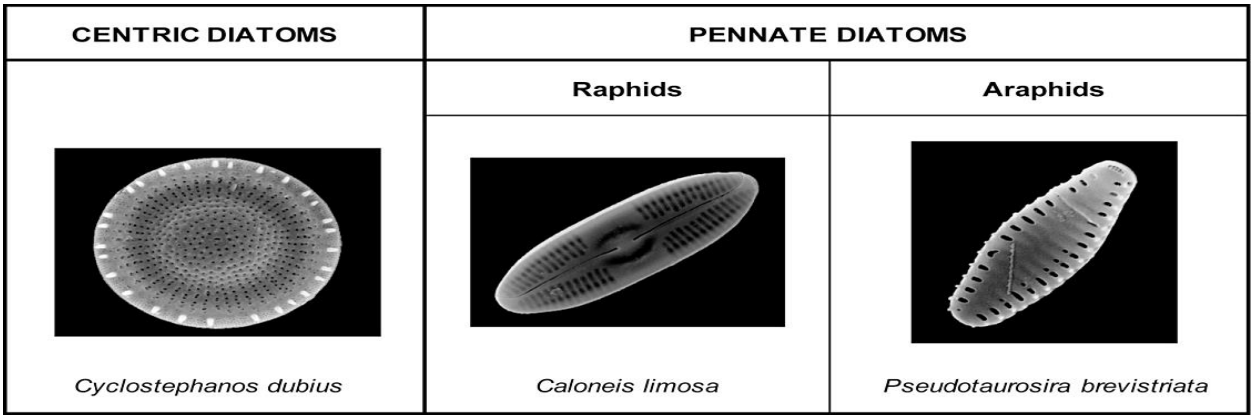



**Figure 3: Examples of algal species of the main diatom groups: centric on the left and pennate on the right** (Kligmann & Calderari, 2012)**.**

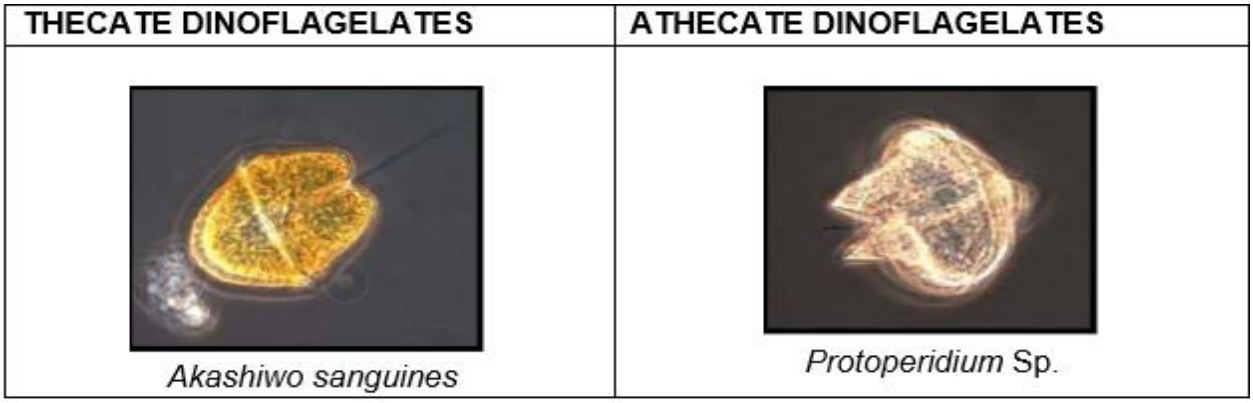



**Figure 4: Example of the major classification of the dinoflagellate's species: Thecate cell on left and Athecate cell on the right** (ICRAM, 2006)**.**

### 1.1.2 Environmental role of phytoplankton

Phytoplankton are responsible for almost half of global net primary production sustaining aquatic food webs in marine and freshwater environment (Field *et al*., 1998). Within the marine environments, phytoplankton serve as important sources of energy initiating the marine food web as primary producers providing food to primary consumers. About a quarter of the world's oxygen is estimated to be produced by phytoplankton (Balkanski *et al*., 1999). They also have an ecological role of nutrient cycling affecting the water quality variables such as turbidity and dissolved oxygen thus influencing many ecosystem processes (Garmendia *et al*., 2013; Otero *et al*., 2020).

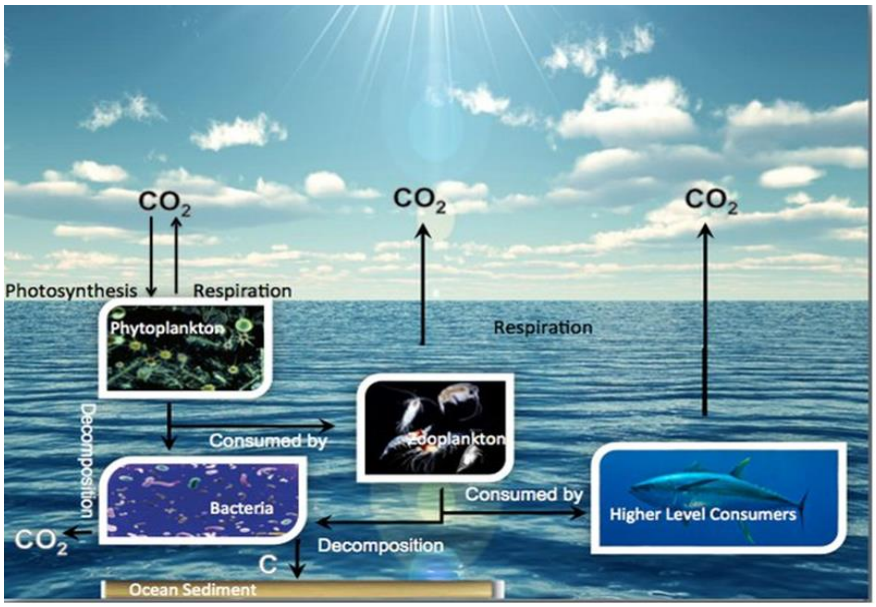



**Figure 5: oxygen cycle and photosynthetic role of phytoplankton as primary producers to other trophic organisms.**

Furthermore, phytoplankton responds to variations in chemical, physical and hydrodynamic parameters and is therefore considered an excellent indicator of change of the trophic state of the water quality, signaling nutrient enrichment that result to an increase in biomass (Bužančić *et al*., 2016; Carstensen *et al*., 2015a; Gobler *et al*., 2017; Trombetta *et al*., 2019). The presence of certain phytoplankton taxa can be considered an indicative of good or bad ecological status. Apart from being used as indicators of water quality, phytoplankton can be used to show changes in climate over a range of period (Fereshteh, 2014). Their population and composition could as well be used as direct indicators of human interference within the marine environment where sources of pollution can be determined.

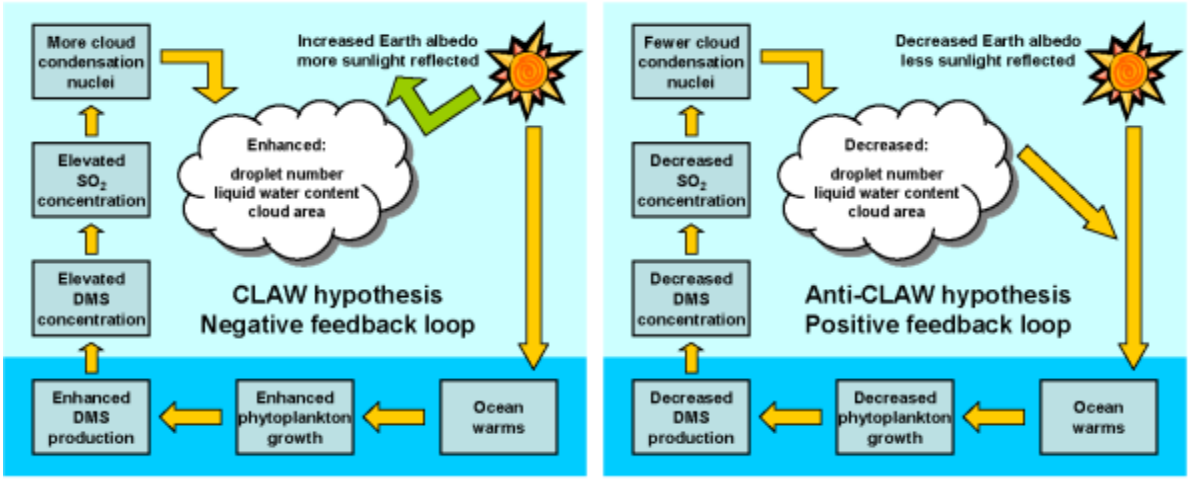



**Figure 6: Diagram of the CLAW hypothesis and the anti-claw hypothesis explaining the role of phytoplankton in climate regulation through excretion of dimethylsulphide (DMS)** (Charlson *et al*., 1987; Lynch, 2008)**.**

### 1.1.3 Factors affecting diversity of phytoplankton

Phytoplankton species occur in different environments. Some species exhibit a high growth in eutrophic environment while other species prefer oligotrophic environments. They also have a seasonal fluctuation with some species being present throughout the year and others only found in a certain period of the year. Their composition, succession and abundance are a function of different hydrological parameters such as nutrient concentrations, salinity, temperature, pH, and solar radiation. These parameters have resulted into the high variability and distribution of phytoplankton. Seasonal succession of phytoplankton could as well be attributed by their relationship with other marine organisms through grazing (Zhu *et al*., 2021).

#### 1.1.3.1 Temperature

As a result of climate change, the average global temperature is predicted to increase by 0.3-0.9°C per decade (Coello-Camba & Agustí, 2017). Temperature is an important parameter that controls physiological rates and affects biological and chemical processes in aquatic environments. Phytoplankton growth rates are related to changes in temperature where an increase or decrease affects the growth rate and reproduction of algae (Eppley, 1972). Phytoplankton species have different responses to temperature, which result in seasonal changes in species composition and biomass. The impacts of

temperature on phytoplankton can be directly by altering physiological processes or indirectly due to changes in grazing activity of zooplankton. Rising temperature because of climate change is expected to be responsive in species evenness and richness. For example, laboratory experiments have reported a loss of species richness at higher temperatures (Burgmer & Hillebrand, 2011) while field studies have reported an increasing number of species richness by immigrating warm adapted species which also are influenced in their global distribution (Beaugrand *et al*., 2010). Studied have shown that dinoflagellate species prefer warmer temperatures while diatoms mostly dominate in temperate cooler regions (Finkel *et al.*, 2010).

#### 1.1.3.2 pH

Worldwide pH levels in the ocean ranges from 7.9-8.4; however, it may vary by 1 or more pH units. Variations may be attributed by changes in temperature, salinity, and biological activities. An increase or decrease in pH in the marine environment is expected to cause a change in the phytoplankton composition. Hinga, (2002) performed a review of twenty-one studies on the effects of pH on marine phytoplankton and found out that different clones of the same species had slightly different relationships between pH and growth rate. Species such as dinoflagellates (*Heterocapsa triquetra*, *Prorocentrum minimum* and *P. micans*), and the diatom *Skeletonema costatum* are found to co-occur with high pH in nature (Hansen, 2002). Laboratory experiments involving monocultures have shown that some cryptophytes, diatoms, dinoflagellates and prymnesiophyte species can grow at pH above 9, and a few species even above pH 10 (Schmidt & Hansen, 2001).

#### 1.1.3.3 Dissolved oxygen

Dissolved oxygen expressed as mg/L in water has a major role in water analysis and its amount can be used as an indication of water quality resources. It is a fundamental requirement for most organisms in metabolism, therefore its concentration in seawater affects the physiology, composition, and abundance of species (Vaquer-sunyer & Duarte, 2008). Climate change is anticipated to increase hypoxic levels further (Meire *et al*., 2013). Hypoxic is common within the European seas attributed by a combination of eutrophication and hydrodynamical conditions. The Black Sea, Baltic sea and the Greater North sea, for instance, have reported hypoxic events while in the Mediterranean, Adriatic

and Aegean Sea some areas are at risk of becoming hypoxic (Capet *et al*., 2016; Carstensen *et al*., 2014; Druon *et al*., 2004; Klein *et al*., 2003; Topcu & Brockmann, 2015).

#### 1.1.3.4 Salinity

Most phytoplankton species are stenohaline, and suffer osmotic stress upon exposure to salinity changes (Lionard *et al*., 2005). Salinity is among the most important property of seawater that influence the growth and survival of phytoplankton. Majority of the species are characterized by a wide salinity tolerance and can grow in a broad range of salinity (Brand, 1984). Estuaries are the ecosystems mostly influenced by salinity where freshwater supplied by rivers is mixed with seawater brought by tides creating an estuarine salinity gradient, having almost pure freshwater near the head of the estuary and seawater near the mouth of the estuary. In such environments, a succession of phytoplankton can be observed, as typical freshwater diatoms like *Cyclotella* and *Stephanodiscus* below 0.5 psu or marine diatoms like *Thalassiosira* species, and *Skeletonema costatum* above 10 psu (Snoeijs, 2017).

#### 1.1.3.5 Nutrients

The supply of nutrients and their concentrations within the marine environments greatly influence the size, taxonomic structure, and abundance of phytoplankton. Phosphates and nitrates are the main limiting nutrients in marine environments and influence marine phytoplankton, while silicates often limit diatom growth. Although available in small concentration within the marine environments, small amounts are sufficient for phytoplankton growth and further increases could results in ecosystem imbalance. Their concentration and distribution are not only useful in predicting phytoplankton abundance and assemblages but also serve as a marker for the status of the general ecosystem. The carbon (C), nitrogen (N) to phosphorus (P) ratio for planktonic algae (106:16:1) for example has been used to indicate nutrient limitation in phytoplankton and its concentration provides a forecast for phytoplankton dynamics and development of management strategies (Redfield, 1934).

Significant efforts have been put to manage nutrients in all EU regional seas, however eutrophication caused by nutrients inputs (nitrogen and phosphorus) remains a large problem in the Baltic Sea, Black Sea, parts of the North-East Atlantic and Mediterranean

Sea. Based on the eutrophication summary status of European seas, about 12.8% Mediterranean Sea regions have their status still unknown (EEA, 2019). Following the use of phytoplankton as an indicator of change in nutrient, data on phytoplankton community structure and its abundances can serve as a major element in assessing eutrophication, filling the knowledge gap for Mediterranean coastal lagoons.

### 1.1.4 Phytoplankton or harmful algal blooms

Although phytoplankton are key primary producers in all aquatic habitats, other forms of aquatic life are affected when their population increases. Bloom formation occurs naturally and could be harmful after long durations. Harmful algal blooms (HABs) signifies ecosystem imbalance that results due to many environmental changes (Watson *et al*., 2015). Many marine phytoplankton species are known to produce endogenous toxins, and when these species accumulate in sufficient numbers, they become harmful to the marine environment. Harmful algal blooms (HABs) can have significant economic, environmental, and social consequences. Biotoxins produced by HAB species can concentrate in the tissues of bivalve shellfish which may be fatal and can give shellfish poising syndromes when consumed by human (Hégaret *et al*., 2009). Huge financial losses to fish and shellfish industries have been reported globally. In 2016, HABs of the dictyochophyte *Pseudochattonella verruculosa* and the dinoflagellates *Alexandrium catenella* resulted into over 40000 tons of farmed salmon death in Chile (Armijo *et al*., 2020) while in Japan massive fish and shellfish mortalities as a result of HABs resulted into over 246 million US dollars (USD) since 1970s (Sakamoto *et al*., 2021).

In the Adriatic Sea blooms have been mainly associated with the dinoflagellates and diatoms groups with total abundances of phytoplankton in a bloom estimated at $40x10^6$ cells/L (Pistocchi *et al*., 2012). According to Tsikoti & Genitsaris, (2021) the diatoms *Skeletonema marinoi, Pseudo-nitzschia* spp. *(P. delicatissima, P. pseudodelicatissima, P. multistriate), Chaetoceros* spp.*,* and *Cylindrotheca closterium* cause seasonal blooms in the northern Adriatic Sea. In addition, different species of dinoflagellates have been associated with HAB event in this area; *Dinophysis* spp*. (D. tripos, D. sacculus,* and *D. caudata)* resulted in Diarrhetic shellfish poisoning (DSP) episodes, *Alexandrium* spp*.* (*A. minutum* related to Paralytic shellfish poisoning (PSP) episodes, and *A. mediterraneum,*

*A. pseudogonyaulax, A. tamutum, A. taylorii)* (Valbi *et al*., 2019). As from 2006 severe blooms of the benthic dinoflagellate *Ostreopsis* cf. *ovata* have occurred in the northern Adriatic sea resulting to aerosolized toxins with effects on human and benthic organisms (Accoroni *et al*., 2011; Monti *et al*., 2007). Marine biologists are globally required to periodically investigate the community structure, growth pattern and seasonal succession of phytoplankton to provide more insights on HABs.

### 1.1.5 Techniques for Quantifying phytoplankton biodiversity

Phytoplankton diversity is a key measurement of the state and activity of the marine environment. Characterization of phytoplankton biodiversity within the marine environments has a long history but questions about how many different species exists in the ocean and how their physiology and behavior vary among species in response to environmental and biological factors still exists. These questions among others have attracted algologists to characterize phytoplankton community structures to understand the state of the environment, possible harmful species, and how organisms relate to each.

#### 1.1.5.1 Microscopy

The development of microscopy by Robert Hooke and Antonie van Leeuwenhoek provided the first detailed characterization of phytoplankton diversity through direct observation. Although it consisted of low optical resolution, it enabled a more detailed view of their morphology allowing an initial characterization of plankton's taxonomic diversity (Johnson & Martiny, 2015). Since then, continued improvements of the light microscope as well as the development of other microscopes (electron and fluorescence ones) has helped to study many of the early groups of marine phytoplankton.

Direct morphological observation of phytoplankton still represents an important technique in characterizing phytoplankton biodiversity, especially for larger cells that have distinguishable morphologies. However, based on literature, characterization of phytoplankton community structure using microscopy has some disadvantages. First, identification of cryptic species is impossible, photosynthetic pico-phytoplankton with <3µm diameter are left out since they lack taxonomically useful external morphological features (Hebert *et al*., 2004). Due to its tiring, errors might happen during identification and can result to oversimplification since often taxa can only be identified to a higher

taxonomic level (e.g., family or genus level). The presence of cryptic taxa and lack of diagnostic features in some developmental stages also makes morphology-based identification not effective (Deagle *et al*., 2017). Huo *et al*., (2020) has pointed out that phytoplankton community composition assessment based on only morphological characteristic could be misleading.

#### 1.1.5.2 Chlorophyll-a

Algae have also been characterized based on colorimetric approaches. Algae have three types of photosynthetic pigments: chlorophylls, carotenoids, and phycobilins. Four types of chlorophyll exist in algae: -a, -b, -c, and -d which are taxon specific and could be used for algae classification. Early studies classified algae into four groups; red, green, brown, and diatoms based on their appearance which formed the foundation for pigment-based classification that is still in use today (Mackey *et al*., 1996).

Chlorophyll-a, a green pigment found in plants including phytoplankton, is a core pigment for photosynthesis through light absorption. Apart from being used as a water quality parameter, chlorophyll-a concentrations have been used as indicators of phytoplankton abundance and biomass in water bodies. Although included in the MSFD (MSFDe2008/56/EC), biodiversity of phytoplankton is very difficult to estimate, and monitoring has usually been limited to certain groups. Johnson & Martiny, (2015) precise identification is challenging since diagnostic accessory pigments are not unique to specific groups. In addition, light, nutrient availability, and other environmental parameters strongly influence pigment ratios. Thus, the limited applicability to quantify specific taxa calls for other better techniques.

#### 1.1.5.2 DNA Metabarcoding in Europe

As new techniques continue to develop, and existing methods become more advanced, emerging molecular genetic approaches, such as DNA metabarcoding with high-throughput sequencing (HTS), are becoming increasingly effective for quantifying phytoplankton diversity and relative abundances (Dowle *et al*., 2015). Metabarcoding is a rapid method of biodiversity assessment that combines two technologies: DNA barcoding and high-throughput DNA sequencing. DNA metabarcoding uses polymerase chain reaction (PCR) primers to amplify a highly conserved gene region (barcode) which

is taxon-specific, and the obtained PCR products are then sequenced. To detect a specific taxon and produce general diversity estimates, sequencies are analyzed by compared with DNA reference databases (Cristescu, 2014).

Metabarcoding together with environmental DNA (DNA extracted from a bulk sample from an environmental matrix) can potentially overcome most of the limitations experienced while using morphology-based taxonomy techniques. The technique is cost-effective and following its high sensitivity, rare taxa can be identified. This technique is also fast, requires less specialists on taxonomic expertise and results into more comprehensive data (Ji *et al*., 2013).

Metabarcoding has been widely applied in different studies within the marine environment using various species. For example, from benthic marine meiofauna to open ocean protists and zooplankton (Deagle *et al*., 2017; Fonseca *et al*., 2010; Keck *et al*., 2021). However, due to the high incompleteness of barcoding reference databases for taxonomic association of metabarcoding data (particularly at lower taxonomic levels), DNA metabarcoding outcomes are still limited nowadays. Currently this necessity represents one of the main drawbacks as some groups of organisms have none or very few publicly available sequences (Abad *et al*., 2016). Additional gene flow might be still possible even between less closely related species leading to intermixture of barcodes (Weigand *et al*., 2019). Copy number variation (CNV) associated with rDNA genes could also affect abundance estimates when using metabarcoding (Kembel *et al*., 2012). The technique is also affected by human during library development which affects the reliability of DNA barcoding to correctly identify specimens to species. These includes identification errors, sequence contamination and inadequate data management (Weigand *et al*., 2019)

In Europe, DNA metabarcoding has been applied in different fields; in freshwater bivalves biodiversity assessment (Valentini *et al*., 2021) in the bioassessment of Mediterranean rivers using diatoms (Mortágua *et al*., 2019; Pérez-burillo *et al*., 2020; Valentin *et al*., 2019) in comparative studies, for example Lin *et al*., (2022) used DNA metabarcoding methods to investigate plankton biodiversity under varying anthropogenic pressures (shipping and bivalve aquaculture) along the Eastern Adriatic coast (the Northernmost

part of the Mediterranean Sea. A recent study conducted at the Venice lagoon and the Gulf of Venice (Northern Adriatic sea) revealed that by the use of metabarcoding approach to assess the composition of protists resulted into many taxa not reported in the area (Minicante *et al*., 2019).

Nevertheless, studies combining traditional microscopy and DNA metabarcoding to fully characterize the phytoplankton community structure in coastal lagoons are still scarce but really important for the development of better monitoring policies

## CHAPTER 2: SCOPE OF THE RESEARCH

Europe's coastal areas have expressed the need to balance economic development with environmental protection. With the vast ecosystem services offered, approximately 40% of the EU's population lives within 50km of the sea. Due to increased anthropogenic activities within coastal environments, biodiversity has declined resulting into ecosystem instability and reduction of ecosystem services. Activities such as shipping, resource extraction, fishing, industrialization, agriculture, and climate change have threatened coastal ecosystem resulting to habitat loss through pollution across Europe. The EU Member State report that only 13% of coastal species are in favorable conservation status while 73% of coastal habitats are assessed as being bad' or 'inadequate' with coastal lagoons termed severely threatened across the EU regions (Silva *et al*., 2017).

Most studies have focused on the loss of biodiversity in larger organisms. Little information is known concerning the effects of biodiversity loss for microorganisms such as algae. Phytoplankton being the base of the food web may result into huge impacts for higher trophic levels in the case of their reduction. Over the last century studies on phytoplankton diversity, abundance and distribution have been based on morphological approaches. Although still in use this technique is challenging and time consuming with small cryptic species remaining undefined requiring expertise which are increasing becoming rare. Out of the global total estimate (over a million species) only 72500 algal species are estimated to have been identified Guiry, (2012). Therefore, to reduce the decline in biodiversity precise assessment of the current biodiversity should be prioritized.

Phytoplankton, the key important primary producers in the marine environment, can provide insights about the status of the ecosystem. Phytoplankton have been widely used as indicators of aquatic ecosystem health conditions because of their small size, rapid growth rates, wide geographic distribution, and their specific sensitivity to variations of a wide variety of environmental conditions. Following the global increase in eutrophication and harmful algae, studies related to the dynamic of phytoplankton community structure have achieved great importance. Comprehensive studies on the annual and seasonal variability of phytoplankton should also be intensified to fully understand the health status

of aquatic ecosystems. However, this is still a challenge due to lack of efficient taxonomic identification techniques.

In the waters of the Adriatic Sea particularly in the Sacca di Goro lagoon, information about phytoplankton community structure has been periodically achieved by traditional morphology-based techniques for taxonomic assessment and made available (i.e. ARPA-EMR, 2013; Carla *et al*., 2017; Ferrari *et al*., 2021). Diatoms dominate in the Sacca di Goro lagoon (ARPA-EMR, 2013; Carla *et al*., 2017; Ferrari *et al*., 2021). However, previous reports have classified over 1,832,460 cells/L under undetermined group (Ferrari *et al*., 2021) due to the use of traditional morphological approaches.

Similarly, in the Venice lagoon, Aubry *et al*., (2021b) reported >40% of the total phytoplankton abundances belonging to the undetermined nano-flagellates. The study also highlights that nano-flagellates are difficult to identify with light microscopy and in routine phytoplankton long-term monitoring such species will always remain undetermined contributing to invalid data. (Caroppo *et al*., 2018; Djakovac, 2009; Draredja *et al*., 2019; Jasprica *et al*., 2022) recorded great percentages of undetermined phytoplankton in their studies within the Mediterranean lagoons. To fully characterize the phytoplankton community structure in the Mediterranean coastal lagoons there is the need to integrate morphological data with other techniques such as the molecular techniques.

Up to date no assessment of phytoplankton in the lagoon based on molecular techniques has been done. Thus, this study aimed to determine the phytoplankton community structure of the Sacca di Goro lagoon by combining (NGS) environmental DNA-metabarcoding with microscopy for a better management of the lagoon waters following its economic value as a clam farm, as well as requirement for the WFD. The data would provide useful information in predicting changes in ecosystem and could also help in the development of an integrated management plan for the sustainable management of the lagoon ecosystem, thus contributing to the achievement of the Sustainable Development Goals (SDGs). Such management plans are crucial for improving and restoring aquatic ecosystems and may provide a scientific basis for further ecological evaluation, and protection of transitional waters.

## 2.1 General objective

To delineate the phytoplankton community structure in the Sacca di Goro lagoon by combining both eDNA- metabarcoding and microscopy.

## 2.2 Specific Objectives

a. To highlight the composition, and spatial-temporal distribution of phytoplankton assemblages.

b. To determine the dominant phytoplankton taxa and evaluate their importance in the assessment of the trophic status of the lagoon.

c. To identify the phytoplankton species that have the potential to form harmful algal blooms (HABs).

d. To compare the effectiveness of the eDNA metabarcoding approach against taxonomic identification through microscopy as fast tool for the assessment of the phytoplankton community structure.

# CHAPTER 3: MATERIALS AND METHODS

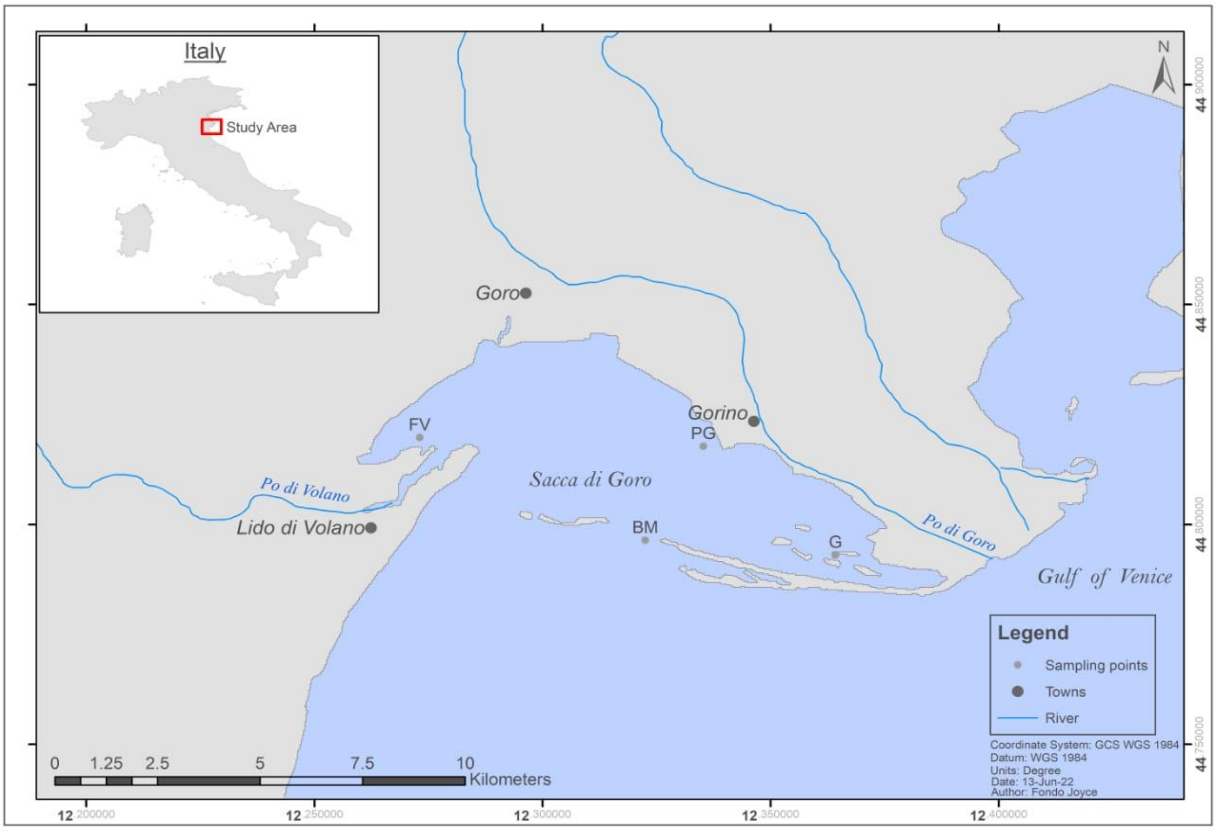



**Figure 7: study area Map and location of sampling stations in the Sacca di Goro lagoon (southern Adriatic Sea).**

## 3.1 Sampling

Samplings were carried out in the Goro lagoon between September 2020 and September 2021 in four sites: Gorino (G) 44°47'35"N, 12°21'51"E located in the eastern part, at the edge of the Valle di Gorino, the shallower and most confined part of the lagoon; Foce Volano (FV) 44°49'11"N, 12°16' 23"E located in the western part and is mainly influenced by freshwater discharged from Po di Volano and Giralda and is therefore characterized by variable salinity; Bocca Mare (BM) 44°47'47"N 12°19'21"E and Porto Gorino (PG) 44°49'04"N, 12°20'07"E ) located in the central area and are influenced more by tidal exchange (Figure 7). Sampling involved randomly collection of three 1L water bottles of

superficial seawater from each site each month. Before sampling, environmental variables were measured as indicated in (table 1). Water samples were placed in an ice box and transported to the laboratory for further analysis.

**Table 1: Environmental variables measured during sampling months for each site**

| Environmental variable | Equipment |
|---|---|
| Depth | Meter and ballast |
| Temperature | Multi parameter probe (HQ30d, HACH-LANGE GmbH) |
| Dissolved oxygen | Multi parameter probe (HQ30d, HACH-LANGE GmbH) |
| Salinity | Refractometer (ATAGO S-10). |

## 3.2 Sample preparations and analysis

### 3.2.1 Chlorophyll-*a* (Chl-a) analysis

Approximately 200-600 mL of seawater from each replicate was filtered through a Glass Fiber GF/F of 0.7µm-porosity with a diameter of 47mm (Whatman). The filters were then immediately stored at -20°C until further analysis.

The analysis of chlorophyll-*a* concentrations (µg/L) and its degradation pigments (phaeopigments) were performed from spectrophotometric measurements on acetone extracts followed by acidification (Lorenzen, 1965). Frozen filters obtained from natural samples were extracted overnight with 10 mL of 90% acetone in centrifuge tubes and kept in a dark and cold place (4-8°C) for 20 hours. Subsequently, the samples and solvents were acclimated to room temperature in a dark place for at least 30min after which another 5mL of 90% acetone was added followed by brief vortex. The samples were later centrifuged for 5-10min at 4000 rpm.

The spectrophotometry was set using fixed wavelengths at 750 and 665nm. Prior to spectrophotometric reading, the autozero and blank were performed with acetone 90%.

3mL of the sample supernatant was carefully pipetted from the centrifuge tubes and transferred into stoppered cuvette for absorbance reading in the spectrophotometric at 750nm ($E_{750}$) and 665nm ($E_{665}$) against the blank. After the run, the sample extracts were subsequently acidified by directly adding 30 µL of HCl (0.66M) followed by mixing and another absorbance reading after 1min of reaction at the same wavelengths. All sample preparations were done under subdued light. Chlorophyll-*a* concentrations and phaeopigments calculations were done using the equations below.

$$chl\ a\ (\mu g/L) = \{26.73 * [Abs(665 - 750) - Abs(665ac - 750ac)] * v.estr(mL)\}/[V.filtr(L) * 1$$

Whereas for phaeopigments, the equation below was used for the calculations.

$$Feop.(\mu g/L) = \{26.73 * [1.7 * (Abs(665ac - 750ac)) - (Abs(665 - 750))] * v.estr(mL)\}/[V.filtr(L) * 1]$$

Where;

Abs (665-750) = absorbance difference 665-750nm before acidification, already subtracted for the blank, Abs (665ac-750ac) = absorbance difference 665-750nm after acidification, already subtracted for the blank, V. estr (mL) = Extraction solvent volume in mL, V. filtr (L) = volume of filtered sample in (L) and 1= Optical path of the cuvette (1cm).

### 3.2.2 Quali- quantitative analysis using Microscopy

The phytoplankton community structure was characterized using the Lugol- fixed bottle samples in the time frame between September 2020 and September 2021 using the Utermöhl sedimentation method (Utermöhl, 1958). About 10-25mL volume from each replicate was allowed to settle before cell counting. To obtain sufficient abundances, for samples with low chlorophyll-*a* concentration and higher salinities, 25mL volume was allowed to settle for 24 hours while samples with high chlorophyll-*a* concentration and low salinities, 10mL volume was settled for about 10 hours. Fixed phytoplankton cells from the settled samples were identified and counted by means of transect count under a Zeiss Germany Axiovert 100x inverted microscope at 320x magnification. Taxonomic identification was carried out with reference manuals (ICRAM, 2006; ISPRA, 2009;

Tomas, 1996). Nomenclature was updated with reference to www.algaebase.org. The total number of cells per liter was calculated using the following formula:

$$Total\ cells/L = Total\ cell\ count/n.transect * 1000/V.sedimentation(ml) * F$$

Where F is a factor obtained by dividing the area of the bottom of the chamber and the visible area of the transect with 320x focus.

A. Sampling

B. water filtration

C. Quantitative analysis

**Figure 8: images illustrating some of the activities carried out during this study. A. water sampling at the study site, B. Water filtration for both chlorophyll-a and DNA analysis, and C. identification of phytoplankton under a microscope.**

### 3.2.3 DNA metabarcoding analysis

About 200mL to 1L water samples from each replicate was filtered through 0.45 µm Nitrocellulose filters of 47 mm diameter. Each filter was separately stored in a sterile 1.5 mL tube at -20°C until DNA extraction.

#### 3.2.3.1 DNA Extraction

DNA was isolated from filters using the DNeasy Power Water Kit (Qiagen, Hilden, Germany), following the protocol provided by the manufacture with a slight modification. The final DNA elution volume of 100µL was aliquoted in three sterile 1.5mL tubes (~27 µl eluted DNA per each tube) for downstream applications, all stored at −20˚C until further processing. All the DNA extractions were performed under a sterile laminar flow hood with sterile tools properly autoclaved before using. Negative extraction control samples were included for environmental contamination checks. The DNA extraction yields were

quantified using the Qubit fluorometer (ThermoFisher Scientific Inc, Waltham, MA, USA) using the broad range dsDNA assay kit.

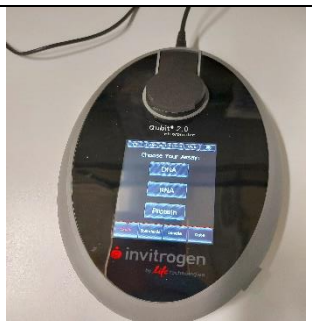
#### 3.2.3.2 PCR Amplification and Sequencing

Polymerase chain reaction (PCR) of the V4 and V5 region of the 18S rRNA gene was performed using universal eukaryotic specific primers; 566F (CAGCAGCCGCGGTAATTCC) and 1200R (CCCGTGTTGAGTCAAATTAAGC) (Hadziavdic *et al*., 2014). Additional 12bp tags were added to allow for sample multiplexing into a single library (each tag has at least 5 differences out of 12 bases). PCR amplification was performed in a final volume of 20 µL: 17µL of AmpliTaq Gold 360 Master Mix (ThermoFisher Scientific Inc, Waltham, MA, USA), 0.5µL of forward primers, 0.5 µL of reverse primers (10 µM working concentration) and 2µL of DNA sample. The PCR cycle consisted in an initial denaturation phase at 95°C for 15min, followed by 35 cycles of 95°C (45s), 60°C (45s), 72°C (1min), and a final elongation step at 72°C for 10min. PCR yields were checked through gel electrophoresis in 1.5% agarose gel withTAE 0.5X:

The PCR products were pooled together in a single Eppendorf tube and homogenized. The pool was purified using NucleoSpin Gel and PCR Clean-up kit (MACHEREY NAGEL, part of ThermoFisher Scientific Inc, Waltham, MA, USA) following the manufacturer's protocol. DNA concentration of the final pool were determined through a Qubit fluorometer using the Broad Range DNA quantification kit. ONT proprietary ligation step was performed using SQK-LSK110 kit for library preparation. The sequencing was performed on a MinION Mk-1C, using a FLO-MIN111 sequencing chip for 12 hours run.

A. DNA extraction

B. Positive control samples




C. DNA quality check

**Figure 9: Images illustrating A. DNA extraction, B. some known algae species used for the positive control and D. Qubit machine with extracted DNA samples for quantification.**

#### 3.2.3.3 Bioinformatics analyses

Basecalling of raw FAST5 files was performed on a HPC (High Performance Cluster) using the Guppy software (ONT), with a quality score threshold of 8. Demultiplexing was performed using OBITOOLS, (Bras *et al*., 2016). Chimera detection and clustering were carried out using VSEARCH and SWARM respectively (Rognes *et al* 2016). The taxonomic association of the final MOTUs table was performed using RDP classifier with SILVA 138 18S Reference Set (Wang *et al*., 2007). The bootstrap thresholds for the taxonomic levels were 0.8 for kingdom, 0.8 for phylum, 0.8 for class, 0.8 for order, 0.85 for family and 0.99 for genus.

### 3.3 Phytoplankton Data Processing and Statistical Analyses

Different types of analysis were performed to meet the objectives of the study. The spatial-temporal distribution of phytoplankton was assessed by calculating the total abundance for each month and relative abundances for all the four seasons. We adopted a convectional division of the seasons defined as follows: (winter: November and December; spring: February, March, April, and May; summer: June, and autumn: September and October), We used this division to individuate the most abundant taxa in each season and highlight those with potential of causing HABs. Phytoplankton community was analyzed in terms of total cells belonging to the main phyla and genera;

data were expressed as the number of cells per liter (cells/L) or as relative abundance (%). Calculations were performed using excel spreadsheets.

Diversity indexes were applied for detecting the random distribution of individuals. Shannon diversity index (H') (Shannon & Weaver, 1964) which takes into account both the abundance and evenness of taxa present in the community:

$$H' = -\sum_{i=1}^{s} p_i - \ln p_i$$

Evenness (Pielou, 1966) which is simply the number of taxa found in a given sample:

$$J' = \frac{H'}{log_2(S)}$$

Margalef's index (M') (R. D. Margalef, 1957) which explains the relationship between the number of taxa detected (R') and a transformation of the total number of individuals counted:

$$M' = \frac{R' - 1}{\ln N}$$

All univariate variables were analyzed by a 2-way crossed ANOVA; the factors considered were the sampling time (fixed, 10 levels for the morphological approach and 6 for the molecular approach) and site (fixed, 4 levels). Levene test was used to check for the homogeneity of variances. When significant main effects or interactions were detected ($P<0.05$), the Tukey's test was used for pairwise a posteriori comparison.

The phytoplankton community structures obtained from the morphological and molecular approach were analyzed by non-metric multidimensional scaling (nMDS) by means of Jaccard similarity. Within the morphological and molecular dataset, phytoplankton abundances among times and sites were analyzed by non-metric multidimensional scaling (nMDS) based on Bray-Curt's similarity. Differences in community structures were assessed by permutational non-parametric multivariate analysis of variance

(PERMANOVA) following the same experimental design adopted for ANOVA. When significant main effects or interactions were detected, the specific procedure provided within PERMANOVA was used for pairwise a posteriori comparison. The analyses were performed using unrestricted permutation of the raw data and 9999 permutations. Significance level was set at 0.05 (5%) for all tests. All analyses were conducted with Past 4.0.

# CHAPTER 4: RESULTS

## 4.1 Physico-chemical parameter recorded in the Sacca di Goro lagoon

### 4.1.1 Temperature

As expected, surface temperature followed a clear seasonal trend with minimum temperatures in winter (14.3-6.5°C November-December-2020) and maximum during summer (26.5°C-27.3°C in June). Site Gorino (G) registered the minimum temperature 6.5°C in both December and February. Maximum temperatures about 28.5°C were recorded in September-2020 (Figure 10).

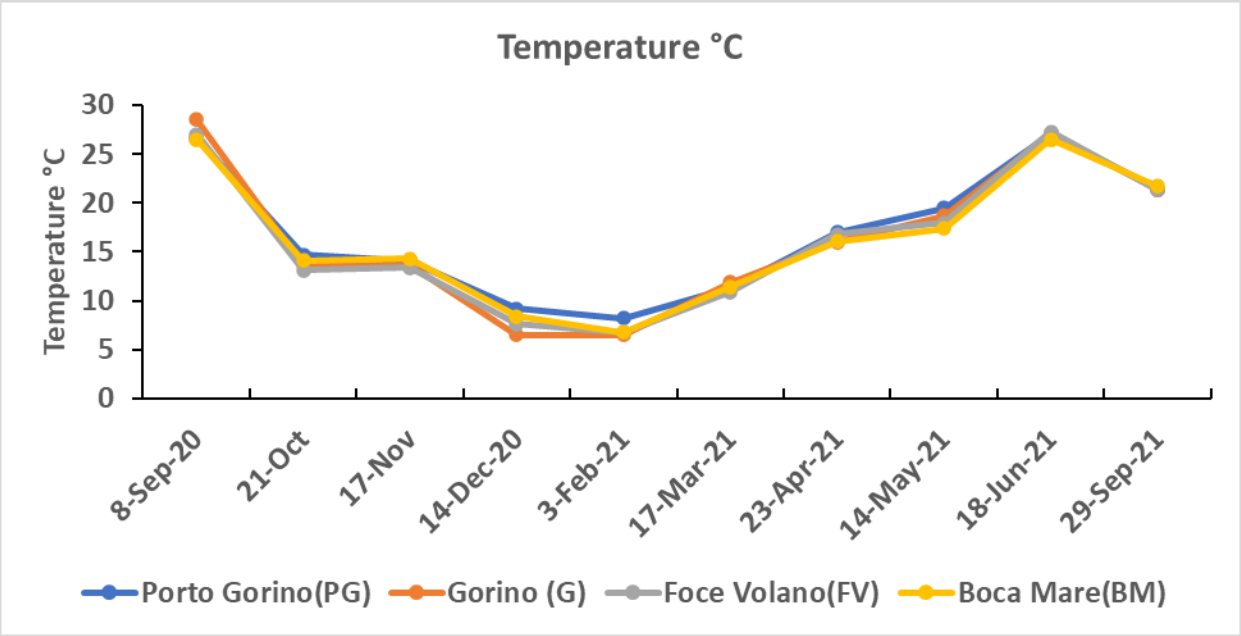



**Figure 10: Spatial and temporal variations of Temperature (°C) recorded during the sampling months within the Sacca di Goro lagoon between September-2020-2021.**

### 4.1.2 Salinity levels

Salinity levels in the Sacca di Goro lagoon fluctuated between 3 and 31psu. Maximum salinity levels were recorded during winter (4-31psu in December) while minimum levels were recorded in June (3-15psu). In all the sampling seasons, site BM and PG recorded highest salinities while site G and FV recorded low salinity levels. December 2020 recorded the highest salinity levels up to 31psu in site BM (Figure 11).

### 4.1.3 Dissolved Oxygen concentrations

Oxygen concentrations (expressed in mg/L) also showed a significant difference among sampling months. Highest concentrations were recorded during spring (May 2021) with a range of 3.74-11.77 mg/L in all the sampling sites, while the lowest concentrations were recorded during summer ranging between (2.81-7.8 mg/L). The highest levels of dissolved oxygen were recorded in site G (11.77 mg/L) in late March 2021 (Figure 12). During the warm season of summer, the lagoon waters were depleted in dissolved oxygen which dropped to 2.81 mg/L in site FV in June 2021. Site FV recorded the lowest oxygen concentrations in almost all the sampling months.

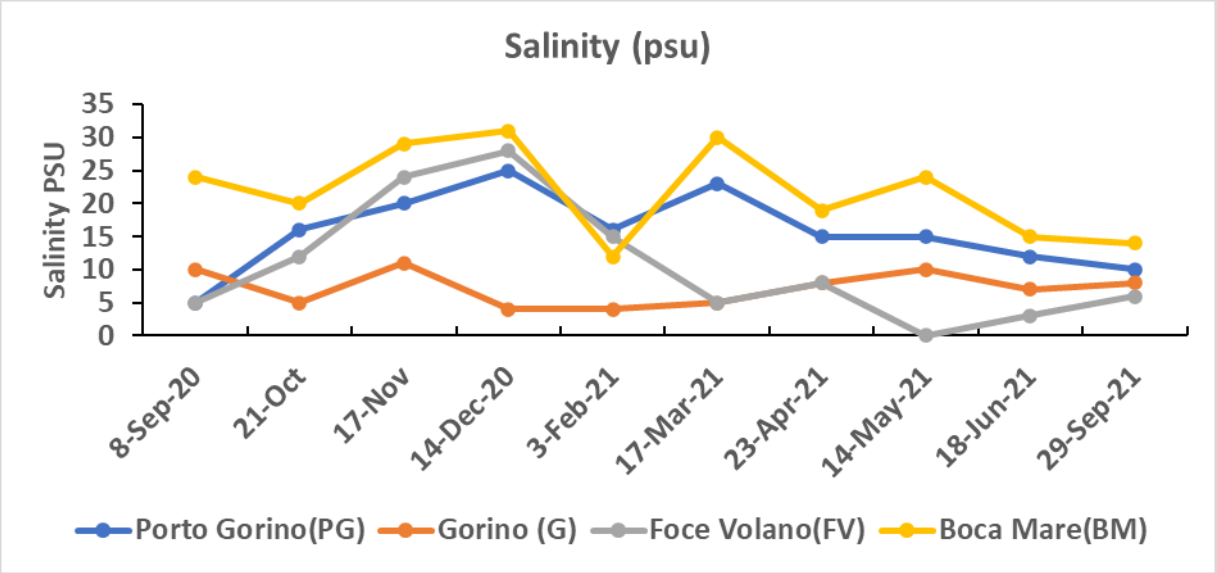



**Figure 11: Spatial and temporal variations of Salinity (psu) recorded during the sampling months within the Sacca di Goro lagoon between September-2020-2021.**

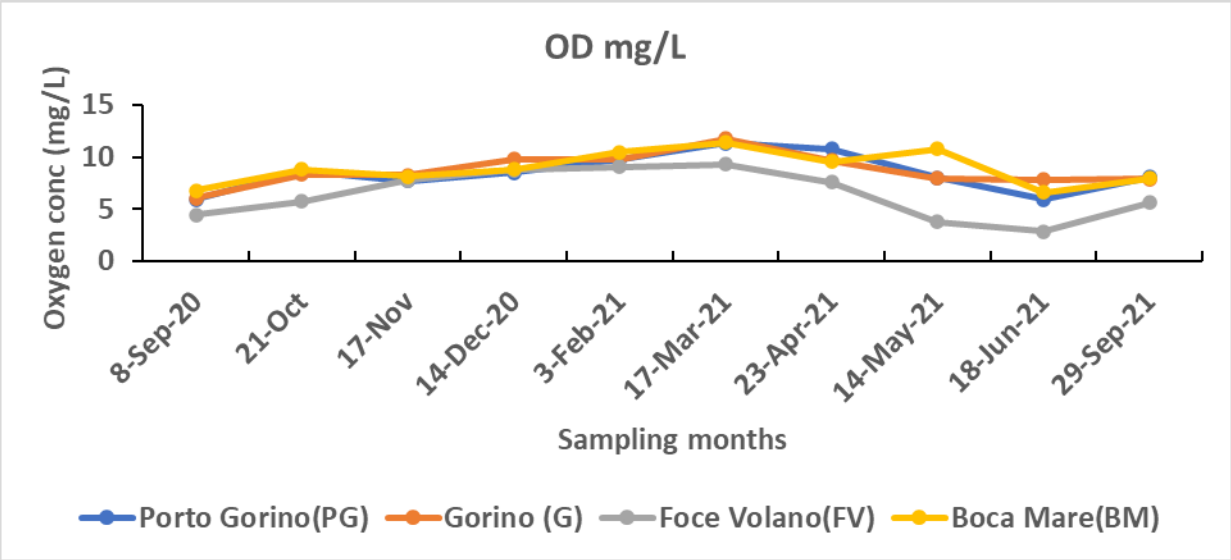



**Figure 12: Spatial and temporal variations of Dissolved oxygen (mg/L), recorded during the sampling moths within the Sacca di Goro lagoon between September-2020-2021.**

### 4.2 Chlorophyll-a and phaeopigments

Biomass was quantified using phytoplankton pigments. The spatial and temporal distribution of chlorophyll-a and its diagnostic phaeopigments for all the sampling months is presented in (Figure 13). The highest average values of both chlorophyll-a and phaeopigments were observed at site FV in all the sampling months. Chlorophyll-a values varied without any significant trend in seasons. Peak values of chlorophyll-a were observed during autumn (September) and winter (December) both at 19.7 µg/L. For the phaeopigments, minimum concentrations were observed during cold months and maximum concentrations in warm seasons with a high peak observed in September-2020 (16.3 µg/L).

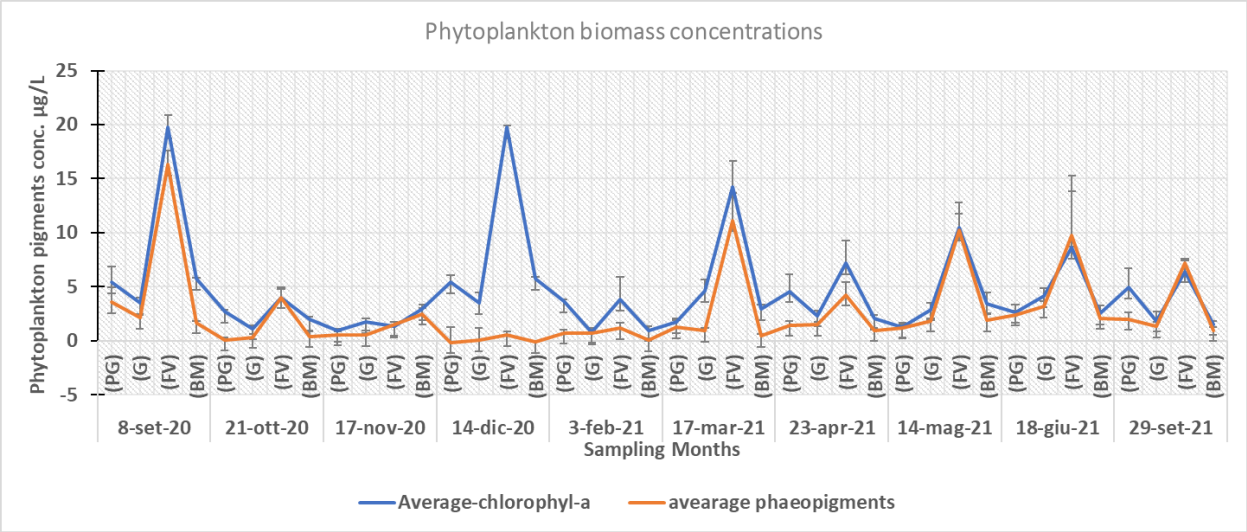



**Figure 13: Spatial-temporal variations of phytoplankton biomass concentration in terms of chl-a and phaeopigments content in the Sacca di Goro lagoon during the sampling months.**

### 4.3 General composition of phytoplankton communities obtained with the morphological and molecular approaches

The phytoplankton composition of the Sacca di Goro lagoon was characterized by a mixture of marine, brackish-water, and freshwater taxa mainly represented by three groups: Bacillariophyta, Chlorophyta and Miozoa. Generally, the taxa were classified into nine major groups (phyla): Miozoa, Bacillariophyta, Chlorophyta, Chryptophyta, Ochrophyta, Euglenozoa, Cyanobacteria, and Haptophyta. In addition, a different group of unidentified forms was counted for species whose morphological features could not be determined.

The morphological approach resulted into a total of 147 taxa identified. Diatoms formed the most dominant group represented by 82 species, Chlorophyta 28, and Miozoa 23 species. The rest of the groups were below <4 total taxa.

DNA metabarcoding resulted into a total of 11751 MOTUs after quality filtering. filtering at 0.8 bootstrap value at phylum level resulted into 8946 sequences. Filtering at 0.99 bootstrap value at genus level resulted into 1736 sequences. Genera were confirmed

using algae base with only two species corrected belonging to the Chlorophyta and Bigyra (i.e., Verdigellas and Oblongichytrium). Sequences belonging to Fungi, Protist and Ciliophora were removed. A total of 6729 algal MOTUs were obtained and classified at phylum level for coherent comparison with the morphological approach. More taxa were present in the samples analyzed with DNA metabarcoding than in those with the morphological approach.

Similar to the morphological approach, diatoms formed the most dominant group represented by 3332 MOTUs, followed by Chlorophyta (1136), Miozoa (685), Chryptophyta (611), Ochrophyta (585), Cercozoa (303), Picophyta (36), Katablepharidophyta (34), Bigyra (7). In comparison the DNA metabarcoding resulted into the identification of some taxa not identified by the morphological approach, while Cyanobacteria, Euglenophyphceae and other species of Chlorophyta were not identified by the DNA metabarcoding approach (Appendix table 1) A total of 158 genera were identified using the molecular approach, while with the microscopy 147 genera were reported. 49 taxa were identified by the molecular analysis and not by the morphological approach; on the contrary, 18 taxa were only detected with the microscopic analysis and were not present in the DNA dataset. In terms of number of taxa more taxa were observed for the DNA as compared to the morphological approach indicating its high efficiency in taxonomy analysis. Regarding the taxa that were only identified in the microscopy-based analysis (Appendix table 2), there could be two possible explanations for their absence in the metabarcoding analysis: the taxa had no representative sequence in the database, or the taxa were present, but not assigned to any reference sequences due to lacks in barcoding databases. The nMDS plot of phytoplankton communities showed a clear separation between samples analyzed by the morphological and molecular approach, indicating as the information obtained with the two approaches are different and could be complementary (Figure.14).

In the nMDS plot of the phytoplankton community assemblages obtained through eDNA analysis the time factor seems to affect the distribution of the samples more than the site, in fact a seasonal trend is observed (Figure 15). PERMANOVA supported this pattern resulting in a significant interaction between times and sites ($P \leq 0.005$; Appendix table 3),

and the post-hoc analysis confirmed a different structure of the phytoplankton communities in BM with respect to the other sites, while within each site, the algal community changed among seasons (i.e., December, March, and June) (Appendix table 4 & 5). Conversely, in the nMDS plot of the samples obtained through the morphological approach, some sites resulted clustered (e.g., PG and FV), while a temporal trend is not evidenced (Figure.16). PERMANOVA supported this pattern resulting in a significant interaction between times and sites ($P \leq 0.001$; Appendix table 6), and the post-hoc analysis confirmed the different structure of the phytoplankton communities at each site, but also among different times (Appendix table 7 & 8).

Based on the two-way ANOVA test a significant difference (ANOVA p-value<0.05) in abundances between sample times and sites was observed as indicated in (table 2). Site PG showed a significant difference in most of the times between site BM and G. No significant difference was observed for site FV among time and sites.

**Table 2: Results of 2-way ANOVA and Pair-Wise comparison tests for the phytoplankton community analyzed through morphological approaches.**

| Interactions between times-sites | | | |
|---|---|---|---|
| | | Q | p-value |
| November 20-PG | February 21-PG | 6.645 | 0.01311 |
| December 20-PG | February 21-PG | 6.853 | 0.008658 |
| February 21-G | February 21-PG | 6.593 | 0.01453 |
| February 21-PG | September 21-PG | 6.405 | 0.0209 |
| March 21-PG | April 21-PG | 8.781 | 0.000143 |
| April 21-BM | April 21-PG | 7.874 | 0.001027 |
| April 21-G | April 21-PG | 8.591 | 0.000216 |

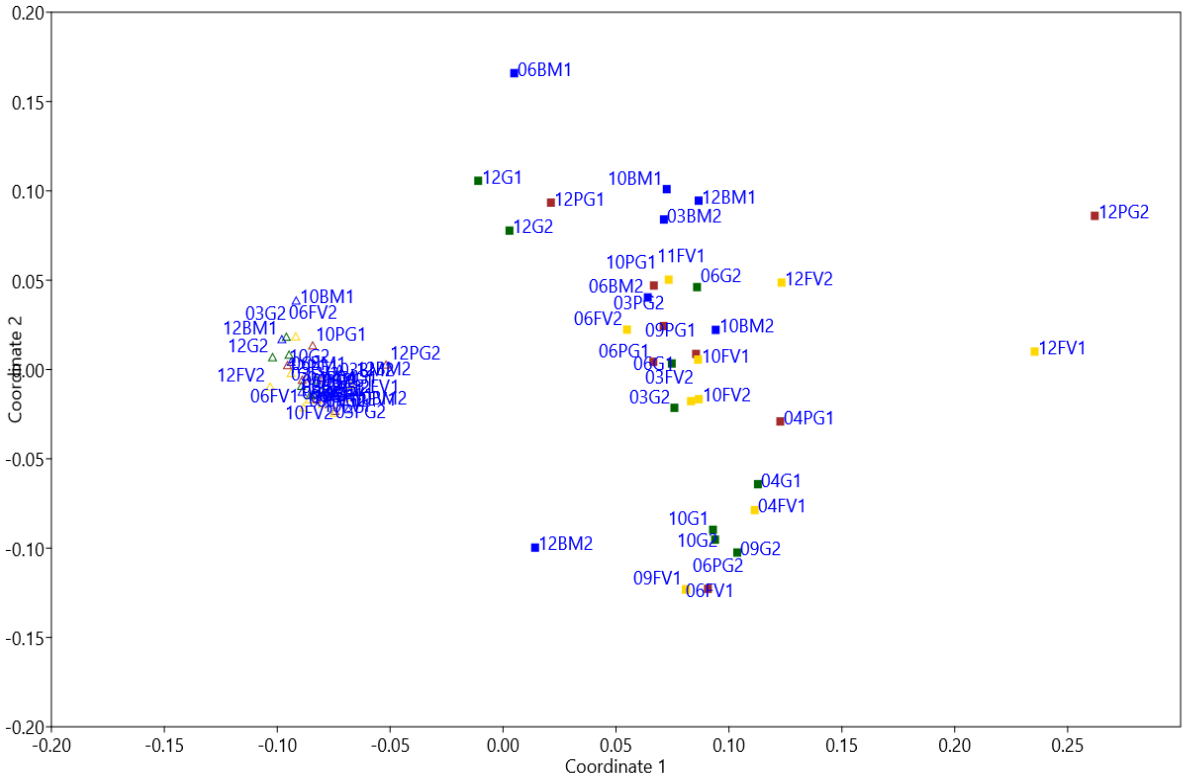



**Figure 14: non-metric multidimensional scaling (nMDS) based on Jaccard similarity (stress=0.179). Phytoplankton community obtained through e-DNA metabarcoding (squares) and based on morphology identification using microscopy (triangles). Colors represent the sample sites.**

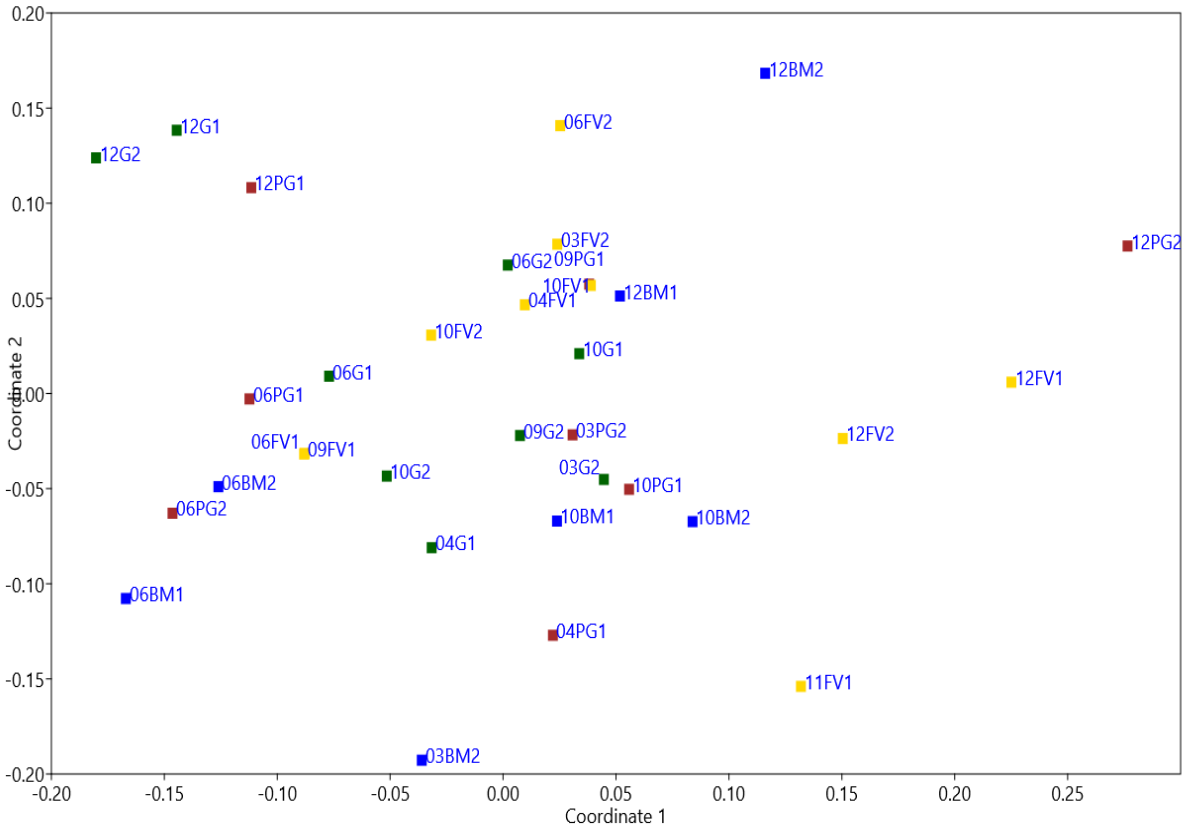



**Figure 15: non-metric multidimensional scaling (nMDS) based on Bray-Curtis similarity for the phytoplankton communities obtained with molecular approach (stress=0.22). Colors represent the sampling sites.**

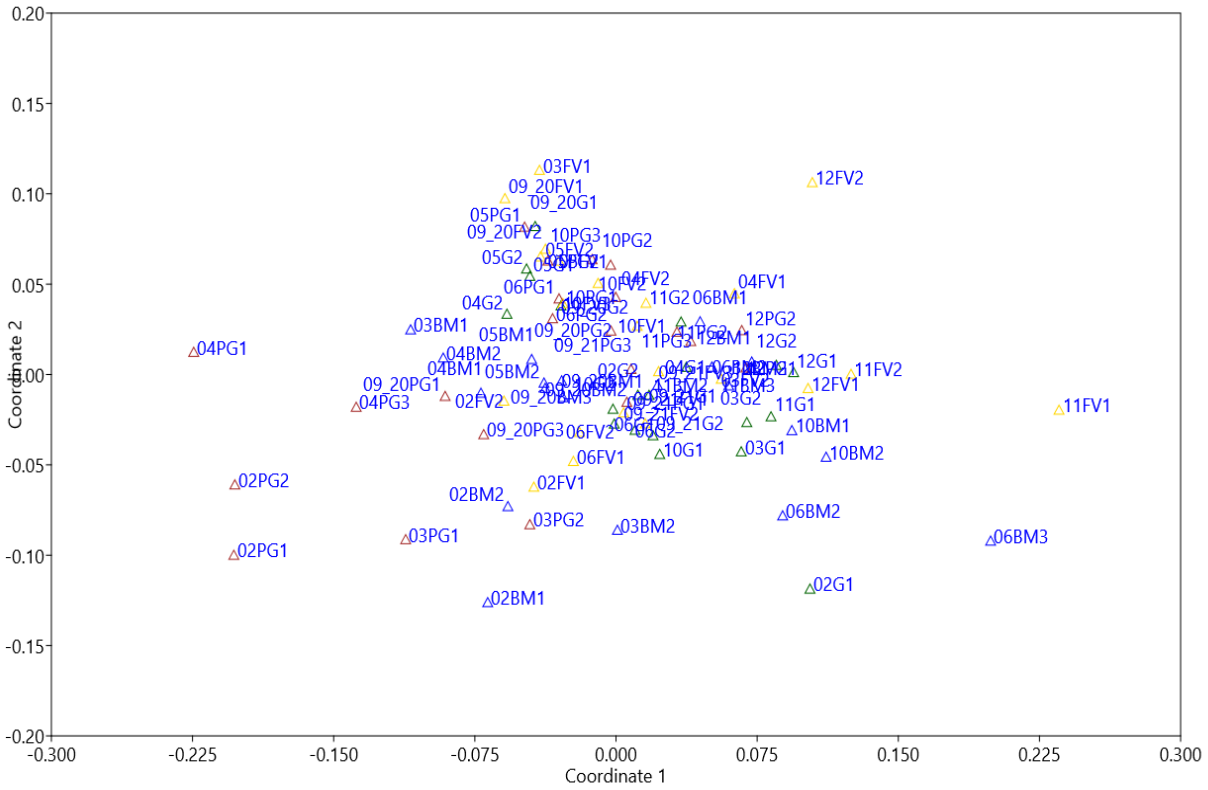



**Figure 16: non-metric multidimensional scaling (nMDS) based on Bray-Curtis similarity for the phytoplankton communities obtained with morphological approach (stress=0.14). Colors represent the sampling sites.**

## 4.4 Spatial-temporal distribution of phytoplankton communities based on morphological approach

The spatial-temporal distribution of phytoplankton in the lagoon was represented in terms of total abundances. Total abundances per sample differed between seasons. The highest abundances were recorded during spring and the lowest in winter in all the sampling sites (Figure 17A, B, C, & D). The range of abundances for each season was as follows; autumn 2.35E+05 to 6.31E+06 cells/L, winter abundances were generally very low ranging from 2.26E+05 to 6.54E+05 cells/L. During spring remarkable high significant abundances of phytoplankton were recorded ranging between 9.17E+05 to 1.12E+07 cells/L while during summer abundances ranged between 4.05E+05 to 1.79E+06 cells/L

(Figure 18). In general, the highest total abundance was in site PG during April-2021 (1.79E+06 cells/L) and the lowest in site FV (2.26E+05 cells/L) during November (Figure 17A &C). However, in most of the sampling months it was observed that significant higher abundances occurred at site FV (Figure 18) with respect to the other sites.

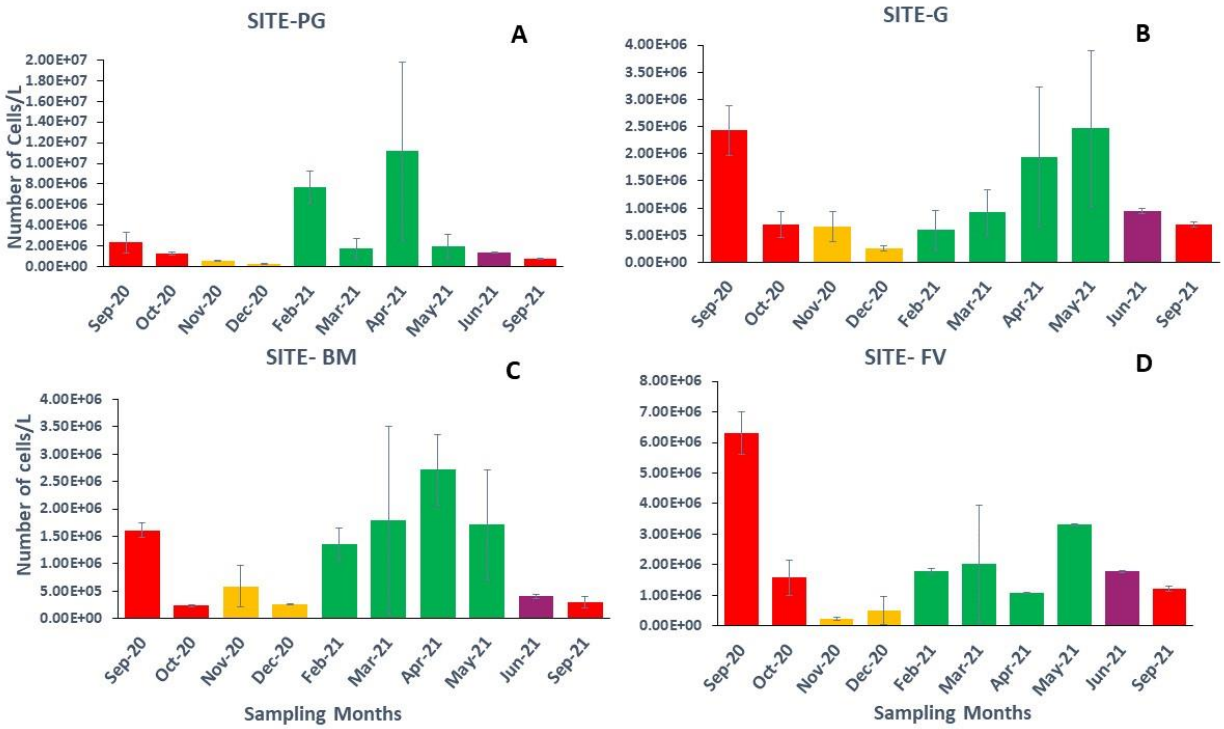



**Figure 17: Spatial variations of total phytoplankton abundances in the different sampling sites A) Porto Gorino, B) Gorino, C) Foce Volano, and D) Boca Mare within the Sacca di Goro lagoon waters sampled between September 2020- September 2021. Colors represent the different seasons (Red-autumn, orange-winter, green-spring and purple-summer).**

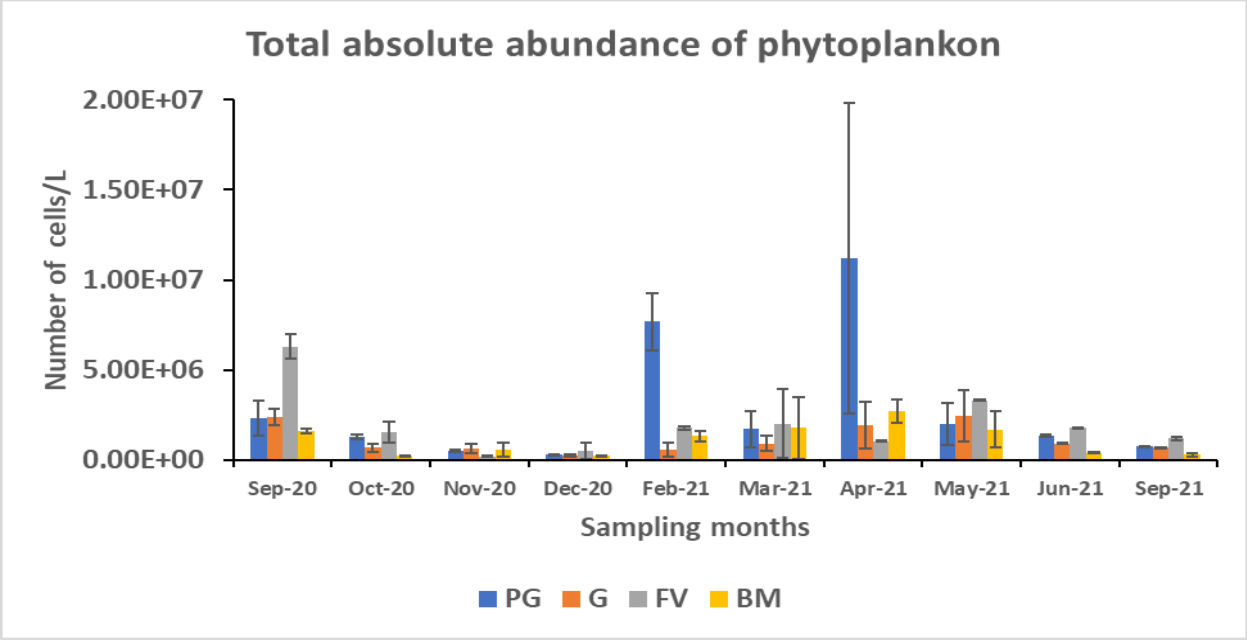



**Figure 18: Temporal variations of phytoplankton absolute abundance in the Sampling lagoon between September 2020-2021.**

### 4.5 Dominant phytoplankton taxa based on morphological approach

Phytoplankton dominant taxa was obtained by calculating their relative abundances in each month for each site. Relative abundances of phytoplankton communities varied greatly in season and site. Bacillariophyta and Cryptophytes were the most abundant in most of the seasons.

#### 4.5.1 Bacillariophyta

During autumn, winter, and summer relative abundances of Bacillariophyta were relatively low in most of the sampling sites. However, at the start of the spring season all through the end of this season a significant increase in relative abundance was observed. Minimum relative abundances were recorded in site FV (0.73%) during December while maximum relative abundances were recorded in site PG (95%) during February (Figure 19).

In site PG, Bacillariophyta were abundant during spring with a remarkable high relative abundance of 95% recorded during February (Figure 20A). The taxa consisted of *Skeletonema* species (*Skeletonema* spp., and *Skeletonema marinoi*) with a relative abundance of 99.24% during February (Appendix Figure1). Towards the end of spring, the relative abundances of *Skeletonema* cells were observed to decrease and they were gradually replaced by *Chaetoceros* spp., with a high relative abundance recorded during October-2020 (92.59%). Other species that characterize this site with relatively minimal abundances included *Cyclotella*, and *Thalassiosira* spp. during autumn. During winter a difference in the composition of Bacillariophyta was observed where the dominant species belonged to the genera *Cocconeis*, *Fragilaria*, *Cymbella*, *Cymatopleura,* Navicula, and *Diploneis*. Spring and summer were dominated mainly by *Skeletonema* spp.*, Chaetoceros* spp. *and Thallassiosira* spp. which appeared in both seasons.

In site G, Bacillariophyta represents the dominant group except for Winter where 39ryptophyte was dominant 60.25% (Figure 20B). In this site the Bacillariophyta consisted of *Pseudo-nitzschia delicatissima* (21.42%), and *Navicula* spp. (50.64%) during winter. Spring, summer, and autumn were dominated by *Skeletonema* spp.*, Chaetoceros* spp., *Thallassiosira* spp, *Cyclotella* spp. and *Cocconeis* sp. This species (*Cocconeis*) appeared to be more abundant in this site with the highest abundances occurring during the warm months (4.75% in September 2021), a situation not observed in other sites (Appendix Figure 1).

Site FV did not show any dominance in Bacillariophyta in most of the sampling seasons, however high abundances could be observed during early spring with relative abundances reaching 48.11% (Figure 20C). Most Bacillariophyta with high relative abundance consisted of the freshwater diatoms (*Cyclotella* spp. with 90% relative abundance in March, *Nitzschia* spp. which characterizes the winter seasons and *Melosira* spp.). Remarkable abundances of *Skeletonema* and *Thalassiosira* were also observed in the warm seasons (Appendix Figure 1).

Bocca Mare located close to the open sea showed high relative abundances in Bacillariophyta during the Spring seasons (85.07% in February, Figure 20D). The

Bacillariophyta mainly consisted of marine diatoms and to some less extent freshwater diatoms. The composition was highly dominated by *Chaetoceros* spp*., Skeletonema* spp. and *Pseudo-nitzschia delicatissima complex* whose high abundances were recorded in spring, summer, and Autumn (Appendix Figure1).

### 4.5.2 Chryptophyta

Chryptophyta exhibited a different trend. The highest abundances were in the seasons of autumn, winter, and summer, while their abundances appeared low during spring. Minimum relative abundances were recorded in site PG (0.28%) during April while maximum abundances in site BM during December (73.01%) (Figure 19).

In site PG, Chryptophyta dominated in winter (December 64%) and summer (June 59.79%). A similar trend was observed for site BM with December having 73.01% and June 60.41% relative abundances. In site G relative abundances appeared high throughout the sampling months with a peak occurring in December (60.25%). Relatively low abundances were observed in all the seasons for FV (Figure 20C). In all sampling sites Chryptophyta was mainly composed of *Cryptomonas* spp., *Hemiselmis* spp., and *Rhodomas* spp.

### 4.5.3 Chlorophyta

Chlorophyta appeared more dominant only in site FV. Abundances appeared high during winter, then a decrease in abundance was observed at the beginning of spring. Towards the end of spring, abundances started to peak again all through summer and autumn seasons. In December maximum abundances were recorded in site FV (71.12%) while in PG and BM no records were observed (Figure 20A, 20C and 20D). The taxa consisted mainly of *Ankistrodesmus* spp., *Scenedesmus* spp., *Crucigenia* spp., *Coelastrum* spp., and *Actinastrum* sp.). The diversity was observed to increase in warm months for all the sites (Appendix figure 2).

### 4.5.3 Miozoa

Miozoa peaked during autumn in almost all sites. During winter all through spring the abundances remained relatively low for all sites. At the beginning of summer their abundances showed a relative increase (Figure 19). Miozoa appeared to dominate mostly in site PG and BM although maximum relative abundances were observed in site G during November (36.01%) (Figure 20B). Miozoa was highly composed by *Heterocapsa* spp., *Protoperidinium* spp., *Gymnodinium* spp., and *Prorocentrum* spp. with high abundances recorded during warm months for all sites (Appendix Figure 3)

### 4.5.4 Euglenozoa

Low abundances of Euglenozoa were observed during Autumn. Then, their abundances decreased all through winter and early spring periods. Towards the end of spring, abundances started to increase with a highest peak (20.92%) observed in site BM during April. After this, low relative abundances were observed all through summer (Figure 19). No dominant characteristic was observed for this taxon, but it occurs mostly in sites BM and PG in both spring and Autumn (Figure 20A & 20B). This taxon mainly consisted of *Euglena* spp., *Phacus* sp., and *Eutreptiella* spp.

### 4.5.5 Cyanobacteria

Cyanobacteria appeared moderately high during early autumn. Afterwards, relatively low abundances were observed from winter to summer (Figure 19). The taxon was prevalent mostly in site FV with the highest peaks observed in September 2020 (19.95%) and September 2021 (13.19%) where temperatures were relatively high with low salinity levels (Figure 20C). The taxon consisted of *Anabaena* sp., and *Merismopedia* sp., while most organisms remained classified under undetermined colonies.

The rest of the phytoplankton groups (Haptophyta and Ochrophyta) were low in abundances <5% in all the sites without showing any significant trend.

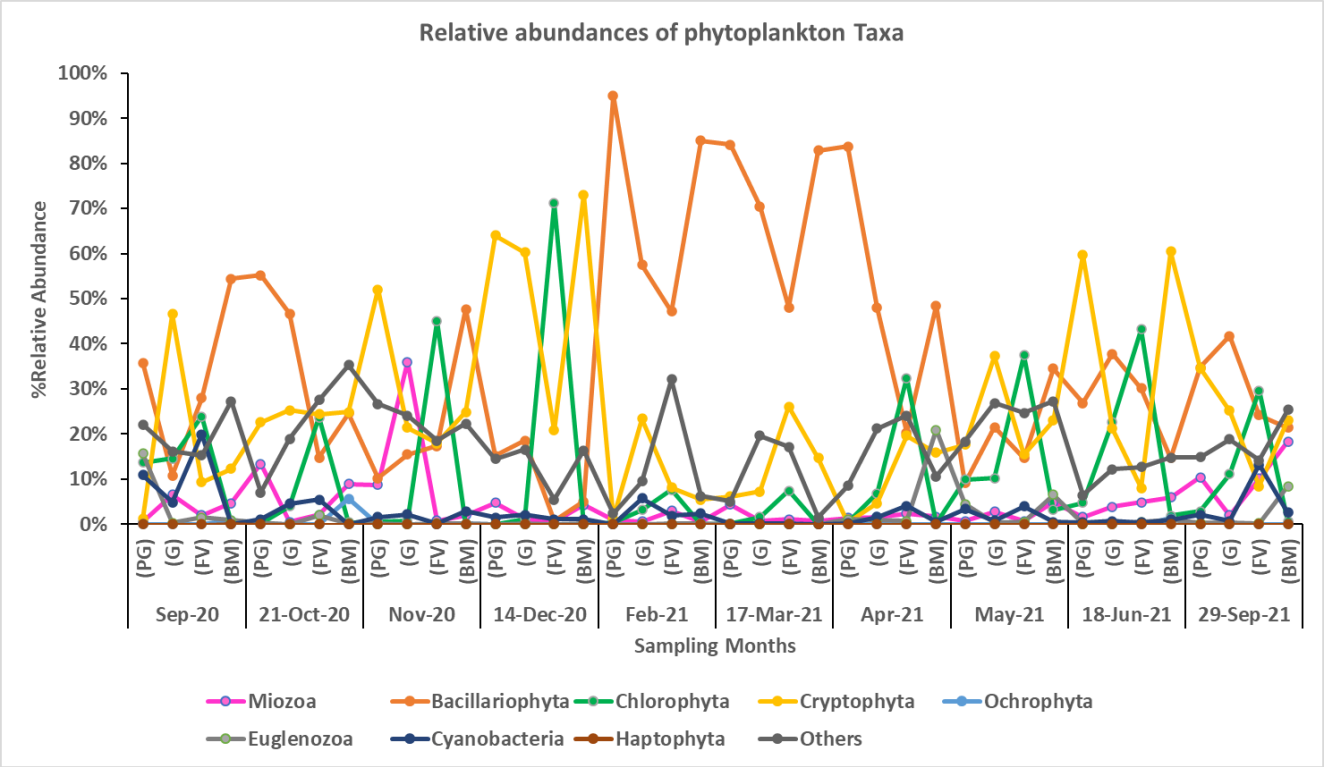



**Figure 19: Spatial and temporal variations of relative abundances (%) of different phytoplanktonic taxa sampled in the Sacca di Goro lagoon waters during 2020 and 2021 using morphological approach.**

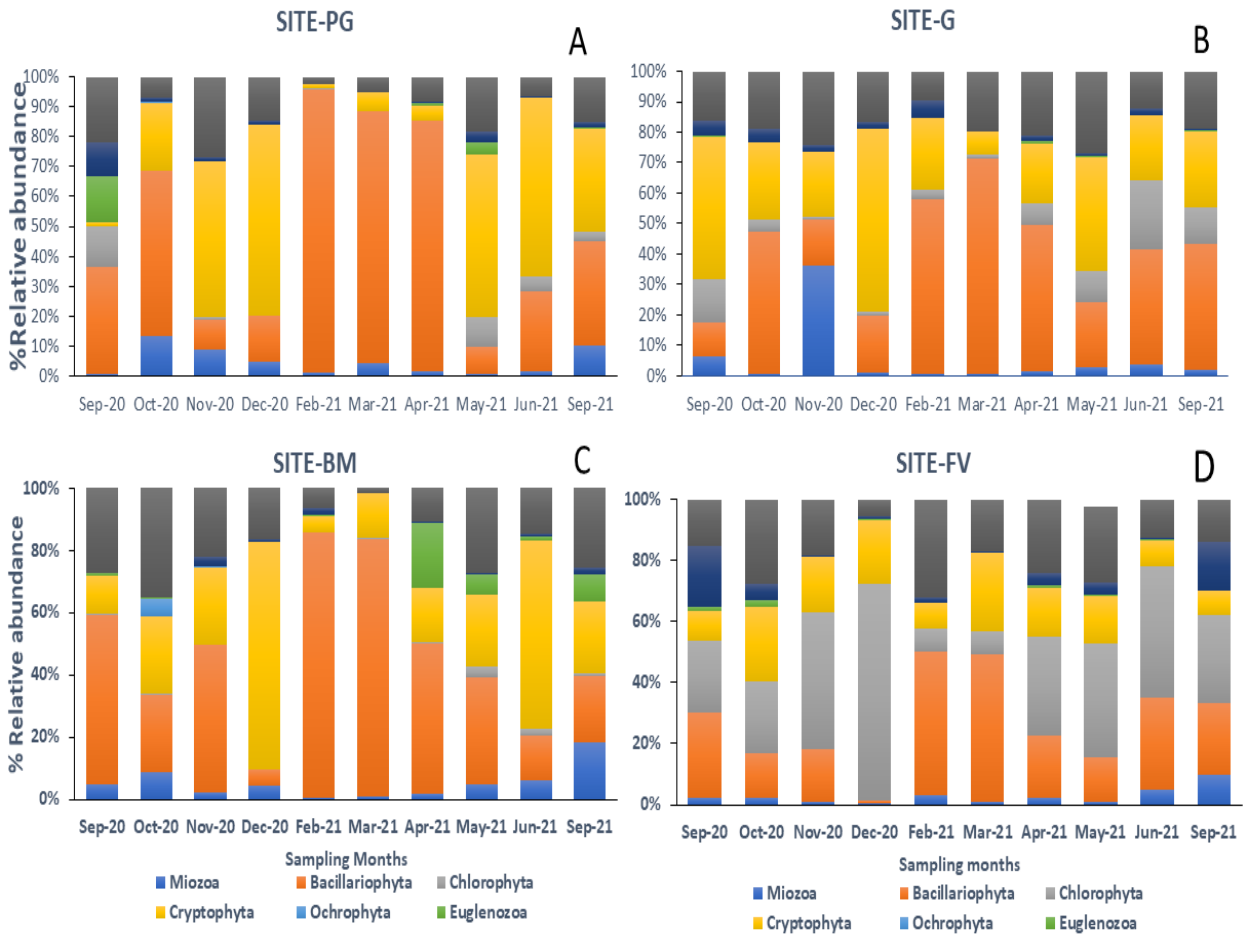



**Figure 20: Spatial-temporal variations of relative abundances (%) of different phytoplanktonic groups sampled in the Sacca di Goro lagoon waters between 2020 and 2021 in site PG (A), G(B), BM (C) and FV (D) using morphological approach.**

### 4.6 Dominant phytoplankton taxa based on DNA Metabarcoding Analysis

As counts cannot be regarded as a straightforward quantitative expression of cell density, they are certainly not independent of autotrophic plankton abundance (as biovolume) as already reported Penna *et al*., (2017), thus we decided to use relative abundances for comparisons among samples. Based on the DNA metabarcoding data, autumn, winter, and summer relative abundances of Bacillariophyta were relatively low in most of the sampling sites. All through the spring season a significance increase in relative abundance was observed except for site BM. Minimum relative abundances were recorded in site FV (5%) during December, while maximum relative abundances were

recorded in site G (92%) March (Figure 21B & 22D). In general, it was observed that sites G and FV had a remarkable high relative abundance of Bacillariophyta which contrasted with what was observed with the morphological analysis. In these sites the prevalent species were *Stephanodiscus* sp. and *Cyclotella* sp., while PG and BM were dominated by species belonging to *Chaetoceros*, *Skeletonema*, and *Pseudo-nitzschia* during spring.

Miozoa relative abundance was observed high during autumn and winter in site BM (the highest relative abundance was (69%) and PG, except for site G where relative abundance was observed high during spring and summer. Significantly low relative abundances were observed in site FV (Figure 22D). In contrast with the morphological analysis, Chlorophyta were more abundant in site BM (56%) (Figure 21C).

The analysis of the eDNA metabarcoding also resulted into remarkable relative abundances of taxa that were recorded in low relative abundances or completely not recorded with the morphological quantification (Haptophyta, Ochrophyta Cercozoa, Bigyra and Katablapharidophyta). Species belonging to Ochrophyta (relative abundance of 83%) were observed in site G, and they were not identified with the morphological approach. The approach also covered most of the species found with morphology-based approach except for the groups of cyanobacteria, some majority of the Chlorophyta except for *Tetraselmis* Sp., whose MOTUs were highly identified with the DNA and species of Euglenophyceae.

Metabarcoding was able to overcome the lack of resolution of microscopy for picoplankton (0.2–2 µm). MOTUs belonging to the smallest size fraction were identified including species of the taxa picophyta (*Codosiga* Sp, *Diaphanoeca* Sp*, Lagenoeca* Sp and *chromera* Sp), species of Haptophyta and Ochrophyta as indicated in (Appendix Table 2). Picoplankton was not identified by the morphological approach.

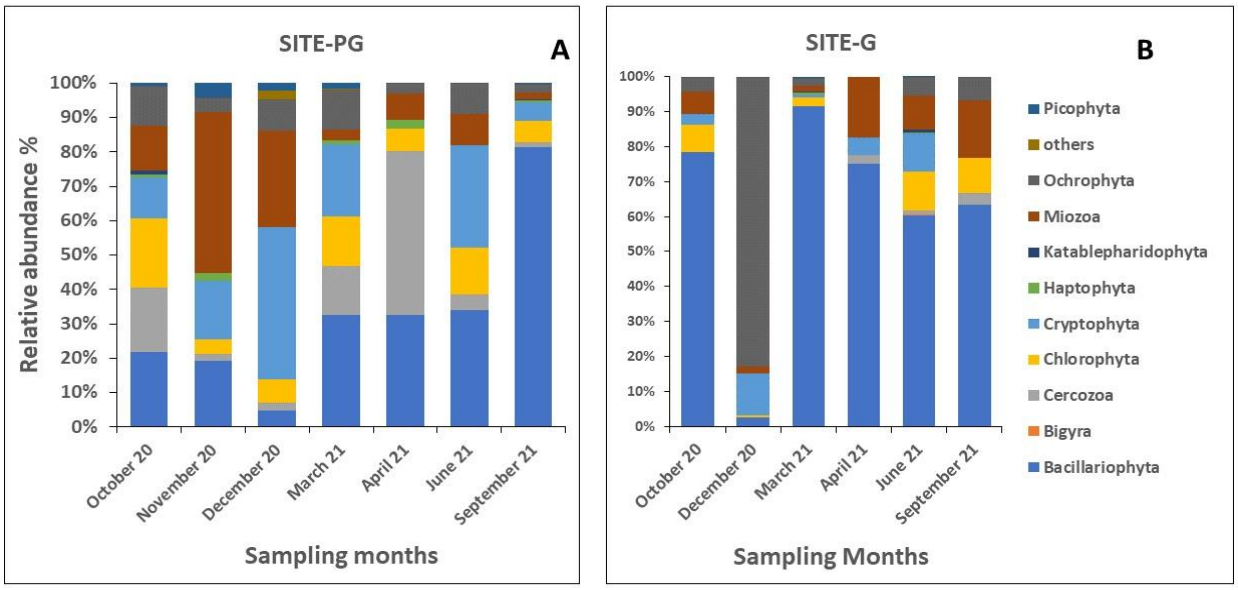



**Figure 21: Relative abundances (%) of phytoplankton phyla in different sampling sites A) Porto Gorino, B) Gorino within the Sacca di Goro lagoon waters sampled between September 2020- September2021 using DNA metabarcoding approach.**

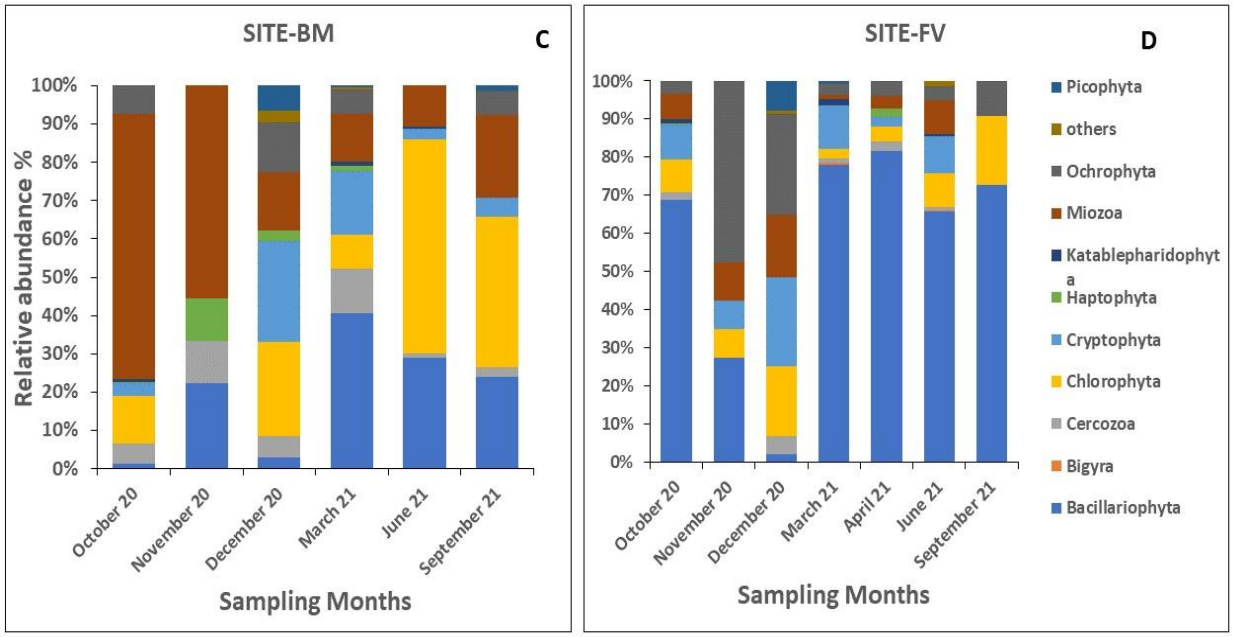



**Figure 22: Relative abundances (%) of phytoplankton phyla in different sampling sites A) Porto Gorino, B) Gorino within the Sacca di Goro lagoon waters sampled between September 2020- September2021 using DNA metabarcoding approach.**

### 4.7 Biodiversity indexes

The number of species (S) in the study area are shown in (Figure 23). In each site, the highest number of species was observed during autumn, towards the end of spring and to a less extent in summer. The maximum (43) and minimum number of species (13) was observed in site FV during September 2020 and December 2020, respectively for the morphological approach, while for the DNA the maximum (17) was observed in site PG during October and only one species was observed at site FV (Figure 25).

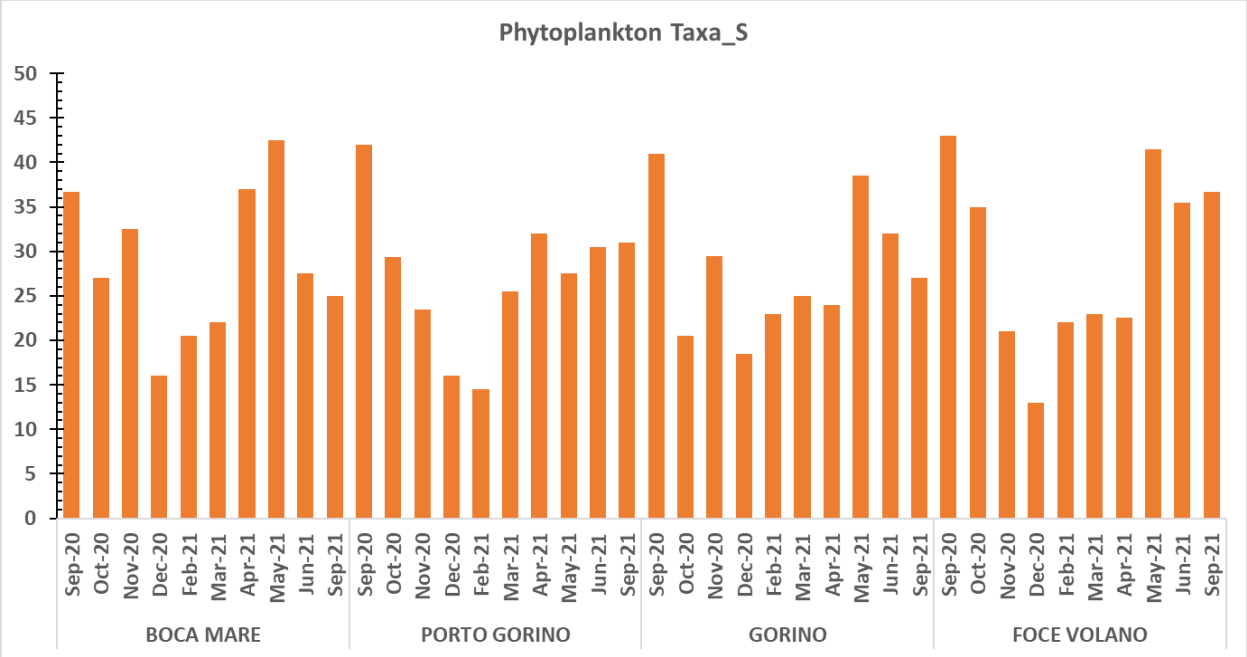



**Figure 23: Spatial and temporal variations of number of species (S) in the Sacca di Goro lagoon obtained by morphological approach**

The Shannon index of diversity (H') values increased in parallel to the number of species (S) and evenness index (J') a trend that characterize stable ecosystem. The highest diversity (H'= 2.77) was observed at site FV during June and September 2021 and the lowest value (H'= 0.7) was observed at the same site during December 2020. Piélou evenness index varied between 0.15 at site PG during February and 0.4 in site FV during June. On the other hand, Margalef varied between 0.8 in site PG during February and 2.6 during May in site FV (Figure 24).

For the DNA metabarcoding the highest diversity (H'= 2.54) was observed at site PG during March and the lowest value (H'= 0) was observed at the same site FV during September 2021. Piélou evenness index varied between 1 at site FV during September and 3.4 in site PG during March. On the other hand, Margalef varied between 0 in site FV during September and 3.4 during March in site PG (Figure 25).

Based on the diversity indices (Shannon index of diversity (H'), and species richness obtained by microscopy data site FV (H'= 2.77) was more diverse while for the DNA site

PG was more diverse species (H'= 2.54). The Piélou evenness index for the DNA analysis was close to 1 in most sites indicating an evenly distribution of phytoplankton communities in the lagoon while for the morphological analysis the distribution was not even as values were far below 1.

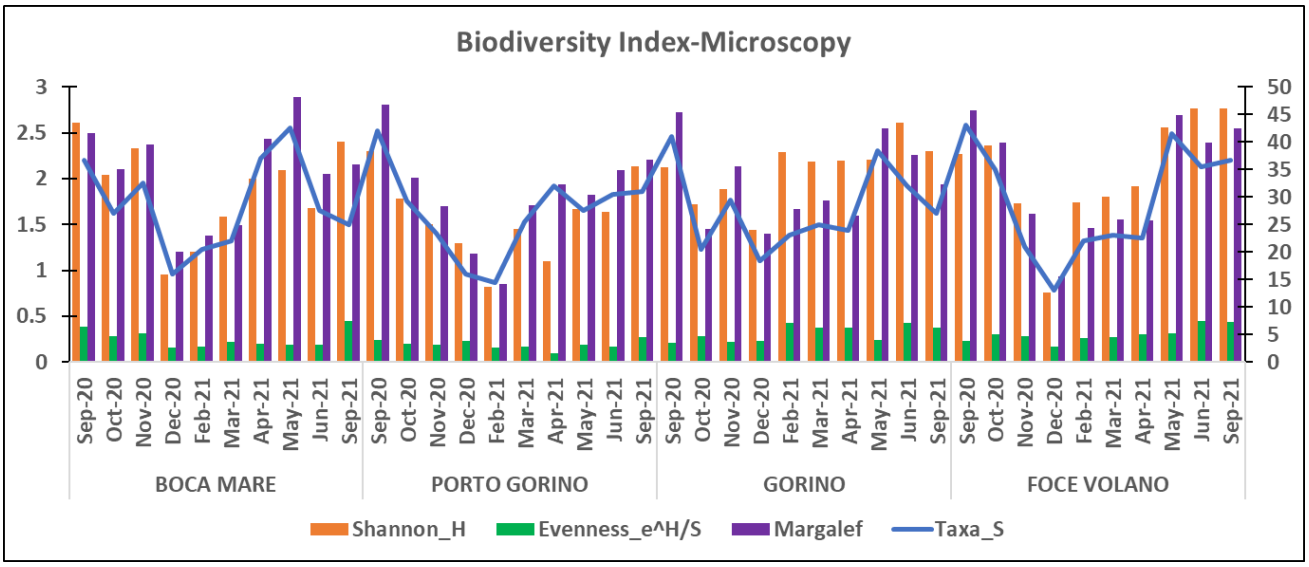



**Figure 24: Spatial and temporal variations of Shannon diversity index (H'), Piélou evenness index (J') and Margalef index of phytoplankton in the Sacca di Goro lagoon waters during 2020-2021 based on morphological approaches.**

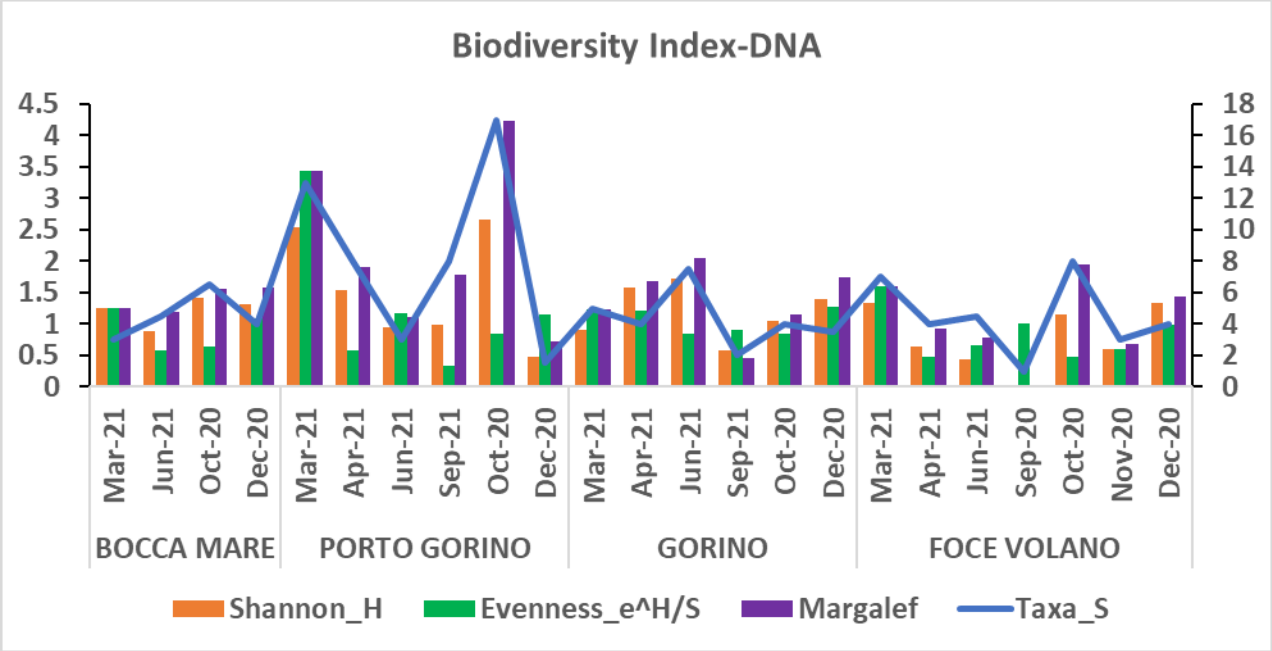



**Figure 25: Spatial and temporal variations of Shannon diversity index (H′), Piélou evenness index (J′) and Margalef index of phytoplankton in the Sacca di Goro lagoon waters during 2020-2021 based on e-DNA metabarcoding analysis.**

### 4.8 Potential harmful algal species observed in the Sacca di Goro lagoon

During our survey, a few potentially toxic species were identified in the Sacca di Goro lagoon through the morphological approach. These included 4 dinoflagellates (*Prorocentrum cordatum* in site PG, G, & FV with the highest cell abundance of 5303 cells/L during February and *Alexandrium* sp. in site BM, G and FV with a high abundance of 3535 cells/L in site FV during September, *Gonyaulax* sp. which was observed in remarkable numbers during autumn in sites BM (2575 cells/L), PG and FV, and *Karenia* Sp. Species belonging to two diatom group (*Pseudo-nitzschia delicatissima complex, Pseudo-nitzschia seriata complex*) mostly occurred in all sites during warm months. Other species whose blooms could results into physical damage to fish but not related to toxins production were also observed, including *Chaetoceros* spp., *Ceratium* spp. (responsible for fish kills), *Protoperidinium* spp. and *Heterocapsa* spp. which is known to cause water discoloration (Figure 26, 27, 28 & 29). From the eDNA analysis only *Chaetoceros* resulted to be present in the samples, while information on the other genera were missing. This result was probably due to their absence in the reference database or by the fact that the

score was low (<0.99) (e.g., *Pseudo-nitzschia* and *Alexandrium* spp.), so data were not considered reliable and MOTUs were deleted during the initial clustering.

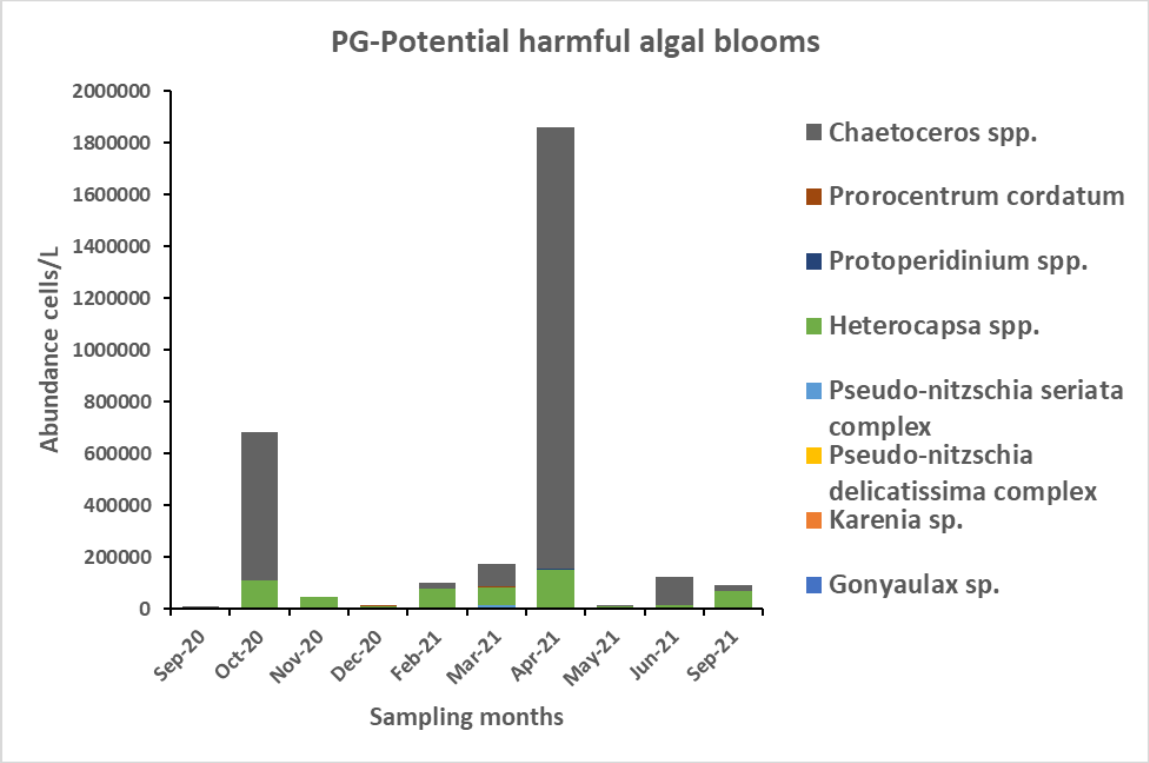



**Figure 26: Abundance of the main potentially harmful species (HABs) sampled in site PG at the Sacca di Goro lagoon waters between September 2020-2021 using morphological approach.**

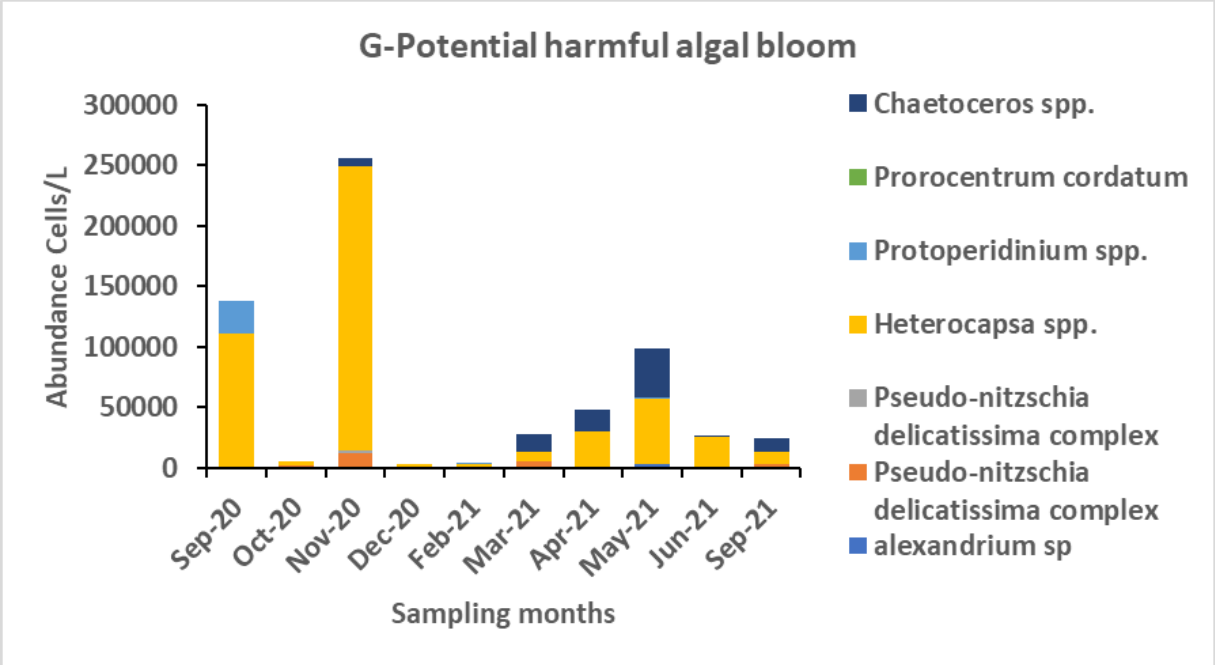



**Figure 27: Abundance of the main potentially harmful species (HABs) sampled in site G at the Sacca di Goro lagoon waters between September 2020-2021 using morphological approach.**

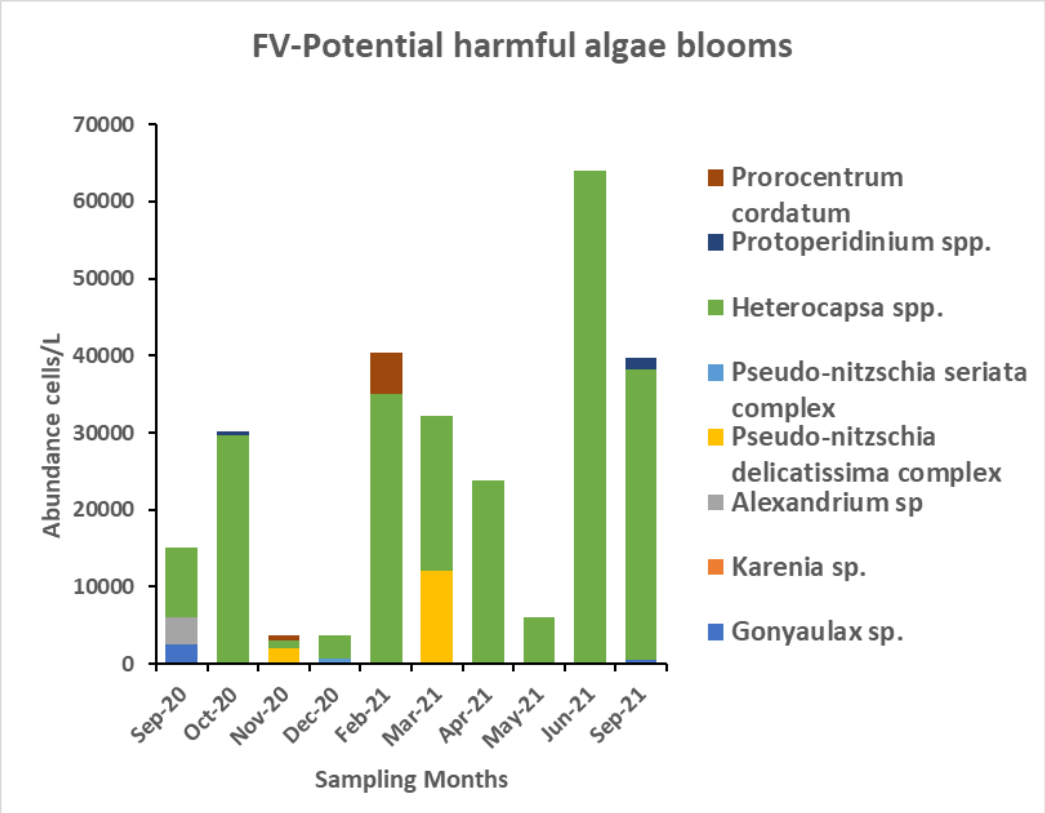



**Figure 28: Abundance of the main potentially harmful species (HABs) sampled in site FV at the Sacca di Goro lagoon waters between September 2020-2021 using morphological approach.**

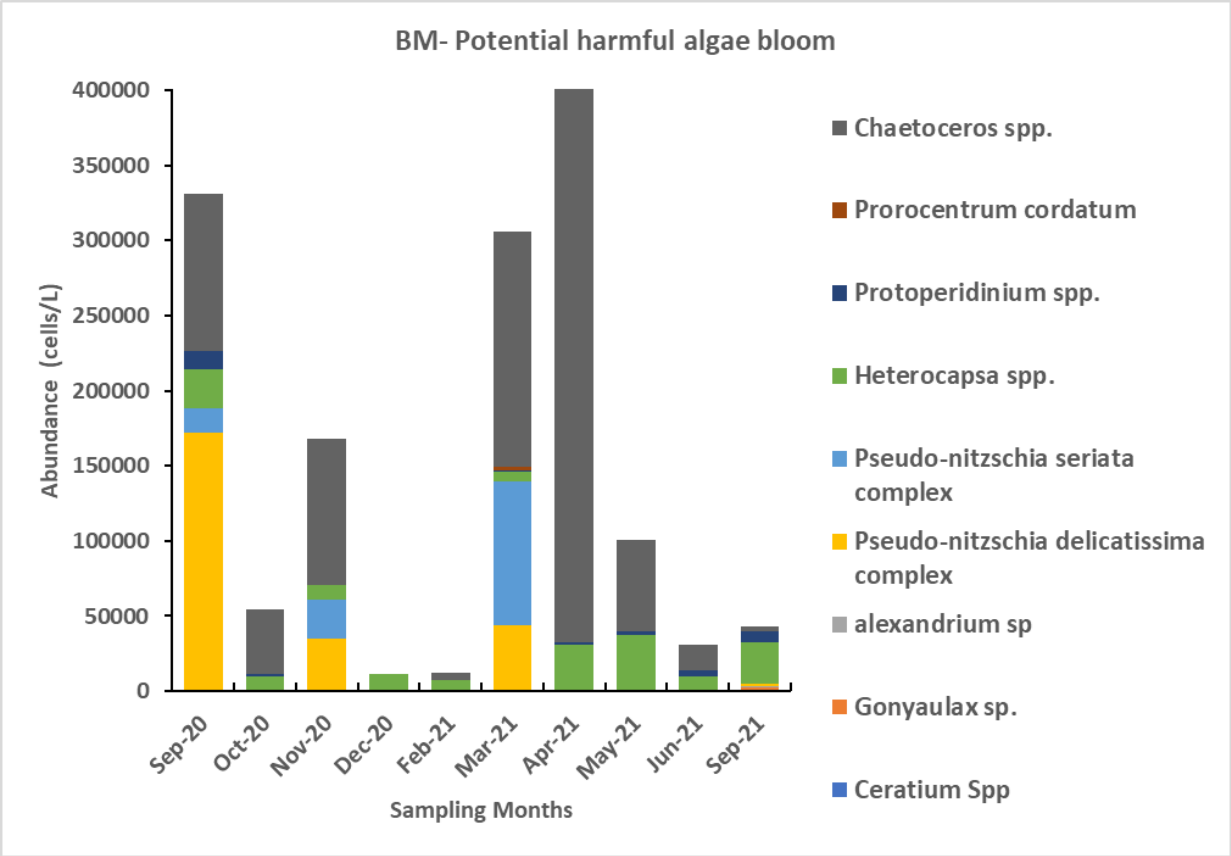



**Figure 29: Abundance of the main potentially harmful species (HABs) sampled in site BM at the Sacca di Goro lagoon waters between September 2020-2021 using morphological approach.**

# CHAPTER 5: DISCUSSION

## 5.1 Environmental data and their relationship with different hydrological conditions

Following its shallow depth about 1.5 m the Sacca di Goro hydrological characteristics are easily impacted by weather conditions, tidal exchange, natural freshwater inflows, industrial and urban wastewaters. In the current study we observed significant fluctuations in temperature and salinity in the Sacca di Goro lagoon as a function of season. Temperature followed a time-related trend typical of the Mediterranean region, with the highest average temperature values recorded in summer. This was similar to previous investigations in the Northern Adriatic Sea (Bernardi Aubry & Acri, 2004; Cerino *et al*., 2019). Similar trend has been observed in several Mediterranean lagoons, among them the Venice lagoon (Aubry et al., 2021b), and Lesina lagoon located in the South of the Adriatic Sea (Caroppo *et al*., 2018). The results were also in conjunction to a study done by (Mistri *et al*., 2001) where the Sacca di Goro lagoon showed minimal and similar temperature values during winter (January), while maximum temperatures were recorded in summer (July).

Salinity was particularly affected by the changes of the hydrological conditions (Riverine and canals output) which resulted into high variability between sampling sites. Generally, salinity values followed a seasonal trend linked to rainy events that could cause freshwater inputs in the lagoon, especially in proximity of G and FV whose low salinity levels are influenced by the presence of the two rivers (Po di Goro and Po di Volano). In site BM on the other hand, high salinities were attributed to water exchanges with sea. Draredja *et al*., (2019) observed that salinity values within a transitional lagoon are greatly influenced by temperature, rainfall, freshwater input from adjacent rivers and water exchanges with open seas. To support the findings of this study, it is important to consider the influence of tides that have a strong impact in this environment and of the hydrodynamic conditions, for instance the sampling in winter (December) occurred during high tide allowing marine water flushing into the lagoon, thus increasing the salinity.

Our results showed that the waters of the Sacca di Goro lagoon are well oxygenated (around 11.77 mg/L). However, site FV showed the lowest oxygen concentrations in all the sampling months. This could be associated with the fact that this site receives freshwater input but less from marine waters, and with the high abundances of phytoplankton recorded in this site. This drop could also have been attributed by the increase in decomposition of the phytoplankton following their massive growth during the spring period.

### 5.2 Phytoplankton Pigments (chlorophyll-a and phaeopigments)

Pigments distribution in the Sacca di Goro lagoon varied greatly. This variability can be linked with both geographic positions of the sample sites and the physio-chemical variables. Bocca Mare, a site located close to the Adriatic Sea, with high salinity levels showed low concentrations in phytoplankton pigments. On the contrary, remarkable phytoplankton pigment concentrations were found at site FV with constant influence from the Po di Volano River water discharge and potential nutrient input. Bužančić *et a*l., (2016) recorded high chlorophyll-a values in stations directly influenced by freshwater inflow and anthropogenic pressure while in stations with no freshwater inflows the chlorophyll-a concentrations were observed to be below 1 mg/$m^3$ in the Eastern Adriatic. A key interest is in the ratio between chlorophyll-a and its degradation products (phaeopigments) which ranges between 1 and 1.7. The ratio between the two has been used as a bioindicator of the health of the phytoplankton communities indicating freshness in terms of age. Low ratio has been associated with older community while high ratios >1.5 indicates the presence of a young active community signifying good status (Lorenzen, 1979). Low ratios observed during December for all the sites indicated the presence of an old phytoplankton communities thus bad status of the lagoon. The measurement of photosynthetic pigments in aquatic ecosystems has been always used to indicate autotroph biomass, potential primary production, and trophic status of the ecosystem (Carlson, 1977).

### 5.3 Spatial-temporal distribution of phytoplankton communities

Phytoplankton community structure in the Sacca di Goro lagoon seems quite similar to the situation previously found in other Mediterranean lagoonal ecosystems (Aubry *et al*., 2021b; Caroppo *et al*., 2018; Djakovac, 2009; Draredja *et al*., 2019; Jasprica *et al.*, 2022; Pestori *et al*., 2018), where a high number of phytoplankton taxa have been observed with a prevalence of diatom species. The main community composite identified by these studies were *Chaetoceros* spp.*, Skeletonema* spp.*, Pseudo-nitzschia* spp.*, Thallasionema* spp.*, Thallassiosira* spp.*, Cerataulina* spp.*,* and *Cyclotella* spp.

The spatial-temporal survey between September 2020-September 2021 of phytoplankton species in the Sacca di Goro lagoon allowed us to observe similar trends. The distribution of phytoplankton abundance generally followed a seasonal trend. The profiles for the various phytoplankton taxa provided a very good confirmation of the importance of temperature and hydrodynamic conditions in structuring phytoplankton communities between seasons. Abundances were low in late autumn-winter (from October to December) where temperatures were minimal, while from early spring temperature levels begin to increase and phytoplankton abundances were observed to increase until summer where a decrease in abundance was observed, resulting in a pattern that characterize most temperate coastal ecosystems with shallow depth and high nutrients concentrations.

The profiles for the various phytoplankton taxa also provided a very good confirmation of the importance of salinity as a critical factor contributing to physiological stress for algae species. In estuarine lagoons, seawater brought in by tides is mixed with freshwater inflows supplied by rivers which creates an estuarine salinity gradient with pure freshwater near the head of the estuary and seawater near the mouth of the estuary. Most phytoplankton species are known to be stenohaline and upon exposure to salinity changes they suffer osmotic stress, a factor that contributes to variability in their composition and succession (Kirst, 1990).

As already discussed, the Sacca di Goro received freshwater inputs from the Po di Volano and the Po di Goro resulting into low salinity levels in both site FV, and site G as compared to the other sites. Following this, a variation of phytoplankton composition was observed varying from typical freshwater diatoms, like *Cyclotella* and *Stephanodiscus* (which tolerate salinities below 0.5 psu) to marine diatoms belonging to the genera *Thalassiosira*, *Skeletonema*., and *Chaetoceros* (typical of salinities above 10 psu). Our results confirm the findings of (Ceccherelli *et al*., 1994) who observed remarkable phytoplankton density and biomass in the center of the lagoon and in the northern area while in the Valle di Gorino phytoplankton showed low values of both density and biomass in most of the year.

In temperate coastal areas, aquatic ecosystems are usually characterized by a spring bloom of phytoplankton. Wind forces in conjunction with the cooling of the water during winter cause a mixture of the water column resulting to a nutrient enrichment of the surface layers. During spring, the increase light intensity coupled with the enriched nutrients water are the main factors which trigger spring blooms (Spilling, 2007). During this period only fast-growing phytoplankton with a tolerance to low temperatures and high turbulence prevails. Since diatoms are characterized by the ability to quickly utilize excess nutrients and transform it to biomass they tend to dominate irrespective of other taxa, as reported in literature (Rothenberger *et al*., 2009). Conversely, diatom richness has been associated with several factors. Some studies (Margalef, 1978; Pielou, 1966) suggested turbulence and fertile environments as key drivers for diatom prevalence, but also diatom richness is related to the ability of diatom species to directly draw nutrients from the water-sediment interface (Bonin, 1988). This could explain the observed prevalence of diatoms in site BM and PG with consistent turbulence caused by the tidal flush. Generally, out of the total 147 taxa identified, a remarkable number consisted of Bacillariophyta (diatoms). Diatoms mostly present in the lagoon were *Chaetoceros* spp*., Skeletonema* spp*., Thallassiosira* spp. and *Pseudo-nitzschia* spp in all the sampling sites except for site FV where the prevalent diatom species was *Cyclotella* spp (freshwater species), while diatoms at the central part of the lagoon (site BM) with constant tidal flushes and site PG were composed mainly of marine diatoms with a high salinity tolerance.

In December, the coldest month, the most prevalent diatom species were *Cymatopleura* sp.*, Navicula* spp.*, Cocconeis, Licmophora* and some diatoms observed during the warm months but in low abundances. These findings agree with those reported from ARPAE in 2014-2019 (ARPA-EMR, 2013; Carla *et al*., 2017). Focusing on their recent reports, ARPAE recorded diatoms as the most abundant species composed by *Chaetoceros, Skeletonema, Navicula* spp. and *Cyclotella* spp*.* In addition, the increased blooms of diatoms in spring in most of the sites was attributed by the increase in surface nutrients following the wet months of winter.

Most of the prevalent diatom species in the Sacca di Goro lagoon are known key species that produce blooms in transitional waters globally (Carstensen *et al.*, 2015b). Diatoms such as *Skeletonema marinoi* with a preference towards nutrient-enriched conditions was found to be the most abundant in winter/spring (up to $2.86x10^6$ cells/L in the Boka Kotorska Bay (South-East Adriatic Sea) (Bosak *et al*., 2012). A similar situation was evidence in our study where >2.4 $x10^5$ cells/L of *S. marinoi* were recorded during the winter/spring months which are considered wet seasons with an increase in freshwater discharge and nutrients input. Other diatoms characterizing eutrophic environments (*Licmophora* spp. and *Thalassionema* sp.) were also recorded although in low abundances (Draredja *et al*., 2019).

Species belonging to the Chlorophyta showed a negative relation with salinity although not statistically significant. High abundances were recorded in site FV, a site with low salinity values especially during the warm months. The most prevalent species included *Ankistrodesmus* spp., *Scenedesmus* spp., *Crucigenia* spp., *Coelastrum* spp., and *Actinastrum* sp., similarly to results found by (Ferrari *et al*., 2021), attesting FV as the site with the most freshwater taxa due to the influence of the Po di Volano discharge.

Cyanobacteria, as well as Chlorophyta, resulted to dominate in low salinity waters and mostly at higher temperatures, as previously observed by Carstensen *et al*., (2015a). Cyanobacteria species, especially the picocyanobacterial, play a major role in nutrient rich transitional ecosystem and may become the prevailing phototrophic planktonic taxa in such sites (Paoli *et al*., 2007).

The increased abundance of cyanobacteria in site FV indicated the role of oxygen as an ecological factor. Whitton & Sinclair, (2016) reported that the success of blue-green algae is characterized by low oxygen levels. Their tolerance to low oxygen levels is a key factor determining the survival of their blooms. Towards the end of spring and summer remarkable abundances of cyanobacteria were recorded, mostly in site FV which reported the lowest dissolved oxygen concentrations.

The distribution of dinoflagellates (Miozoa) showed similarity trend as described for diatoms, however their abundances were lower compared to those of diatoms. Out of the 23 taxa recorded, above the 50% were found in site PG and BM during autumn and late summer, differently from the findings of Drakulović *et al*., (2017) where abundances were highest in November in the South-Eastern Adriatic sea. The prevalent Miozoa taxa were *Heterocapsa* spp*., Prorocentrum* spp*., Protoperidium* spp*., Gymnodium* spp*.,* and *Gonyaulax* spp*.* The prevalence of dinoflagellates in sites BM and PG suggest dinoflagellates as species dominating in warm saline waters.

In particular, remarkable abundances of dinoflagellates were observed after the spring bloom of diatoms supporting the hypothesis that high numbers of diatoms are often associated with low numbers of dinoflagellates. Tremblay *et al*., (2002) reported that when the upper layer is depleted of nitrate and silicate, diatom bloom ends, and a bloom of dinoflagellates follows whose development do not necessitate high nutrient concentration and silicate. A similar trend was observed here although dinoflagellates did not proliferate creating a bloom. The absence or minimal abundances observed in site FV could be attributed by the influence of freshwater inflows. *Prorocentrum micans* which appeared in all sites but mostly in site BM has been suggested to indicate eutrophic environments (Draredja *et al*., 2019).

Euglenophyta with only 3 species were highly recorded in site BM during June and September after an increase in surface temperature. Selina *et al*., (1999) attested as euglenoids species have been used as a biological indicator of organic pollution and characterize most diluted, warm and eutrophicated waters. Our findings corroborate with those of ARPAE where great abundances of Euglenophyta occur during the warm months when temperatures are high. However, ARPAE recorded most abundances in site FV

with average abundances of 2.5 x$10^5$ cells/L in 2018 while 9.1 x$10^5$ cells/L were recorded in site G during 2019, contrarily to our findings (Ferrari *et al*., 2021). Additionally, clam farming in the Sacca di Goro induces deep alterations of nutrient dynamics through the filtration of suspended particulate organic matter (Viaroli *et al*., 2010). Such alteration can contribute to changes in the seasonal variation of species whose growth is positively related to nutrients loads.

### 5.4 Harmful algal species

During our survey, a few potentially toxic species were identified in the Sacca di Goro lagoon. These included dinoflagellates (i.e., *Prorocentrum cordatum* in site PG during October and *Alexandrium* sp. in site BM during September and diatoms (i.e., *Pseudo-nitzschia delicatissima complex, Pseudo-nitzschia seriata complex*) mostly occurring in all the sites during warm months. Some species of *Alexandrium* are known to produce toxins such as Paralyzing Shellfish toxins (PST) that can be harmful to humans when toxic mollusks are consumed (Armijo *et al*., 2020). Heil *et al*., (2005) reported *Prorocentrum cordatum* blooms mostly in brackish environment associated to eutrophication. In the north-eastern part of the Adriatic Sea, diatom blooms are mainly attributed to *Pseudo-nitzschia* spp., mostly the *P. delicatissima* group where densities of up 1.2x$10^6$ cells results in the accumulation of Amnesic shellfish poisoning (ASP) in shellfish (Tsikoti & Genitsaris, 2021). Although Sacca di Goro showed low abundances of HABs species, warming effects linked to climate changes could potentially result in bloom formation of these species causing significant impacts on the trophic structure of this lagoon in the coming decades. Following the ongoing aquaculture activity in the farm, such blooms can result to potential intoxication of seafood with great threat to human health. Therefore, monitoring programs are important to provide understandings on the development of toxic species and their potential associated toxins.

### 5.5 Comparison between microscopy and DNA metabarcoding for phytoplankton monitoring

To our knowledge, this is the first study performed in the Sacca di Goro lagoon which aimed at analyzing the phytoplankton community by employing the traditional morphology-based (microscopy) approach and the more innovative molecular (DNA

metabarcoding) one. Based on the findings of this study, the qualitative composition of the phytoplankton community obtained by both approaches appeared quite similar. However, some differences were observed with DNA metabarcoding, where some taxa were missing and at the same time more diversity was seen than with traditional techniques. Based on the MOTUs, metabarcoding uncovered a vast taxonomic diversity exceeding the 147 taxa identified by microscope with less effort and taxonomic expertise required, proving it to be an efficient method for biodiversity estimates. From a quantitative point of view, DNA metabarcoding is less informative than microscopy, as MOTUs cannot be regarded as a straightforward quantitative expression of cell density, although in a certain way they depend on autotrophic plankton abundance, as previously reported by Penna *et al*., (2017). Results could be thus analyzed considering the relative abundance of each taxon as number of reads. It must be considered that the relative abundance of each taxon is strongly influenced by the composition of the phytoplankton community, which in turn depends on what is present in the reference database that has been used for the processing of the DNA results. It is known as the lack of representative sequences for some organisms in the current databases could result into a different spatial and temporal trend resulting from metabarcoding and morphological (microscopic) taxonomic analysis, as previously reported (Abad *et al*., 2016). This could be also the reason of a different spatial and temporal distribution of our samples when using the two approaches. It is reported that similar spatial and temporal trends of taxonomic diversity were observed for metabarcoding and microscopic studies of zooplankton, but not for phytoplankton, mostly attributable to the lack of representative sequences for phytoplankton species in current databases (Abad *et a*l., 2016). In addition, due to PCR and sequencing errors an overestimation of species diversity can sometimes occur (Kunin *et al*., 2010).

The results were in conjunction to other studies previously done in various regions. Keck *et al*., (2021) performed a meta-analysis of all available studies that compared traditional methods and DNA metabarcoding to measure and assess biological diversity of aquatic organisms and found that DNA metabarcoding provides diversity estimates that are consistent with those obtained using traditional methods. However, some differences

were observed with DNA metabarcoding where some taxa were missing and at the same time detecting diversity that were not seen with traditional techniques.

# CHAPTER 6: CONCLUSION

The Sacca di Goro lagoon is characterized by strong intra-annual variations related to its shallowness and climatic conditions. During the wet period, the lagoon receives freshwater input which results into lower salinities and increase in nutrients availability. During summer, rainfall become scarce and due to increased temperatures, the salinity levels could increase. These strong environmental variabilities are the major contributing forces that resulted into the variability of the phytoplankton community structure and its diversity between seasons and sites.

Regarding this, Foce Volano (FV) represents the site with the most phytoplankton diversity due to the abundance of freshwater species, while Boca Mare (BM) represents the site with less phytoplankton diversity and most of the species being marine diatoms and dinoflagellates. High Bacillariophyta abundances occur during the transitional phase between spring/summer as a consequence of an increase in daylight and nutrients concentrations due to heavy rainfall. This study also confirms the presence of eutrophic related taxa. It highlights that, management measures should integrate all the possible drivers of nutrient loads and their concentrations should be regularly monitored in the lagoon, especially on site FV and G with high influence of freshwater input. The presence of some potential HAB-related species indicates the importance of a monitoring program to detect the toxic species and their cell densities and protect the high value services offered by the lagoon (aquaculture, fishing, and tourism).

Microscopy techniques are still very common in the study of phytoplankton diversity but require expertise, are time consuming, and based on the findings of this study result into less biodiversity. However, microscopy is more informative since identification can be performed at a low taxonomic level (species level) and more harmful algae species could be detected by this technique, overcoming the limitation of the lack of representative sequences for some phytoplankton species in current databases.

On the contrary metabarcoding approach is a good candidate for routine applications because of its technical potential. As observed in this study, the technique is fast, results into more diversity including the small size picoplankton, without requirement of taxonomic expertise. The correspondence in community composition between the two techniques also suggests a semiquantitative potential for metabarcoding. However, to

turn that potential into significant advances, the gap between sequence reads and ecologically meaningful entities must be adequately bridged. Although changes in the relative abundance of MOTUs closely matched the seasonal dynamics of phytoplankton reported for the lagoon based on microscopy, which indicated relative abundance data based on read counts are ecologically meaningful, the incompleteness of the reference library influenced our results. Currently, this necessity represents one of the main drawbacks since some groups of organisms have none or very few publicly available sequences and could not be detected by the metabarcoding approach.

Finally, integrating microscopy with metabarcoding provides a powerful approach for studying phytoplankton dynamics in aquatic systems and shows promise for long-term whole community monitoring.

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**List of appendices**

**Table 1:** Differences between A. DNA-Metabarcoding and Morphological approach in terms of Taxa and species composition highlighting the main differences. Numbers in brackets represent reads for DNA while for morphological represents frequency of counts across all samples.

**A. DNA-Metabarcoding**

| | | | |
|---|---|---|---|
| **Ochrophyta** | | | |
| *Aureococcus (1)* | *Ciliophrys (3)* | *Merotricha (1)* | *Polypodochrysis (1)* |
| *Bolidomonas (1)* | *Dinobryon (1)* | *Ochromonas (3)* | *Poteriospumella (2)* |
| | | *Paraphysomonas* | |
| *Chattonella (2)* | *Ectocarpus (1)* | *(7)* | *Psammamonas (2)* |
| *Chlorobotrys (1)* | *Epipyxis (15)* | *Pedinella (103)* | *Pseudopedinella (4)* |
| *Chromulina (1)* | *Fibrocapsa (8)* | *Phaeoplaca (5)* | *Pteridomonas (2)* |
| *Chrysolepidomonas* | | | |
| *(117)* | *Gonyostomum (1)* | *Pseudopedinella* | *Spumella (43)* |
| | *Halothrix (1)* | *Tessellaria (38)* | *Trachydiscus (1)* |
| *Chrysonephele (10)* | *Helicopedinella (16)* | *Mallomonas (1)* | *Uroglena (123)* |
| *Chrysoxys (5)* | *Heterosigma (39)* | | *Vacuoliviride (2)* |
| **picophyta** | | | |
| *Codosiga (3)* | *Lagenoeca (1)* | *Chromera (2)* | |
| *Diaphanoeca (1)* | *Picomonas (36)* | | |
| **Cercozoa** | | | |
| *Cavernomonas (10)* | *Ebria (58)* | *Heteromita (2)* | |
| *Cryothecomonas (151)* | *Eocercomonas (1)* | *Minorisa (2)* | |
| **Katablepharidophyta** | | | |
| *Katablepharis (31)* | *Leucocryptos (2)* | | |
| **Bigyra** | | | |
| *Bicosoeca (5)* | *Oblongichytrium (1)-worms* | | |
| **B. Morphological approach** | | | |

| | | | |
|---|---|---|---|
| **Chlorophyta** | | | |
| *Coelastrum* sp. *(31)* | *Pediastrum* sp *(58)* | *Crucigenia* spp. *(15)* | *S. acuminatus (21)* |
| *Monoraphidium* spp. *(24)* | *Scenedesmus* spp. *(37)* | *C. tetrapedia (9)* | *S. protuberans (4)* |
| *S. dimorphus (5)* | *ankistrodesmus* spp *(41)* | *Actinastrum (15)* | *S. quadricauda (36)* |
| **Euglenophyceae** | | | |
| *Euglena* spp. *(58)* | *Phacus* sp. *(6)* | *Eutreptiella spp. (14)* | |
| **Cyanobacteria** | | | |
| undefined colonies (61) | *Anabaena* SP (5) | *Pseudoanabaena (2)* | *Merismopedia (18)* |

**Table 2:** Genus and Operational taxonomic units obtained after clustering 80% score of the sequence reads (Numbers in brackets represents their frequency reads).

| | | | |
|---|---|---|---|
| **Bacillariophyta** | | | |
| Achnanthidium (1) | Discostella (146) | Minutocellus (9) | Nitzschia (6) |
| Arcocellulus (3) | Entomoneis (2) | Navicula (3) | Pleurosigma (5) |
| Aulacoseira (3) | Epithemia (1) | Palmerina (2) | Pseudostriatella (3) |
| Bacillaria (1) | Falcula (9) | Pseudo_nitzschia | Rhaphoneis (2) |
| Catacombas (7) | Fistulifera (2) | Roundia (640) | Rhopalodia (2) |
| Cerataulina (8) | Fragilariforma (4) | Skeletonema (128) | Tabularia (30) |
| Chaetoceros (234) | Grammonema (7) | Stephanodiscus (611) | Thalassiosira (201) |
| Cyclotella (1061) | Guinardia (3) | Synedropsis (1) | Placus (1) |
| Cylindrotheca (7) | Lithodesmium (4) | Licmosphenia (3) | Cymbellonitzschia (37) |
| **Miozoa** | | | |
| Aduncodinium (1) | Lessardia (7) | Nematodinium (17) | Warnowia (4) |

| | | | |
|---|---|---|---|
| Alexandrium (20) | Duboscquella (10) | Neoceratium (2) | Woloszynskia (3) |
| Amoebophrya (37) | Glenodinium (1) | Ornithocercus (8) | Stoeckeria (24) |
| Amphidinium (3) | Goniodoma (3) | Paragymnodinium (31) | Pyrodinium (3) |
| Ansanella (12) | Gymnoxanthella (4) | Pfiesteria (3) | Roscoffia (21) |
| Apicoporus (6) | Gyrodinium (24) | Podolampas (112) | Scrippsiella (2) |
| Biecheleria (6) | Haplozoon (16) | Polykrikos (16) | Sinophysis (87) |
| Blixaea (1) | Heterocapsa (13) | Protodinium (16) | Ptychodiscus (3) |
| Chytriodinium (1) | Karenia (3) | Spiniferodinium (12) | Preperidinium (5) |
| Cochlodinium (1) | Karlodinium (2) | Takayama (32) | Tintinnophagus (16) |
| Colponema (1) | Katodinium (1) | Thoracosphaera (27) | |
| Diplopsalis (4) | Lepidodinium (4) | | |
| **Chlorophyta** | | | |
| Bathycoccus (29) | Floydiella (131) | Micromonas (11) | Tetraselmis (526) |
| Chaetopeltis (59) | Hormotilopsis (51) | Nephroselmis (2) | Verdigellas(1)-worms |
| Crustomastix (5) | Mamiella (12) | Ostreococcus (218) | Pedinomonas (2) |
| Dolichomastix (1) | Uronema (9) | | |
| **Cryptophyta** | | | |
| Chroomonas (3) | Geminigera (96) | Teleaulax (38) | |
| Cryptomonas (3) | Hemiselmis (25) | Plagioselmis (3) | |
| **Haptophyta** | | | |
| Chrysocampanula (1) | Diacronema | Haptolina (4) | Pseudohaptolina (2) |
| Chrysochromulina (1) | Exanthemachrysis (226) | Jomonlithus (2) | Scyphosphaera (4) |
| **Ochrophyta** | | | |
| Aureococcus (1) | Ciliophrys (3) | Merotricha (1) | Polypodochrysis (1) |
| Bolidomonas (1) | Dinobryon (1) | Ochromonas (3) | Poteriospumella (2) |
| Chattonella (2) | Ectocarpus (1) | Paraphysomonas (7) | Psammamonas (2) |

| | | | |
|---|---|---|---|
| Chlorobotrys (1) | Epipyxis (15) | Pedinella (103) | Pseudopedinella (4) |
| Chromulina (1) | Fibrocapsa (8) | Phaeoplaca (5) | Pteridomonas (2) |
| Chrysolepidomonas (117) | Gonyostomum (1) | Pseudopedinella | Spumella (43) |
| | Halothrix (1) | Tessellaria (38) | Trachydiscus (1) |
| Chrysonephele (10) | Helicopedinella (16) | Mallomonas (1) | Uroglena (123) |
| Chrysoxys (5) | Heterosigma (39) | | Vacuoliviride (2) |
| **picophyta** | | | |
| Codosiga (3) | Lagenoeca (1) | Chromera (2) | |
| Diaphanoeca (1) | Picomonas (36) | | |
| **Cercozoa** | | | |
| Cavernomonas (10) | Ebria (58) | Heteromita (2) | |
| Cryothecomonas (151) | Eocercomonas (1) | Minorisa (2) | |
| **Katablepharidophyta** | | | |
| Katablepharis (31) | Leucocryptos (2) | | |
| **Bigyra** | | | |
| Bicosoeca (5) | Oblongichytrium (1)-worms | | |
| Total OTU 6729; Species 158 | | | |

**Table 3**: Two-way PERMANOVA Permutation N:9999 for nMDS DNA

| Source | Sum of sqrs | df | Mean square | F | p |
|---|---|---|---|---|---|
| TIME | 3.68648 | 6 | 0.61441 | 1.0939 | **0.0001** |
| SITE | 1.81286 | 3 | 0.60429 | 1.0759 | **0.0006** |
| Interaction | 4.23522 | 18 | 0.23529 | 0.41892 | **0.0555** |
| Residual | 3.36991 | 6 | 0.56165 | | |
| Total | 13.104 | 33 | | | |

**Table 4**: Two-way PERMANOVA Permutation: POST-HOC-Site nMDS DNA

| | BM | FV | G | PG |
|---|---|---|---|---|
| BM | | **0.005** | **0.001** | **0.0372** |
| FV | **0.005** | | 0.4201 | 0.3225 |
| G | **0.001** | 0.4201 | | 0.2 |
| PG | **0.0372** | 0.3225 | 0.2 | |

**Table 5**: Two-way PERMANOVA Permutation: POST-HOC-Time nMDS DNA

| | MAR | APR | JUN | SEP | OCT | NOV | DEC |
|---|---|---|---|---|---|---|---|
| MAR | | 0.6512 | **0.0419** | 0.263 | 0.3101 | 0.1985 | **0.0125** |
| APR | 0.6512 | | 0.3128 | 0.6986 | 0.6036 | 0.2513 | **0.0115** |
| JUN | **0.0419** | 0.3128 | | 0.3515 | 0.0515 | 0.1103 | **0.0002** |
| SEP | 0.263 | 0.6986 | 0.3515 | | 0.5119 | 0.2571 | **0.0137** |
| OCT | 0.3101 | 0.6036 | 0.0515 | 0.5119 | | 0.1194 | **0.0004** |
| NOV | 0.1985 | 0.2513 | 0.1103 | 0.2571 | 0.1194 | | 0.2225 |
| DEC | **0.0125** | **0.0115** | **0.0002** | **0.0137** | **0.0004** | 0.2225 | |

**Table 6:** Two-way PERMANOVA: Permutation N: 9999, quantitative analysis

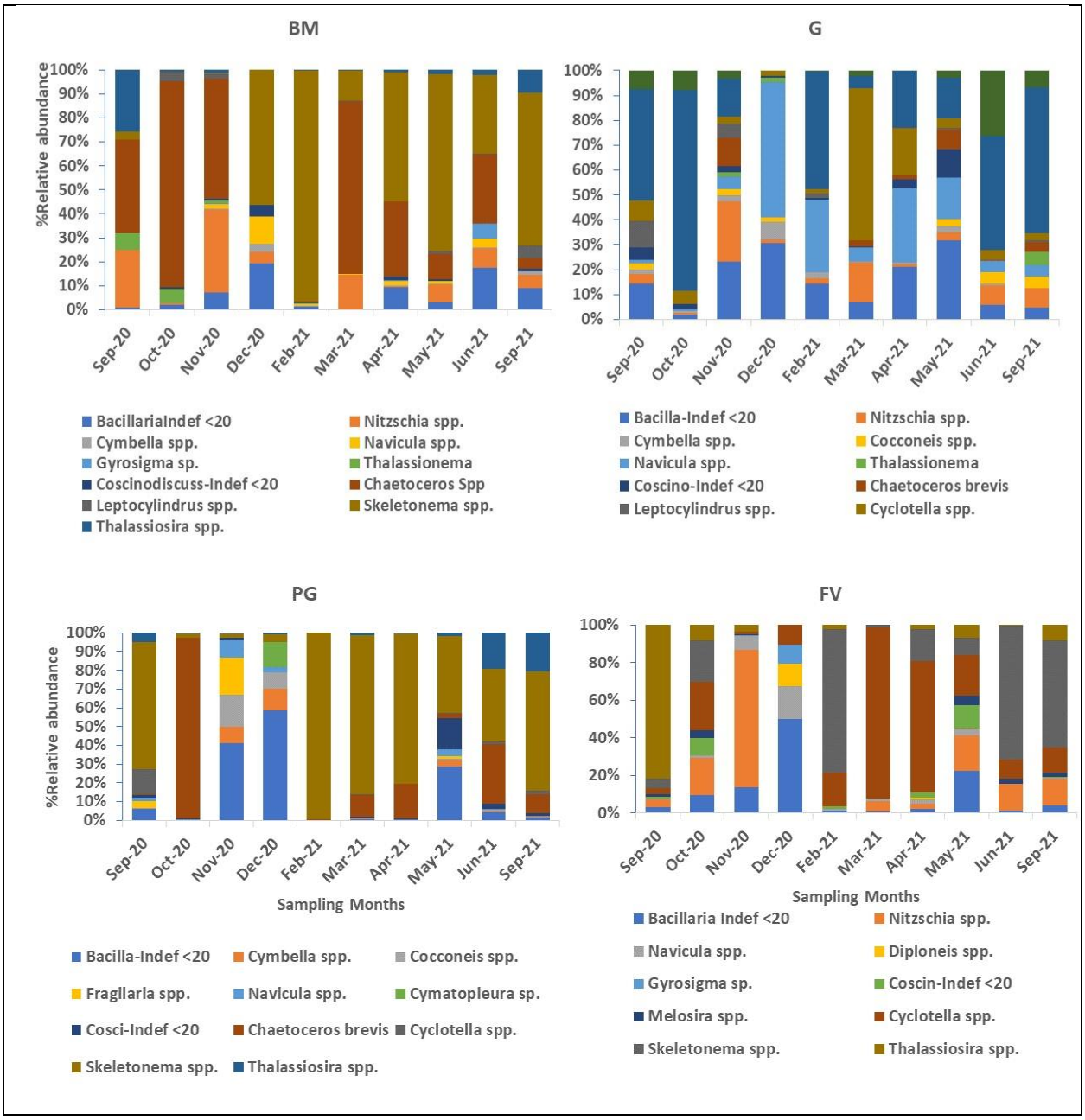
| Source | Sum of sqrs | df | Mean square | F | p |
|---|---|---|---|---|---|
| site | 2.53552 | 3 | 0.84517 | 8.8662 | **0.0001** |
| time | 7.01572 | 9 | 0.77952 | 8.1775 | **0.0001** |
| Interaction | 9.00987 | 27 | 0.3337 | 3.5006 | **0.0001** |
| Residual | 4.28965 | 45 | 0.095326 | | |
| Total | 22.851 | 84 | | | |

**Table 7**: One-way PERMANOVA Permutation: POST-HOC-Time, quantitative analysis

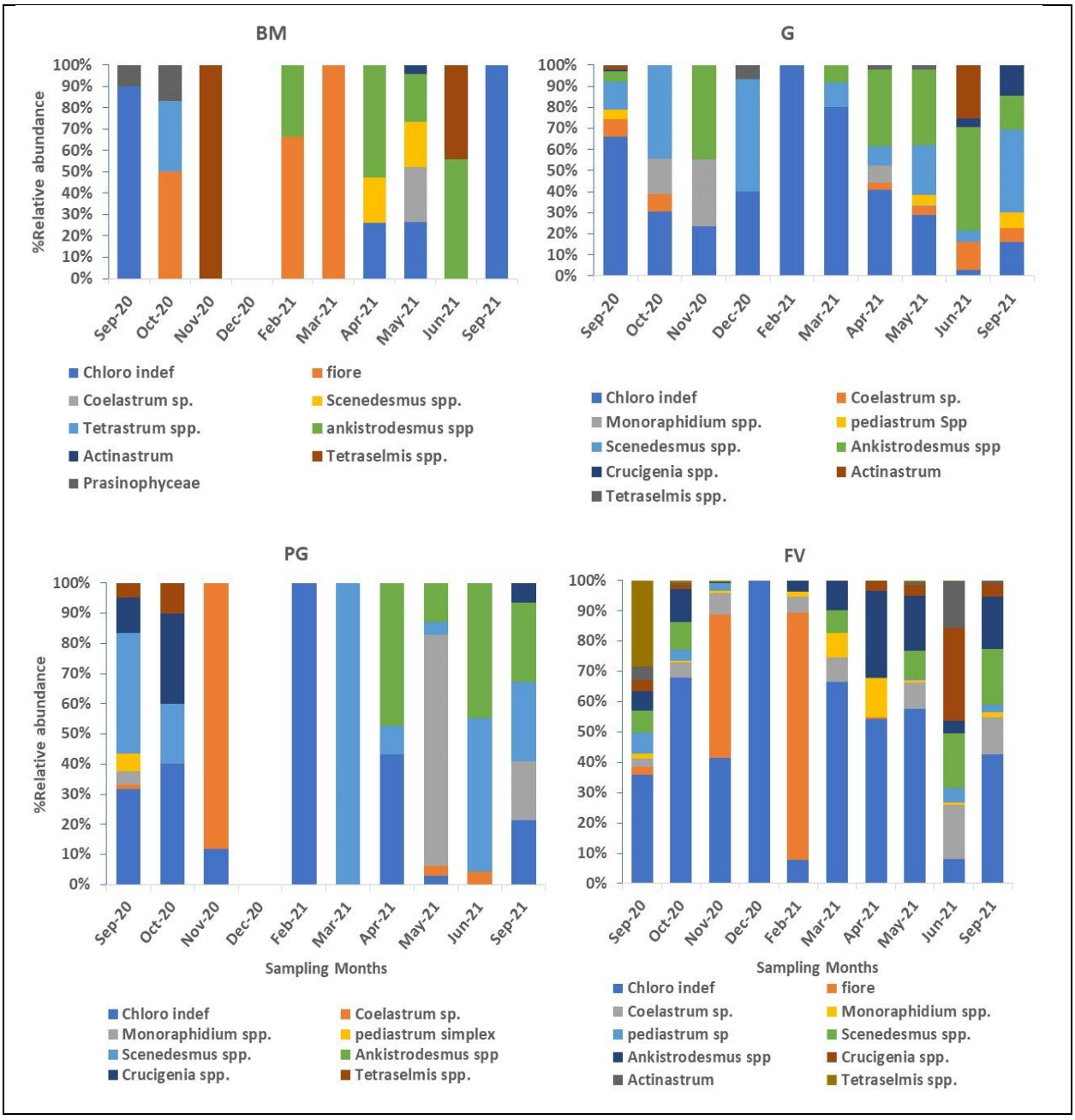
| | sep20 | oct20 | Nov-20 | dec20 | Feb-21 | Mar-21 | Apr-21 | may21 | jun21 | sep21 |
|---|---|---|---|---|---|---|---|---|---|---|
| sep20 | | **0.0054** | **0.0002** | **0.0001** | **0.0004** | 0.0216 | 0.1101 | 0.035 | **0.0046** | **0.0016** |
| oct20 | **0.0054** | | 0.1101 | **0.0008** | **0.0008** | 0.0783 | **0.0307** | **0.0014** | 0.1119 | 0.0742 |
| Nov-20 | **0.0002** | 0.1101 | | **0.0022** | **0.0011** | **0.0037** | **0.0002** | **0.0001** | **0.0003** | **0.0017** |
| dec20 | **0.0001** | **0.0008** | **0.0022** | | **0.0007** | **0.0006** | **0.0002** | **0.0001** | **0.0008** | **0.0006** |
| Feb-21 | **0.0004** | **0.0008** | **0.0011** | **0.0007** | | 0.122 | 0.166 | **0.0003** | **0.0013** | **0.0008** |
| Mar-21 | 0.0216 | 0.0783 | **0.0037** | **0.0006** | 0.122 | | 0.1676 | **0.0016** | **0.0398** | 0.0279 |
| Apr-21 | 0.1101 | **0.0307** | **0.0002** | **0.0002** | 0.166 | 0.1676 | | **0.0277** | **0.0047** | **0.0004** |
| may21 | **0.035** | **0.0014** | **0.0001** | **0.0001** | **0.0003** | **0.0016** | **0.0277** | | **0.0007** | **0.0001** |
| jun21 | **0.0046** | 0.1119 | **0.0003** | **0.0008** | **0.0013** | **0.0398** | **0.0047** | **0.0007** | | 0.1904 |
| sep21 | **0.0016** | 0.0742 | **0.0017** | **0.0006** | **0.0008** | **0.0279** | **0.0004** | **0.0001** | 0.1904 | |

**Table 8:** One-way PERMANOVA Permutation: POST-HOC-Site, quantitative analysis

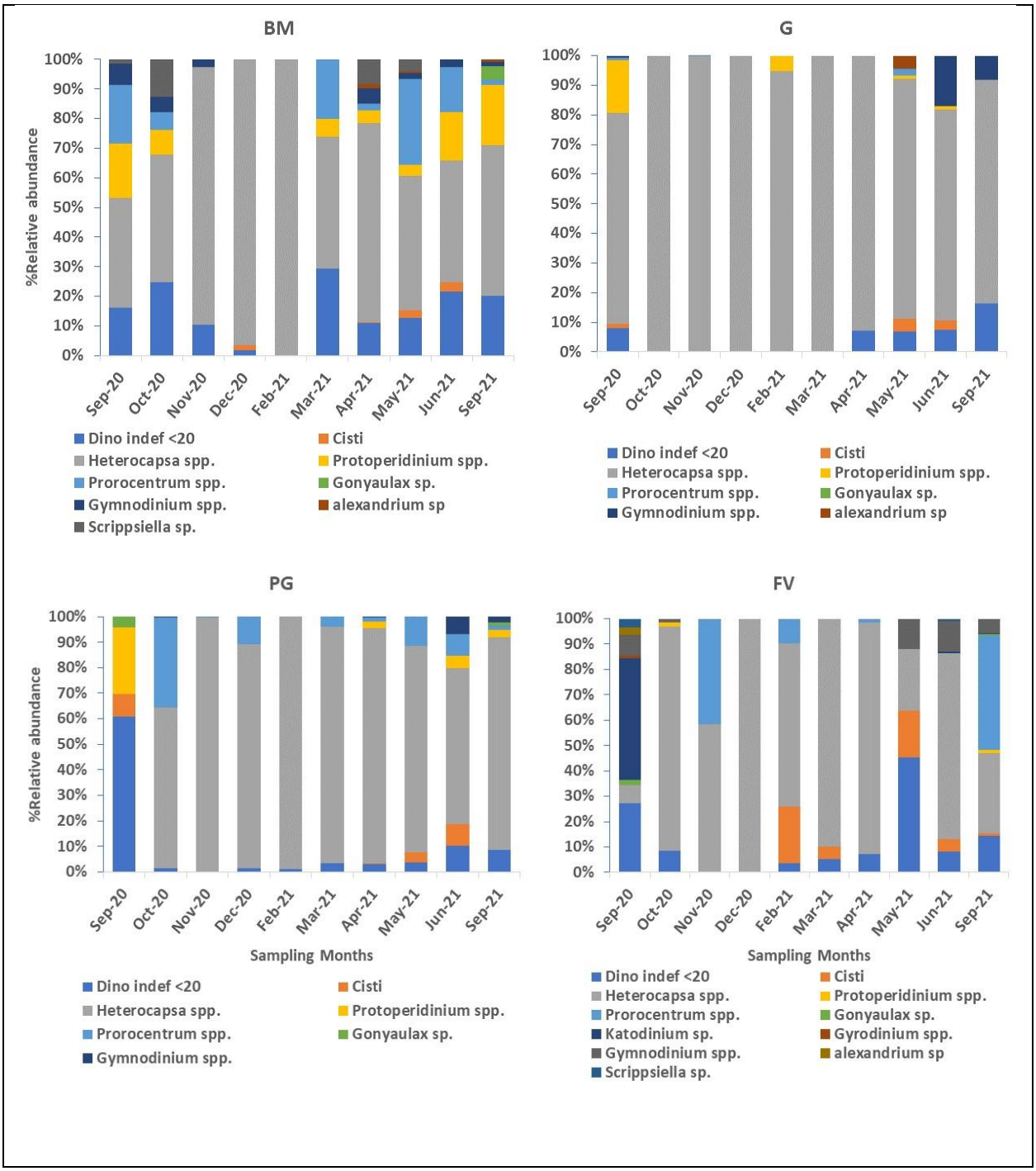
| | BM | G | PG | FV |
|---|---|---|---|---|
| BM | | **0.0025** | **0.0372** | **0.0001** |
| G | **0.0025** | | **0.0065** | **0.0035** |
| PG | **0.0372** | **0.0065** | | **0.0001** |
| FV | **0.0001** | **0.0035** | **0.0001** | |



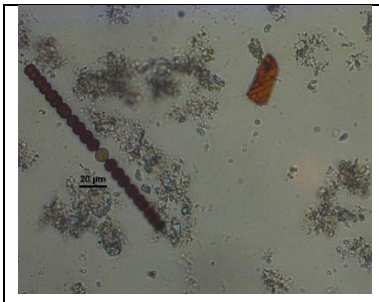
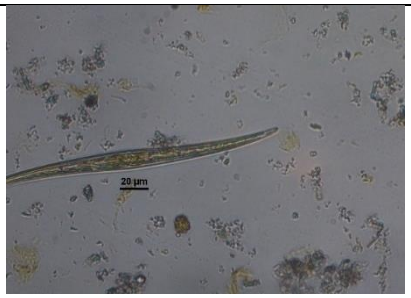
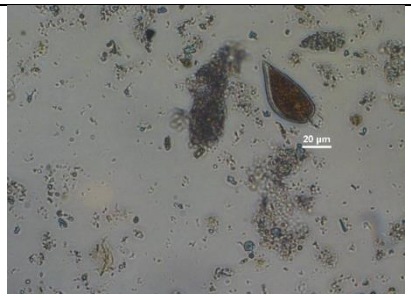
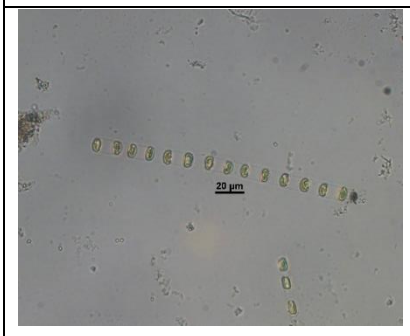
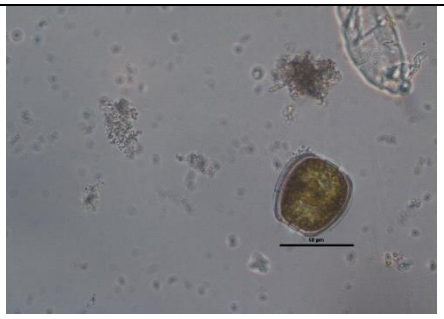
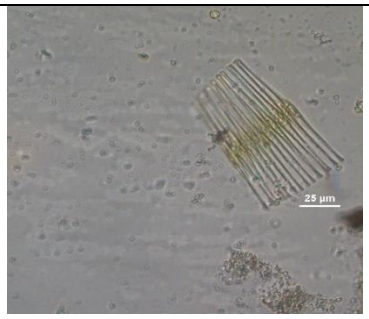
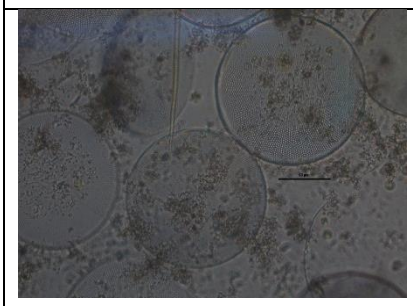
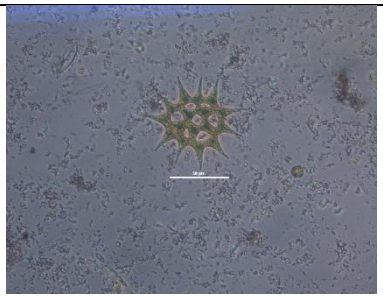
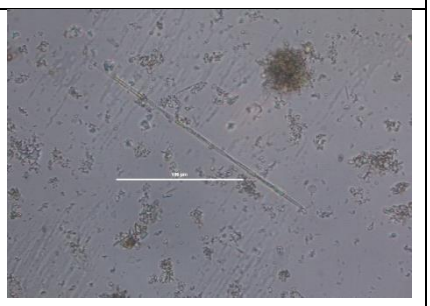

**Appendix Figure 1:** Bacillariophyceae dominant phytoplankton communities from different sites BM (Boca Mare), G (Gorino), PG (Porto Gorino) and FV (Foce Volano). (Species dominant >5% contribution to the total relative abundance) for each month.




**Appendix Figure 2:** Chlorophyta dominant phytoplankton communities from sites BM (Boca Mare), G (Gorino), PG (Porto Gorino) and FV (Foce Volano). (Species was considered dominant >5% contribution to the total relative abundance) for each month.




**Appendix Figure 3:** Miozoa dominant phytoplankton communities from different sites BM (Boca Mare), G (Gorino), PG (Porto Gorino) and FV (Foce Volano). (Species dominant >5% contribution to the total relative abundance) for each month.

| | | |
|---|---|---|
|  |  |  |
| **Cyanobacteria *anabaena* sp.** | ***Gyrosigma* sp.** | ***Prorocentrum micans*** |
|  |  |  |
| ***Skeletonema* sp.** | ***Coscinodiscus* sp.** | ***Fragilaria* sp.** |
|  |  |  |
| ***Coscinodiscus centralis*** | ***Pediastrum simplex*** | ***Pseudo-nitzschia* sp.** |

**Appendix Figure 4**: Images of some phytoplankton cells identified by morphological approaches.