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Understanding environmental and anthropogenic factors affecting dinoflagellate communities in the Black and Caspian seas

Understanding environmental and anthropogenic factors affecting dinoflagellate communities in the Black and Caspian seas

By

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Abstract

Biodiversity loss worldwide is linked to human activities and climate change, this trend is threatening the function and health of ecosystems globally. In aquatic ecosystems, studying the biodiversity of key trophic groups at regional and local scales and including coastal and estuarine areas is essential for understanding how such ecosystems respond to perturbations. The Black Sea and the Caspian Sea both have highly variable environmental conditions with steep salinity gradients and are experiencing strong anthropogenic activities in places. These basins are part of the Pontocaspian region which, as a consequence of its geological and climatic history, hosts unique biological assemblages adapted to variable environmental conditions.

Dinoflagellates are a key component of aquatic systems and an ideal group for biodiversity studies on short and longer timescales, e.g. from current monitoring to millions of years. They respond to and are therefore used as proxies for changing water conditions including pollution, and provide a rich fossil record based on their resting cysts (dinocysts). Previous studies on dinoflagellates in the Pontocaspian region are fragmented by geographically uneven sampling efforts. They are also, almost exclusively, based on traditional taxonomic observations under light microscopy. This methodology is time-consuming, requires considerable expertise to undertake and may not be suitable for identifying cryptogenic and parasite species.

This study targeted both dinocysts and dinoflagellates, adding substantial new data to previously poorly studied areas of the Pontocaspian, using both traditional morphological identification and new genetic methods to infer the biodiversity and biogeography of the group.

Statistical analyses of new dinocyst data generated from this project confirm that temperature, salinity and primary productivity are the main drivers of dinocyst distribution and assemblages in the Pontocaspian basins. This conclusion was also achieved by analysing water samples from the different Black Sea environments. The results demonstrate that the main factor affecting the diversity of dinoflagellates and limiting their distribution is salinity, followed by temperature, rather than spatial factors; indicating that dinoflagellate communities in the Black Sea are mostly dependent on niche-based processes. This new dinoflagellate diversity and distribution data were obtained by applying Next Generation Sequencing (NGS) and DNA barcoding methods. These techniques recovered a high diversity of dinoflagellates including taxa recorded for the first time for this region and those known to be hard to identify by light microscopy. Dinocyst assemblages analysed in this thesis also reflect nutrient-enriched conditions in the north Caspian Sea and the western Black Sea. Moreover, there is a significant correlation between shipping activities and dinocyst assembles in the Caspian Sea. In addition, new dinoflagellate cultures isolated from the Pontocaspian region were established to test the effect of dominant environmental factors on dinoflagellates under controlled conditions. Culture experiments with *Gymnodinium aureolum* suggest that this species is adapted to Black Sea water conditions, indicating that it is probably a long-established population, which has the potential of developing harmful blooms.

This study is the first to test statistically the impact of shipping activities on dinocyst assemblages, and the first work to implement NGS and ITS barcoding to study diversity and biogeography of dinoflagellates in the Pontocaspian region. In addition, new cultures of HAB species of dinoflagellate were established expanding the knowledge of these species in the region. This work also complements and confirms the findings from previous studies in the region and provides new data and information to support further ecological, diversity and monitoring studies.

Acknowledgements

Throughout the writing of this work, I have received a great deal of support and assistance. First and foremost, I would like to express my gratitude to my supervisors Suzanne Leroy, Rachel Flecker, Alexandre Anesio and Anne Lockyer. Suzanne, thank you for trusting me to carry out this project, your constant input, for guiding me through palynology, a completely new field for me, and for sharing your invaluable expertise in the Pontocaspian region. Alex and Anne, I am thankful for your patience, positive input and guidance in all of the biodiversity and molecular aspects of this thesis. I have learnt many valuable approaches, concepts, methods and techniques useful for my career. Rachel, I am grateful for your endless encouragement that helped me to weather every storm, not of scientific nature exclusively, I faced during this PhD. I can say without any doubt that your support has been essential for finding the motivation and strength to accomplish this work, and for that, I will be always thankful.

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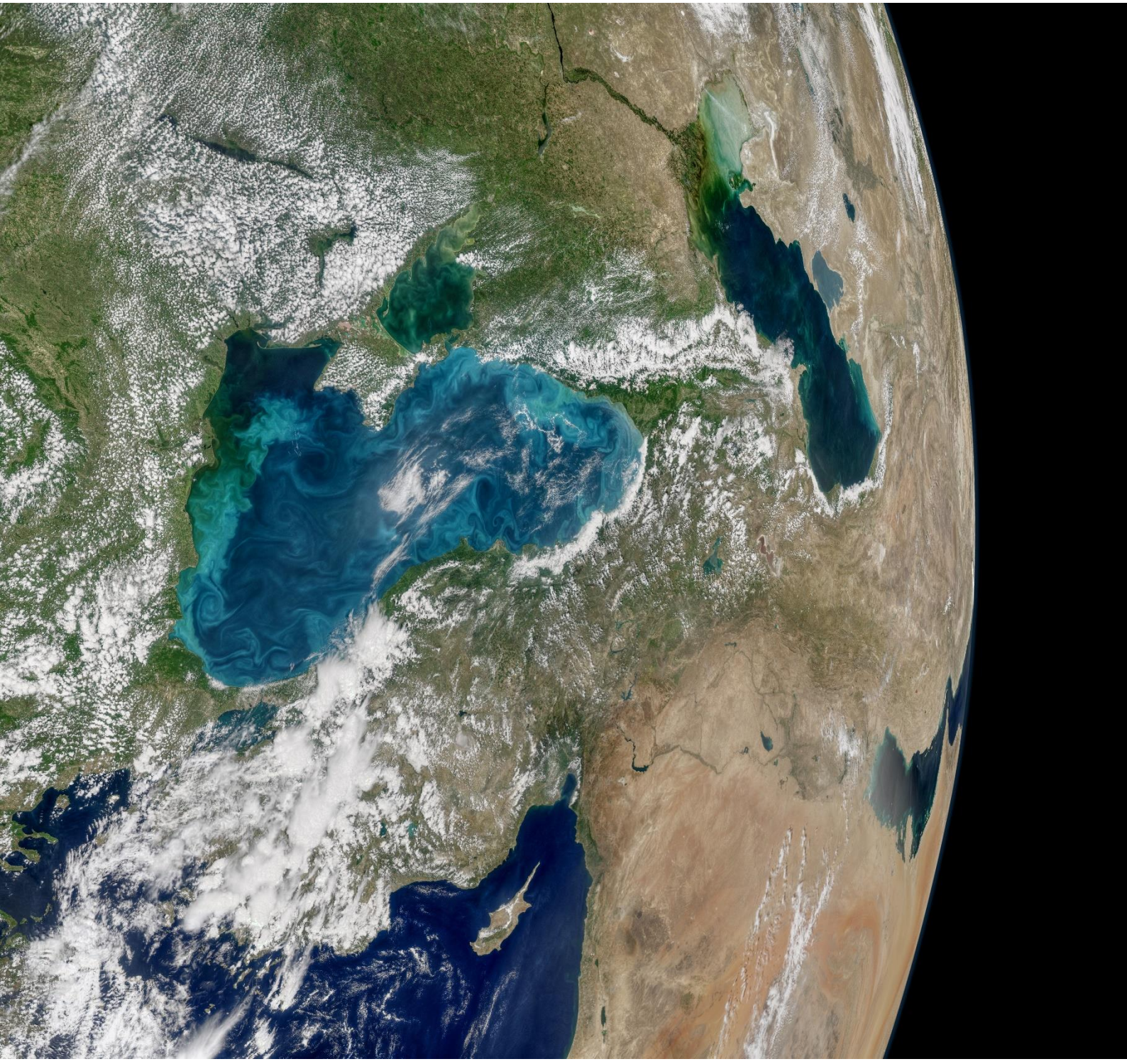
Author's declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: ..... DATE: 18 March 2021

“I know of no pleasure deeper than that which comes from contemplating the natural world and trying to understand it.”

— Sir David Attenborough



NASA image by Norman Kuring,
NASA’s Ocean Biology Processing Group
https://earthobservatory.nasa.gov

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CHAPTER 1

INTRODUCTION

1.1 Thesis motivation

Ecosystem functioning is highly dependent on interactions between both abiotic factors, such as geology and climate, and biotic drivers, such as interactions between species (Humbert and Dorigo, 2005). All processes involved in ecosystem functioning are characterized by the diversity of functional biotic groups, which are defined as a set of species present in an ecosystem showing either similar responses to environmental conditions or comparable influence on ecosystem processes (reviewed by Hooper et al., 2005). Therefore, the ecosystem functioning is determined by the functional features of species present in that ecosystem, the distribution and abundance of such species and their biology (Naeem and Wright, 2003). Ecosystem health highly depends on ecosystem functioning (Tett et al., 2013) and the latter provides essential services for society including freshwater and food, biological products, nutrients and pollutant recycling, protection against coastal erosion and sea-level rise and regulation of infectious diseases (WHO, 2005; Sala and Knowlton, 2006). Human activities together with climate change have precipitated a global loss of biodiversity loss that is threatening ecosystem functioning worldwide (Loreau et al., 2001; Covich et al., 2004). This trend can be observed at the largest scale of marine ecosystems, classified as large marine ecosystems (LMEs), these are large ocean extensions ($\leq$ 200,000 km^2) covering from coastal areas to the offshore limit of the continental shelves, including river basins and estuaries areas (Sherman and Duda, 1999). These regions provide essential ecosystem services i.e. over 80% of the yield of global fisheries in the last 50 years have been produced in LMEs. Figure 1.1 shows that the trend of collapsed fish and invertebrate taxa recorded since 1950 has increased to 65% in these regions worldwide (Worm et al., 2006). LMEs group numerous ecosystems of which coastal and estuarine areas are economic and ecologically valuable areas and the most likely to suffer perturbations from human activities due to their fragile equilibrium (Halpern et al.,

2008). Ecosystems with richer species diversity are predicted to be more resilient to perturbations showing lower rates of collapse (Worm et al., 2006). Biodiversity indicators, such as species richness and community structure (Gray and Elliott, 2009), are commonly used to assess ecosystem health and to study trophic levels of the marine ecosystems (Birk et al., 2012). Therefore, assessing biodiversity at regional and local scale, such as coastal and estuarine areas is essential for understanding how ecosystems respond to perturbations (Hooper et al., 2005; Geist, 2011).

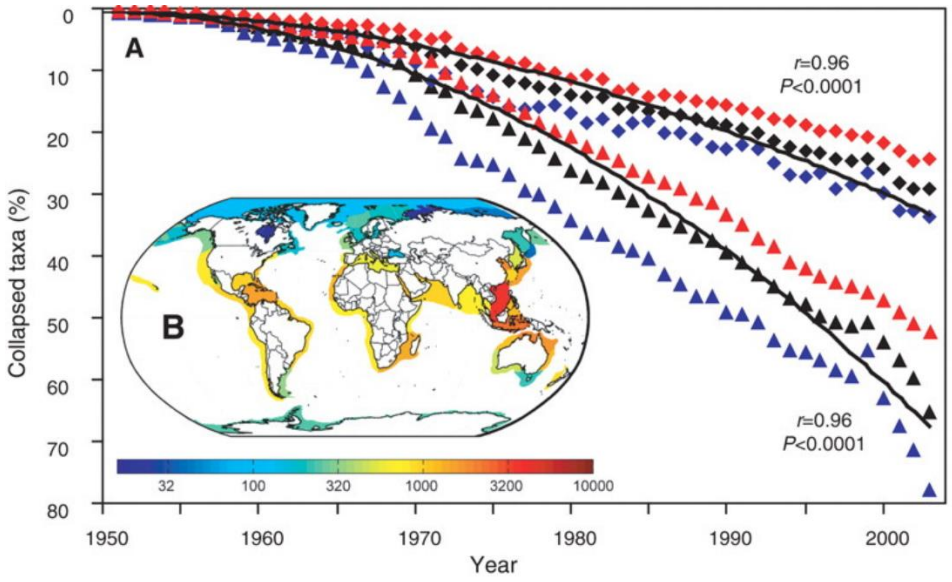

Fig. 1.1: Global loss of fish and invertebrate species from LMEs. (A) Fall down fish and invertebrate taxa over the past 50 years (diamonds, by year; triangles, cumulative collapses). Black symbols represent all species, blue symbols represent LMEs with <500 species and red symbols represent LMEs with > 500 species. (B) Map of all 64 LMEs, colour-coded according to their total fish species richness. Source Worm et al. (2006).

The Pontocaspian biogeographical region (Fig. 1.2, see details in section 1.3), which includes the Black, Caspian, Aral, Azov, and Marmara seas as well as the S-SW Anatolian lake basins, was part of the intercontinental Paratethys Ocean (Popov et al., 2006). The geological history of these basins since the late Miocene included multiple isolation and reconnection events between each other and with the global ocean, e.g. Mediterranean Sea (Aksu et al., 2002; Yanko-Hombach et al., 2007; Badertscher et al., 2011). As consequences of these geological events, the environmental conditions of these basins have changed substantially over time, with highly variable salinity that ranges from freshwater to nearly totally marine conditions (see sections 1.3.1 and 1.3.2). Although today most of these water bodies are isolated from each other, because of their common history, the basins share environmental (e.g. unique

salinity regimes) and biological (e.g. high number of endemic species) characteristics (Dumont, 2000; Mudie et al., 2002; Bahr et al., 2005; Son, 2007; Marret et al., 2009). Assemblages in the Black and Caspian seas host freshwater, marine and brackish species adapted to the particular environmental fluctuations that these basins have experienced, including but not limited to a wide range of salinity and temperature and sea-level changes (Zaitsev and Mamaev, 1997; Krijgsman et al., 2019; Leroy et al., 2020). Autochthonous communities in the Pontocaspian region are adapted to these environmental conditions and alterations of these conditions can produce a cascade effect in the ecosystem, e.g. the introduction of the comb jelly *Mnemiopsis leideyi* collapsed the pelagic system of the Black Sea in the 1990s producing the decline of the main fisheries (Dumont, 1994; Kideys, 2002; Karpinsky, 2005).

Fig. 1.2: Map of the Pontocaspian region, showing the inland seas in blue, the major rivers in light blue (text in italics), and 11 neighbouring countries (bold letters). R: Lake Razim; OB: Odessa Bay; NC: North Caspian Sea sub-basin; MC: Middle Caspian Sea sub-basin; SC: South Caspian Sea sub-basin. Modified after Mudie et al. (2017). * Kara-Bogaz Gol.

Both climate change and human activities are deteriorating ecosystems worldwide (Danovaro et al., 2008; Capinha, et al., 2013; Kernan, 2015), altering the physico-chemical balance in aquatic systems which is probably causing turnover in autochthonous communities, changes in dominance and distribution of key species and substantial loss of biodiversity (Moncheva and Kamburska, 2002; Roohi et al., 2010; Lattuada et al., 2019). The Pontocaspian region offers excellent natural conditions to investigate these events because: 1) the region is characterized by steep environmental gradients, i.e.

salinity range, at medium spatial scale due to the strong influence of the two main basins, the Black and Caspian seas, catchment areas and its geological and climate history (see section 1.3); 2) the Pontocaspian biota is adapted to this unique range of environmental conditions that delimited their distribution and abundance (see section 1.3); 3) if Climate change causes the conditions to change, then we can map that through distribution and abundance shifts in the biotic assemblages. In less unusual settings, this is not possible because the species are more likely to go extinct, or the change in their distribution is less clearly related to specific climate-driven environmental factors. This means that in the Pontocaspian region, once the relationships between climate, environmental conditions and the Pontocaspian biota are established it is possible both to interrogate the fossil record for the history of past climatic influence on biodiversity change, and predict future changes to the distribution and abundance of these assemblages. In addition, this region contains some coastal areas heavily affected by human activities, such as the impact of shipping, eutrophication or industrial pollutants (see section 1.3.1 and 1.3.2).

To understand changes in biodiversity over time, it is therefore essential to investigate the diversity of groups that can be both monitored in the present and tracked in the fossil record. Dinoflagellates (see more details in section 1.2.1 and 1.2.2) are an excellent example of a group that has a ubiquitous and informative fossil record which makes them an ideal proxy for long-term studies and for investigating changes in the biological community due to environmental and anthropogenic perturbations (see sections 1.2.2 and 1.4.1). This project focuses on studying the present-day dinoflagellate diversity and the natural and anthropogenic forces that may drive changes to it using different approaches. This, together with the studies on the dinoflagellate fossil record, will help us understand future changes to the diversity and distribution of these taxa.

Microbial eukaryotes, or protists such as dinoflagellates, are important components of all aquatic ecosystems, i.e. marine, brackish and freshwater, playing diverse and important roles for ecosystem functioning including acting as primary producers, predators, decomposers and parasites; and linking trophic levels in aquatic food webs (DeLong, 2009; Caron et al., 2012; de Vargas et al., 2015). Taxa dominance and distribution are strong indicators of the state of ecosystem health and how anthropogenic

pressures are affecting them (Pospelova et al., 2002; Winter et al., 2011; Garmendia et al., 2013; Wasmund et al., 2017). The biogeography and diversity of phytoplankton taxa such as dinoflagellates reflect adaptive capacity, tolerance and resilience of the taxa within ecological niches defined by environmental conditions (Smayda, 2002; Smayda and Reynolds, 2003; Jeong et al., 2010a). Traditionally biogeography has been studied in depth in macroorganisms. However, the rapid development of molecular tools in the last decades has allowed an improved knowledge of microbial diversity and distribution. Biogeography of microorganisms was thought to be less affected by geographic variation than macroorganisms distribution (Fenchel and Finlay, 2004). The idea was that microorganisms present ecological and biological traits such as high dispersion mechanisms, short generation time and large population size that define the ability to reach new habitats and establish new populations, smoothed out any diversity gradient (Dolan, 2005; Izaguirre et al., 2015). However, recent studies of aquatic microeukaryotic communities have shown that spatial patterns in microorganism distribution and diversity do exist (Stomp et al., 2011). These are driven by physico-chemical as well as anthropogenic factors (Nogales et al., 2010; Bazin et al, 2014; Rasconi et al., 2015; Vajravelu et al., 2018). In addition, aquatic microeukaryotic communities can be influenced by spatial factors at local and intermediate scales due to site-specific variations related to coastal hydrodynamics (Chen et al., 2017; Sun, 2020). The Pontocaspian region presents environmental gradients at local and intermediate spatial scales (see section 1.3) which make this region ideal to investigate spatial, environmental and anthropogenic factors influencing local microbial communities. This would help to constrain how key taxa respond to changing environmental conditions, e.g. rapid changes in salinity, as well as to anthropogenic perturbations such as eutrophication.

Biodiversity and biogeography of protists such as dinoflagellates have been studied in the Pontocaspian region mainly using traditional taxonomy i.e. microscopy observations, with a few exceptions in recent years (see section 1.4). However, many studies have shown that the use of Next Generation Sequencing (NGS) and DNA barcoding techniques can reveal additional diversity of protist groups not visible through microscopic analysis alone (de Vargas et al., 2015). The ecology of dinoflagellates, a group with up to 20% of the species showing more than one life stage, requires the study of different stages

of their life cycle in order to comprehend the diversity of the group and its distribution (Bravo and Figueroa, 2014). This approach has only been used in a few studies in the Pontocaspian region to date (Dzhembekova et al., 2017; 2018).

The overarching aims of this thesis are to:

1. add substantially to the knowledge of dinoflagellate species in the Pontocaspian region by using both traditional taxonomy and NGS together with DNA barcoding;
2. to culture-specific dinoflagellate species under different environmental conditions to constrain their ecological baseline for adaptation and potential growth performance;
3. to identify the main environmental factors influencing dinoflagellate community structure and their distribution patterns and to test dinoflagellates as indicators of anthropogenic perturbations and environmental conditions.

1.1.1 PRIDE

This PhD was funded by the PRIDE (drivers of Pontocaspian RIse and DEmise) Project, a European Union Marie Curie Initial Training Network (ITN). The PRIDE programme is a multidisciplinary project that combines geological and fossil data, climate-lake modelling with molecular and ecological analyses, to identify environmental drivers of past biodiversity change in order to identify and discriminate between their potential causes, e.g. anthropogenic drivers such as microplastic and heavy metal pollution, the introduction of invasive species and sea-based activities like maritime traffic and dredging. The project aimed to evaluate both the Pontocaspian biodiversity and the likely impacts on it of future climatic and anthropogenic activities through the investigation of the causes of natural biodiversity fluctuations both today and reflected in the recent fossil record. These results are incorporated into the development of conservation strategies that support the present Pontocaspian biodiversity.

The PRIDE network comprises fifteen early-stage researchers based in four European countries, all of whom carry out PhD projects in different natural science disciplines including sedimentology, micropalaeontology, geochemistry, climate modelling and molecular biology. In addition, PRIDE has

the support and contribution of local project partners around the Black and the Caspian seas, who bring their expertise and facilitate the work in the region. PRIDE uses a holistic approach to understanding biodiversity and its drivers in the Pontocaspian region with different projects sharing and combining results.

1.2 Dinoflagellates

Dinoflagellates (division Pyrrhophyta, class Dinophyceae) are one of the main groups of phytoplankton in marine and freshwater ecosystems, with more than 2000 species and more than 200 genera (Gómez, 2012). Dinoflagellates as a group of alveolates, together with apicomplexans and ciliates, present on their cellular cortex flattened vesicles known as alveoli. Dinoflagellates are a phylum of unicellular eukaryotes (Fig. 1.3), mostly 10-100 μm in size. They are characterized by two flagella; a belt-like transverse flagellum surrounding the body and a second flagellum longitudinal from the cell (Fig. 1.4). The free-living cells can be found covered either by a unique cell covering (thecate) or without cell covering, athecate (Fig. 1.4; Lin, 2011; Gómez, 2012). Photosynthetic species present, among other pigments, unique xanthophylls, e.g. peridinin, dinoxanthin, and diadinoxanthin that give typical golden-brown dinoflagellate colour (Hackett et al., 2004). Dinoflagellates have a nucleus organization that differs from any other Eukaryotes showing permanently condensed chromosomes attached to the nuclear membrane and having nucleoproteins of viral origin instead of typical eukaryotic histones (Gornik et al., 2012).

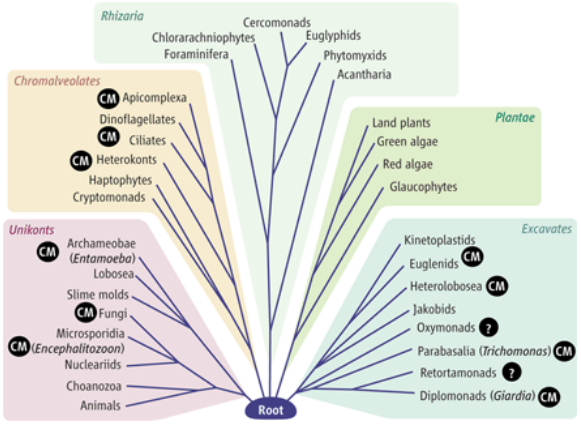

Fig. 1.3: Eukaryotic evolution tree. Dinoflagellates are located in the Chromalveolates clade. CM and question mark symbols are from the original figure but not discussed in this work. From Keeling (2007).

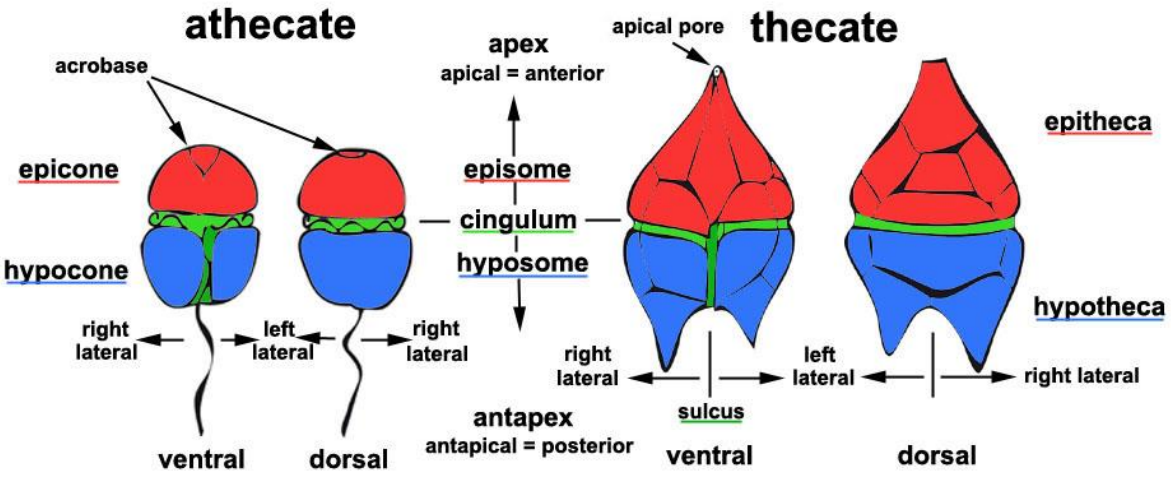

Fig. 1.4: Diagram of athecate and thecate dinoflagellate cells indicating morphological terminology commonly used for these taxa. © Mona Hoppenrath. Source: Tree of life (www.tolweb.org).

1.2.1 Ecological importance

Dinoflagellates are adapted to an extensive range of habitats, which is reflected by an enormous diversity in living forms (free-living cells, parasites and symbionts) and nutrition strategies (photosynthetic, heterotrophs and many species have been proved mixotrophic). They act as both primary producers and consumers of the trophic chain, depending on the environmental conditions such as prey availability and mixotrophy is widespread among dinoflagellates (Stoecker, 1999). Most dinoflagellates species are motile cells that can gain dominance under unfavourable conditions for many non-motile phytoplankton species, such as diatoms, a success partially as a reason of their behaviour patterns such as diurnal vertical migration through the water column on a 24-h cycle (Hackett et al., 2004). Free-living dinoflagellates are an essential component of the planktonic food web, contributing up to 50% of the phytoplankton biomass in some cases, e.g. North Sea (Leterme et al., 2006), serving as food for other heterotrophic protists and zooplankton species including the larvae of commercial fish and shellfish species (Leterme et al., 2006). Moreover, dinoflagellates play an important part in nutrient recycling, as decomposers, as more than half the described dinoflagellate species are heterotrophic or mixotrophic organisms. These species are major players in controlling the population of primary

producers with a key role as grazers and can achieve higher grazing coefficients than those attributed to species of copepods, such as *Acartia* spp. (Jeong et al., 2010a).

Both beneficial and parasitic associations between dinoflagellates and invertebrates have also been found. Symbiotic species of dinoflagellates can be found in a number of marine invertebrates such as members of the class Anthozoa, which includes anemones and cnidarian species (Rodriguez-Lanetty et al., 2003; Cunning and Baker, 2014). Parasites make up only a small fraction of the total known dinoflagellate species compared with free-living cells with around 100 described species. However, the parasite community can rapidly increase and trigger a cascade effect that may decrease the population of its host resulting in changes in the ecosystem functioning (Chambouvet et al., 2008). As a result, these species can be critical controls on ecosystem functioning and some dinoflagellate species are identified as key players in the decline and termination of phytoplankton bloom dynamics (Choi et al., 2017).

In addition, dinoflagellates are known for being the main group populating Harmful Algal Blooms (HABs) events. These events are commonly formed by species of dinoflagellate that can produce secondary metabolites such as toxins, which can adversely affect ecosystems, services and human health (Hallegraeff, 1993; Anderson et al., 2012). Toxin-producing species may develop blooms that can affect the ecosystems, including fish or even mammal mortality events and shellfish poisoning that threaten human health and produce large economic losses. In the U.S.A., it is estimated that HABs resulted in an average annual loss of US$ 75 million over the period 1987 to 2000 (Anderson, 2009). These events have also been reported as well from the Pontocaspian region, e.g. a red-tide event during 2011 of the dinoflagellate *Noctiluca scintillans* from the southeast coast of the Black Sea (Kopuz et al., 2014) and HAB events of *Nodularia spumigena* offshore Iran during 2006 and 2010 (Ramezanpour, 2014).

For all these reasons, therefore, efforts to understand the diversity and distribution of dinoflagellates species are essential for appropriate ecosystem management. This work brings together different approaches that provide a deeper understanding of the dinoflagellate community across the Pontocaspian region and data that support monitoring and management efforts.

1.2.2 Life cycle and evolution of the group

Dinoflagellates have a complex life cycle (Fig. 1.5) involving several different forms and occurring in different ecological niches. Besides the asexual reproduction by binary division of the vegetative cells (planktonic phase), some species of dinoflagellates, under unfavourable environmental conditions, have shown sexual reproduction by gamete conjugation, resulting in a resting non-motile cyst (dinocyst). There are approximately over 2000 species of marine dinoflagellate described of which more than 10% (this number may increase up to 28% in temperate areas) of these species are known to have cysts as part of their life stages (Persson et al., 2000; Bravo and Figueroa, 2014). Dinocysts are known to last in the sediment for months and some studies have shown that some species may survive for decades (McQuoid et al.,2002) until environmental conditions (such as light, temperature, salinity, oxygen and nutrients) are favourable again (Matsuoka and Fukuyo, 2000). These resting stages (dinocysts) play a key role in the ecology of dinoflagellates, allowing these species to survive unfavourable conditions for vegetative growth and, re-inoculate the water column from the sediment when the environmental conditions are favourable again. Moreover, Klais et al. (2011) suggested that the massive production of cysts after dinoflagellate blooms fuels their dominance in the phytoplankton community in the following year. This massive production of cysts allows dispersal by currents. It also enables them to gain dominance over fast growing organisms such as diatoms due to the highly concentrated inoculum into the water column at the beginning of the blooming season. Other ecological functions related to cyst production reviewed by Matsuoka and Fukuyo (2000) are the recombination of chromosomes, which increases the genetic variability of the population, and the termination of bloom events because of a decrease of cell number through sexual fusion of two gametes to form a zygote.

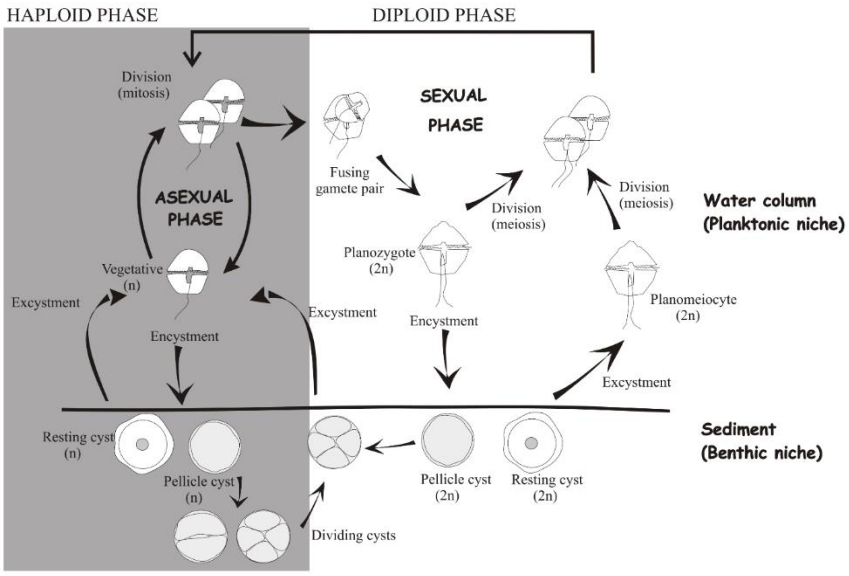

Fig. 1.5: Life cycle of dinoflagellates, including all possible transitions (Bravo and Figueroa, 2014).

Fossil evidence of dinoflagellate cysts was found first in early Triassic sediments (245–208 Ma). A dramatic increase in both numbers and diversity of dinoflagellates occurred in the Jurassic (208–144 Ma) and Cretaceous (144–66 Ma) periods, although the number of species is declining today (Fensome et al., 1999 and reviewed by Hackett et al., 2004 - Fig. 1.6). Dinocysts have been widely used for palaeoenvironmental reconstruction through the recognition of the environmental affinities of the cyst-producing dinoflagellates, which are then fossilised in the sediments. However, there are a number of limitations to the use of dinocysts for reconstructing past environmental conditions:

- Not all cysts are demonstrably linked to a specific planktonic species. A good example where the cyst is known, but no planktonic form has so far been identified is *Spiniferites cruciformis* (Kouli et al., 2001).
- Cysts can sometimes be transported long distances by currents before their deposition on the seafloor. They, therefore, in certain cases, may not represent the sea conditions *in situ* but the environmental conditions where they were formed (Dale, 2001a);
- Only a few species with motile affinity can be found through the fossil record at geological timescales (Ma), e.g. *Lingulodinium machaerophorum,* and in some cases, these species have

a cosmopolitan distribution reflecting their wide ecological range which means they cannot be used to detect small changes on the water conditions (Dale, 2001a).

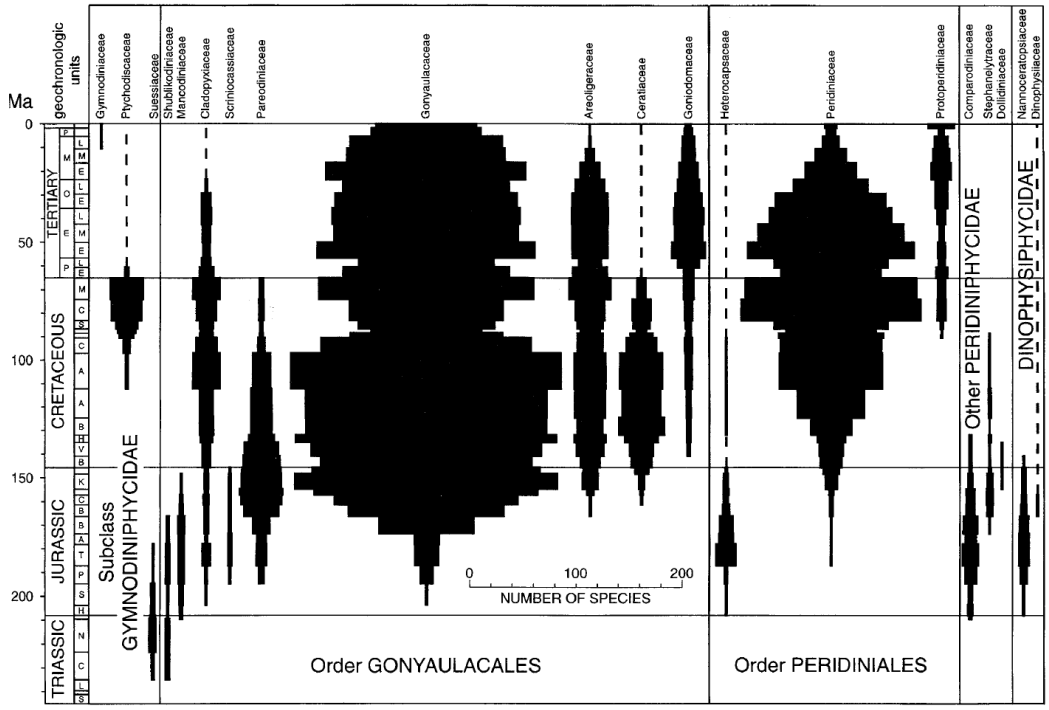

Fig. 1.6: Spindle plot showing the number of species per family in a time interval. Source: Fensome et al. (1999).

Nevertheless, fossil shifts in dinocyst diversity and taxa dominance have been suggested to be accurate proxies of anthropogenic eutrophication (Dale, 2001b; Pospelova et al., 2005; Price et al., 2017) and industrial pollution (Dale, 2001b; Liu et al., 2012; Lu et al., 2017). In addition, dinoflagellates are one of the main proxies extensively used in the Pontocaspian region for this purpose because their preservation is better than many other taxa, e.g. carbonate fossils (see section 1.4.1).

1.3 The Pontocaspian region

The Pontocaspian biogeographical region (Fig. 1.2) was part of the intercontinental Paratethys Basin until the Alpine orogenesis during the Neogene divided the basin into separate sub-basins (Müller et al., 1999; Popov et al., 2006). Since then, the Pontocaspian basin has experienced multiple geological and climatic events that have produced sudden changes in lake connectivity and environmental conditions, particularly in salinity (Aksu et al., 1995; Mudie et al., 2002; Yaltirak et al., 2002; Bahr et al. 2005; Yanko-Hombach et al., 2007). Figure 1.7 illustrates several different configurations of the

Pontocaspian region's geological history, which are the result of tectonic and/or climatic changes. For example, during the last several million years various lake basins have been at times isolated from each other and the global ocean and others connected to both the Mediterranean and Arctic seas (Aksu et al., 2002; Chepalyga, 2007; Yanko-Hombach et al., 2007; Badertscher et al., 2011; Piper and Calvert, 2011). Climate change can impact ice cover, fluvial input and evaporation rate all of which can influence the geometry and connectivity between sub-basins. A good example of this occurred at ~ 10 ka during the Last Glacial when the north Caspian sub-basin was covered by ice and the Caspian Sea was substantially smaller than today as a result of a sea-level fall between - 80 to -113 m (Krijgsman et al., 2019). Drying and desiccation like that seen impacting the Aral Sea today are also seen in several Pontocaspian sub-basins on various occasions (Gillet et al., 2007). The geological history of the main Pontocaspian basins exhibits fluctuations in the sea level of the Black and the Caspian seas over very short periods, e.g. The Caspian Sea level decreased about 1.7 m from the late 1920s to the late 1970s followed by a rise of almost 2.5 m in the next 20 years (Baldina et al., 1999; Leroy et al., 2020). These rapid changes are associated with global and regional climate variations leading to rapid variations in environmental parameters, such as temperature and salinity, of the Black and Caspian seas (Konikov, 2007; Leroy et al., 2020).

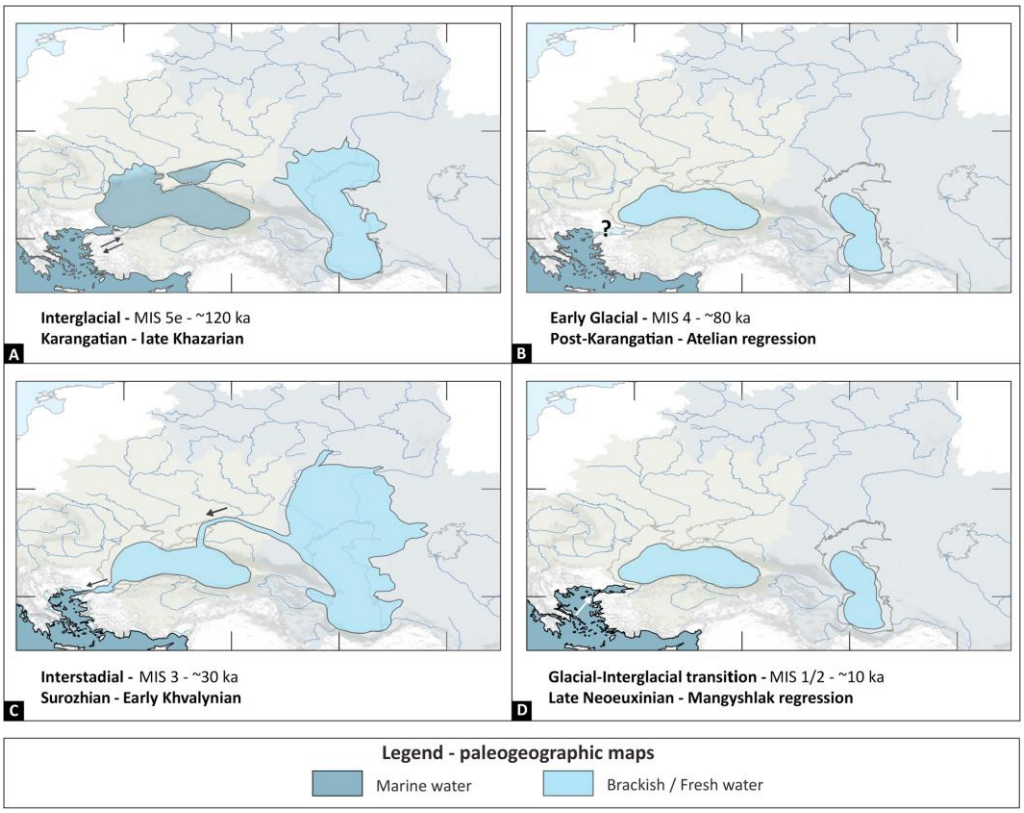

Fig. 1.7: Maps representing the main basins of the Pontocaspian region at different times during the late Pleistocene. Arrows indicate the water flow direction. Krijgsman et al. (2019).

This highly dynamic environment has been the stage for the evolution of a unique fauna and flora assemblage. The Pontocaspian region hosts many endemic species and these are well adapted to the unique salinity regimes of the lakes and seas across the region. The Pontocaspian endemic biota has its origins in species from different locations including the ancient Paratethys Sea, high latitude seas, the Mediterranean Sea and species from freshwater origin (Zaitsev and Mamaev, 1997). This unique Pontocaspian biota includes fish with many species of goby (Gobiidae; (Matthew et al., 2009) the Kilka (*Clupeonella* spp.; Fazli et al., 2007) and sturgeon (Acipenseridae; Dudu et al., 2014); bivalves such as dreissenids (Dreissenidae), *Adacna* and *Monodacna*; snails such as Hydrobiids and species of *Theodoxus spp;* (Wesselingh et al., 2019) and planktonic organisms such as dinoflagellates (*Caspidinium rugosum* and *Impagidinium caspienense;* Marret et al., 2004). Many of these unique taxa have limited distribution or occur in isolated patches across the Pontocaspian region. A good example are the endemic species of gobies (e.g. *Mezogobius batrachocephalus* and *Neogobius melanostomus*), which only occur in low salinity waters and are therefore found limited to estuarine areas near the main

rivers (Zaitsev et al., 2001; Keskin, 2010). Other taxa that in the past have shown a wide distribution across the Pontocaspian region have become isolated in one of the basins. One example is the dinoflagellate *C. rugosum*, which currently only occurs in the Caspian Sea today (Leroy in Mudie et al, 2017), but whose cysts exist as fossils in 8-10 ka in the Black Sea sediments, a period when the Black Sea had similar sea surface conditions to the Caspian Sea today (Shumilovskikh et al., 2013). Some endemic Pontocaspian species have become successful invasive species in other regions of the world, e.g. zebra mussel (*Dreissena polymorpha*; Carlton, 2008).

Over the last century, however, the Pontocaspian biota has been threatened. Coastal ecosystems, lagoons and estuaries have been polluted, silted up or converted into land for human use and artificial water-ways have been opened reducing the suitable habitat for endemic populations which have been displaced by invasive species (Dumont, 1995; Cociasu et al., 1996; Vadineanu et al., 1997; Bakan and Buyukgungor, 2000; Lattuada et al., 2019). Pontocaspian basins have suffered from eutrophication (Zaitzev, 1992; Bakan and Buyukgungor, 2000; Moncheva et al., 2001) that has increased phytoplankton biomass and the frequency and intensity of algal blooms (see section 1.3.1); and in both the Black and Caspian seas, the introduction of invasive species has caused the collapse of the endemic biota. For example, the accidental introduction of *Mytilaster lineatus* into the Caspian Sea produced a displacement of the autochthonous mollusc community traditionally composed of *Dreissena, Didacna* and *Monodacna* species from the coastal benthic habitats to deeper areas where the invader is not present (Zaitzev, 1992; Dumont, 1995; Zarbaliyeva et al., 2016; Wesselingh in Leroy et al., 2018).

1.3.1 The Black Sea

The Black Sea is a eutrophic semi-enclosed sea that covers 1175 km E-W (27°27’ - 41°42’E) and approximately 800 km N-S (46°33’ - 40°56’ N). It has a total area of ≈ 436,400 km^2 and occupies a total volume of 547,000 km^3. The Black Sea is open in the south to the Marmara Sea through the Bosphorus Strait, which is on average 1.6 km in width, 36 m depth and has a total length of 31 km. In the north, it connects through the Strait of Kerch to the Sea of Azov (Fig. 1.2). The northern Black Sea receives freshwater discharges from three of the major European rivers: Danube, Don and Dnieper. The hydrographic regime is characterized by a strong density stratification where low-salinity surface water

of fluvial origin sourced from the major rivers situated in the North-west of the Black Sea overlies high salinity deep-water (35-37 psu) of Mediterranean origin which flows through the Bosphorus Strait. Consequently, the Black Sea presents a permanent halocline located between 100 and 200 m below the surface preventing vertical mixing of water below this point. This favours the development of permanent anoxic conditions below 200 m depth (Oguz et al., 2000; Stewart et al., 2007, Fig. 1.8). The Black Sea has a mean depth of about 1,300 m and a maximum depth of 2,258 m (Yanko-Hombach et al., 2007). Its bathymetry consists of two well-differentiated areas: a deep anoxic part and a wide continental shelf in the northwest of the basin. Mean surface temperature shifts annually from 16 °C in the southernmost part to 11 °C in the northwestern of the Black Sea; while mean surface salinity is ~18.0 psu during winter and are typically 1.0 ± 1.5 psu higher during summer. Salinity values of about 14-16 psu are frequently observed in the northern part of the Black Sea due to river discharge and runoff (Yanko-Hombach et al., 2007).

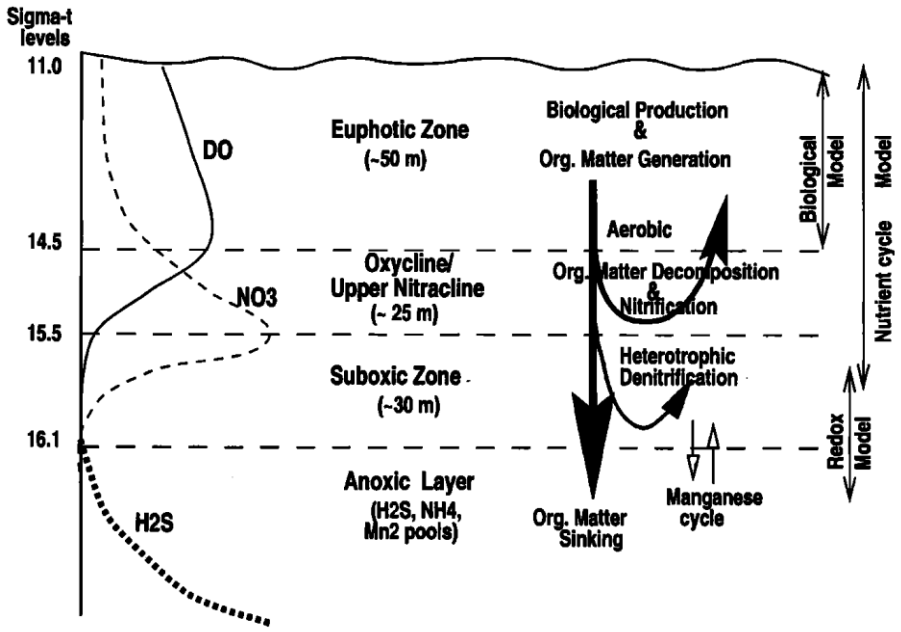

Fig. 1.8: Schematic diagram of the Black Sea water column. Source: Oguz et al. (2000).

Over the last few centuries, the anthropogenic pressure on the Black Sea's coastal biological communities has increased, with examples of eutrophication (Moncheva et al., 2001; Yunev et al., 2007; Mirzajani et al., 2010), industrial pollution (Simeonov et al., 2000; De Mora et al., 2004), invasive species (Kideys, 2000; Roohi et al., 2008), and oil pollution (Bakan and Buyukgungor, 2000; Tolosa et

al., 2004). Among the most serious outcomes of human activities is the increasing eutrophication of the Black Sea basin due to nutrient discharge from rivers, mostly the Danube, as a result of the increasing agriculture and development of coastal cities (Alexandrov and Zaitsev, 1998; Bakan and Buyukgungor, 2000). Eutrophication has been identified as the cause for many changes in the Black Sea ecosystem producing a significant decline of biodiversity, e.g. number of some common planktonic organisms declined by two orders of magnitude since the 1960s (Zaitsev, 2001). Although eutrophication is a common phenomenon in coastal and semi-enclosed marine areas worldwide, the Black Sea is one of the areas most badly impacted seas by this pressure (Zaitsev, 2001). The most heavily eutrophicated area of the Black Sea is in its north-western region because its large rivers flowing through vast agricultural and industrial areas as well as large settlements (Oguz and Velikova, 2010). Continuous nutrient discharges have caused a substantial increase in phytoplankton mass in the north-west Black Sea (NWBS) e.g. average phytoplankton biomass (mg/m^3 of seawater) increased from 670 in the 1960s to 30,000 in the 1980s. Eutrophication impacts have also been observed in species composition as well. For instance, the abundance of the dinoflagellate *Prorocentrum cordatum* has increased 45-fold in the water column. Consequently, the number of phytoplankton bloom events has increased and the area of the Black Sea affected by these events has been widened during the last few decades (Zaitsev, 2001). For example, only eight blooms with densities higher than 5×106 cells L^{-1} were recorded between 1960 and 1970 with the number of species limited to five, while in contrast, 45 blooms with cell concentrations $>5 \times 10^6 L^{-1}$ during 1980 and 1990, 2-8 blooms per year, of 15 different species, (Cociasu et al., 1996). Phytoplankton blooms produce a cascade effect in the ecosystem, reducing the oxygen in deeper layers and producing anoxia at the seafloor due to increased organic carbon exported from photic layers, discolouring the water and as a result limiting light penetration (Daskalov, 2002; Kemp et al., 2005). These phytoplankton blooms also affect commercial and recreational activities in the sea (see section 1.2.1).

1.3.2 The Caspian Sea

The Caspian Sea is the largest enclosed basin in the world and occupies a vast depression in the earth's crust located inside the Eurasian continent. The sea level today is below the level of the World Ocean

by - 28 m (Cretaux in Leroy et al., 2020). However, the Caspian level fluctuates substantially and rapidly. Over the last century, it dropped 1.7 m during the 1930s and rose 2.5 m between 1978 to 1995 (Arpe and Leroy, 2007). The area the Caspian Sea covers today is more than 390,000 km^2 with a N-S dimension of ~1030 km and 200 to 400 km E-W. Its water volume reaches 78,000 km^3 at a mean depth of 208 m and a maximum depth of 1,025 m in the south. Today, around 80-90% of the total runoff of the Caspian Sea basin is discharged through the Volga River (Cretaux in Leroy et al., 2020). The Caspian Sea is divided into three different sub-basins: a northern, shallow sub-basin (average and maximum depth 6 and 20 m respectively), a middle sub-basin (average 190 m and maximum 788 m) and a southern sub-basin (up to 1,025 m; Kosarev, 2005; Fendereski et al., 2014). The North sub-basin of the Caspian Sea has an estuarine-like environmental setting with salinity changes from 0 psu near the Volga delta to 10 psu at its southern limit. Its annual surface temperature varies annually from freezing temperatures to 26 °C in summer. The middle and southern sub-basins experience an oscillation in surface water salinity ~11-12 psu and ~12-13 psu respectively, while temperature varies from 3-25 °C and 7-30 °C respectively (Zonn et al., 2010, Fig. 1.2). Analyses included in this work were performed with data obtained from the three sub-basins covering the whole Caspian Sea environmental range.

In parallel with the Black Sea, disturbances from human activities have increasingly impacted the Caspian Sea over the last two centuries (Dumont, 1995). However, The Caspian Sea is landlocked which, by comparison with the open ocean, limits its capacity to absorb pollution. As a result, oil spills, for example, can remain localized, affecting marine biota for longer periods than those dispersed and diluted by rough seas (Jafari, 2010). Heavy metal and organic compounds pollution derived from oil industries as well as maritime transport reached a maximum concentration of total petroleum hydrocarbons (TPH) and heavy metals maximum concentration in the middle 1980s, with the highest values found in North Caspian sediments (Kostianoy et al., 2010). The main stresses disturbing the Caspian aquatic biota come from two different sources: 1) the commercial use of the sea such as for oil and gas extraction, fishing and maritime transport; and 2) human activities carried out on the coast as well as in the catchment area that accumulates in the Caspian Sea through river and runoff discharge

(Aladin & Plotnikov, 2004). Among the impacts produced by human activities, the Caspian Sea ecosystem has been heavily threatened by the introduction of invasive species. For example, the invasion by the ctenophore *Mnemiopsis leidyi* in 1996 has produced a cascade effect on the planktonic trophic web in the Caspian Sea, where the resulting decline of zooplankton has produced an augment in phytoplankton biomass in the south Caspian Sea, characterized by low phytoplankton biomass due to the low nutrient input from land i.e. Chl-*a* levels has shown this trend for the South Caspian Sea (Roohi et al., 2010; Nasrollahzadeh et al., 2014).

1.3.3 Differences between the current Black and Caspian seas

The Black and Caspian Seas share a common geological and climatic history (see section 1.3). However, today the two basins show different environmental settings and are therefore affected differently by human pressures. The clearest contrast between the two basins is that the Black Sea is still connected to the global ocean and has been since the early Holocene, while the Caspian Sea has been isolated from the Black Sea since ~2.6 Ma with sporadic reconnections during highstands and floodways and the last time that was connected to the Arctic ocean was during the late Pliocene (Fig. 1.7, Badertscher et al., 2011; Forte and Cowgill, 2013; Naderi Beni et al., 2013; Richards et al., 2018). This explains the two different salinity regimes that can be observed currently in both seas (see more detailed section 1.3.1 and 1.3.2). Despite these environmental dissimilarities, Pontocaspian taxa can be found in both basins and some species can dominate assemblages in both seas despite their different environmental settings. This project seeks to explore how the wide range of environmental parameters that can be found currently across the Black and the Caspian seas drive the distribution and biodiversity of dinoflagellate species in the region. In addition, it investigates how different anthropogenic factors may affect this group.

1.4 Dinoflagellates in the Pontocaspian region – Previous studies

Dinoflagellates have been the focus of study in the two main basins of the Pontocaspian region. Previous studies have used both dinoflagellate cysts (e.g. Mudie et al., 2001; 2011; Marret et al., 2004; Atanassova, 2005; Mertens et al., 2012; Leroy et al., 2013; Balkis-Ozdelice, 2016) and dinoflagellate

motile forms (e.g. Kideys et al., 2001; Gómez and Boicenco, 2004; Terenko, 2005; Bagheri et al., 2012; Krakhmalny et al., 2012; Agirbas et al., 2017; Yasakova et al., 2020) to assess the distribution and diversity of the group across this region. Although these records are extensive in the Pontocaspian region, there are unbalanced sampling efforts between different areas. For instance, the northwestern part of the Black Sea has been mainly studied using dinocysts as well as by plankton surveys, but not many data are available for the northeastern Caspian Sea. In addition, the majority of these records are based on traditional morphological features observed under the microscope, which require a high level of expertise and time to be accurate. Even in this scenario, some cryptic and morphologically different types of the same species are documented (Montresor et al., 2003).

1.4.1 Dinoflagellate cysts

Dinoflagellate cysts (dinocysts) are abundantly preserved in sediments across all the basins of the Pontocaspian region. The environmental characteristics of these basins, brackish water regime and low oxygen bottom layers (see section 1.3.1), favour the accumulation and preservation of dinocysts in the sediments (Marret et al., 2017). As a result, dinocysts are one of the main proxies used to understand changes in the surface water conditions using variation in dinoflagellate diversity and distribution. In the last decades, numerous studies have used dinoflagellate cysts in the Pontocaspian region for understanding either past environmental conditions, using fossil dinocyst records (Wall et al., 1973; Aksu et al., 1995; Mudie et al., 2001, 2002 and 2004; Leroy et al., 2007, 2010, 2011, 2013 and 2014; Marret et al., 2009) or recent environmental or anthropogenic changes, using modern dinocyst assemblages (Balkis-Ozdelice, 2016; Mudie et al., 2017). Despite the numerous studies addressing the distribution and diversity of dinocysts in both the Black Sea and the Caspian Sea, the availability of data across the Pontocaspian region is fragmented. Figure 1.9 shows the distribution of available data of both modern and fossil dinocysts across the Pontocaspian region (Mudie et al., 2017). This well-documented review shows that there are still areas with very low or poor records available e.g. the northeast Caspian Sea. To understand fully the factors driving dinoflagellate distribution and diversity, a complete record of their distribution is a requirement, especially in areas with strong environmental gradients such as the Black and Caspian seas. Complete records can complement the environmental

conditions associated with dinocyst taxa are commonly used in palaeoenvironmental reconstructions (Zonneveld et al., 2013; see section 1.2.2). In the Pontocaspian region, numerous studies have used dinocysts for studying water conditions. One example demonstrated the use of *Lingulodinium machaerophorum* length processes as an accurate proxy for sea surface salinity (Mertens et al., 2009). As well as being an excellent proxy for water column conditions, dinocysts can also be used as proxies for anthropogenic impact such as eutrophication and metal pollution (see section 1.2.2). However, only a few studies focused on the Marmara Sea have addressed the use of dinocyst to evaluate the anthropogenic disturbances in the Pontocaspian region (Marret et al., 2009; Bradley et al., 2012). Some of these studies discuss the importance of shipping in the past in the introduction of new dinoflagellates taxa into the Black Sea; however, to my knowledge no study has statistically addressed the impact of shipping activities on dinocyst assemblages.

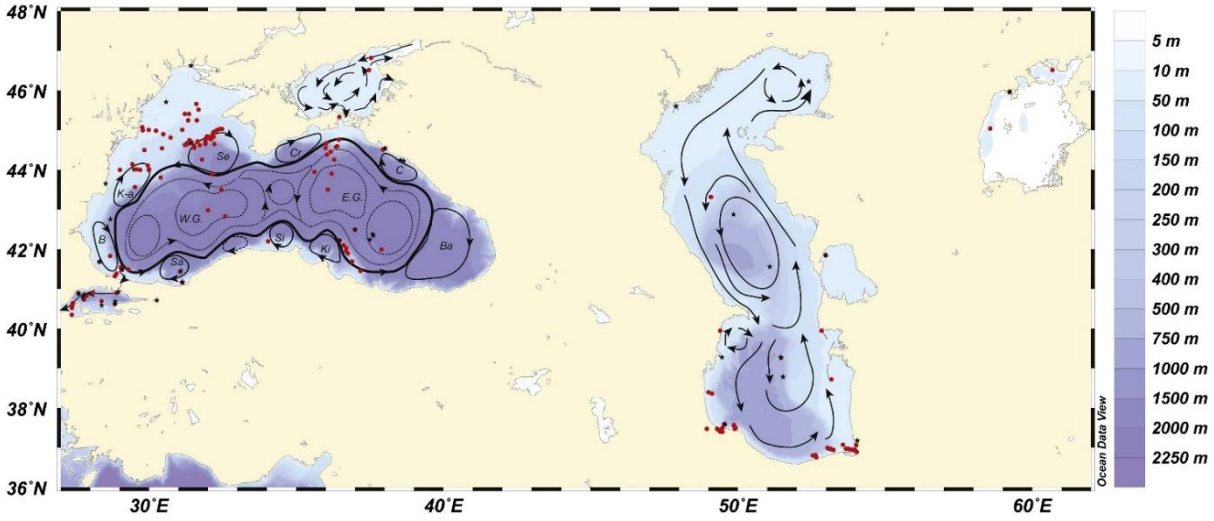

Fig. 1.9: Bathymetric map of the Pontocaspian main basins, the Black Sea, the Caspian Sea and the Aral Sea showing the location of published cores (black dots) and surface sample (red dots). Black lines indicated water circulation (Mudie et al., 2017).

1.4.2 Dinoflagellate motile cells

Phytoplankton is a key component of any aquatic system. As such monitoring is essential for assessing the status of the ecosystem and to understand potential changes due to climatic or anthropogenic disturbances. With more than 200 potential harmful and toxic species, the distribution and diversity of dinoflagellates as a group requires special focus (Gómez and Boicenco, 2004). The vast majority of

diversity assessments on dinoflagellates for this area has been based on traditional taxonomical studies performed at a national or local scale under light microscopy (e.g. Tarenko, 2005; Bagheri and Fallahi, 2014; Agirbas et al., 2017; Yasakova et al., 2020). This sometimes makes data difficult to compare between regions due to different methodologies, besides, cryptogenic and morphologically challenging species can introduce bias in the results. Gómez and Boicenco (2004) reviewed the published diversity studies across the Black Sea showing many uncertain records (Table 1 in Gómez and Boicenco, 2004) regarding the accuracy of the identification of the species. An example of uncertainty is given by the species *Gymnodinium aureolum* and *Gymnodinium mikimotoi* complex, which until the year 2000 were identified as the same species (Hansen et al., 2000; see more details in Introduction in Chapter 4). New molecular methods such as high-throughput sequencing (HTS) tandem with DNA barcoding approaches, has been proven to reduce sampling processing time and increase reliability and resolution of taxon identification for biodiversity studies (de Vargas et al., 2015). These techniques have not been commonly applied across the Pontocaspian with only one report using massively parallel sequencing (MPS) to study potential harmful phytoplankton species in the Bay of Varna (Dzhembekova et al., 2017). These authors identified dinoflagellate species such as *Cochlodinium polykrikoides*, *Karenia bicuneiformis*, and *Karlodinium veneficum*, which had been overlooked in previous phytoplankton surveys, demonstrating the advantages of complementing traditional phytoplankton identification with Next Generation Sequencing techniques.

Although previously there has been much interest in studying dinoflagellates' distribution and diversity in the Black Sea and the Caspian Sea, the available data do not cover homogeneously all the ecologically different areas in these two basins. This is a result of the unbalanced sampling effort, where monitoring programs have been carried out at national level without harmonized methodology, which prevents in some cases comparison and sharing of data between countries. This is hindered further by the socio-political situation across the region, which comprises eleven countries with different languages and unequal access to international journals (Mudie et al., 2017). Therefore, despite numerous studies in the region, there are still areas, especially areas with low salinity that are poorly studied and where no or very limited data are available from the literature. In a region where steep environmental gradients, as

well as different anthropogenic disturbances sources, can be detected in small to medium spatial scales (see section 1.3), it is of key importance for understanding ecosystem functioning to have consistent data across the region. Additionally, the use of new techniques such as DNA barcoding and NGS can complement the already available data for the taxon overcoming traditional taxonomical studies limitations.

1.5 Research goals

This work aims to identify the main environmental factors driving the dinoflagellate community and composition and their distribution patterns. In addition, the use of dinoflagellates as indicators of anthropogenic perturbations is tested. There are two main objectives for this thesis:

1) to assess the biodiversity and biogeographic patterns of dinoflagellate communities in the Black and Caspian seas and

2) to identify the factors that drive these patterns.

1.5.1 Patterns of dinoflagellate diversity and biogeography in the Black and Caspian seas

Many previous studies have addressed the distribution and community structure of dinoflagellates in both the water column and sediments, for this area. However, these studies traditionally have targeted only one of the phases in the dinoflagellate cycle, focusing either on resting cysts or on motile cells. In addition, most of these studies use traditional taxonomical identification methods. This thesis aims to study the distribution of both dinoflagellate phases to complement and draw together previous studies and to generate new datasets, particularly in some of the more poorly studied areas using both traditional taxonomy and next-generation sequencing tandem with DNA barcoding methods to reveal the group's previously invisible diversity. The specific goals that this work will try to address are:

1) to describe the differences in dinocyst assemblages relative to their environmental settings complementing previous studies and enhancing data from areas previously less studied in order to enrich the Pontocaspian dinocyst database

2) to investigate the distribution and diversity patterns of dinoflagellates in different ecological settings across the Black Sea at an intermediate spatial scale using NGS and DNA barcoding methods to study dinoflagellate assemblage changes along environmental gradients.

1.5.2 Factors affecting dinoflagellate diversity and distribution

It is well known that the diversity and distribution of microeukaryotic organisms are controlled by a number of environmental parameters including temperature, salinity, nutrients availability and light as well as anthropogenic factors such as eutrophication (section 1.2). Nevertheless, studies statistically testing which of these parameters affect both diversity and distribution of dinoflagellates in the Pontocaspian region and how this influence is achieved, are lacking. In this thesis, I address this research gap by testing the effect of environmental parameters using new data obtained by next-generation sequencing methods. This work will address the following specific aims:

3) To test dinocyst assemblages as proxies for physico-chemical and shipping variables of the Black and Caspian Seas
4) To explore the relationship between environmental variables and dinoflagellate biogeography
5) To study the contribution of environmental factors and geographical distance in controlling the dinoflagellate community composition
6) To test the effects of temperature and salinity on the growth of newly established dinoflagellate cultures obtaining novel understanding in the adaptation and acclimatization ability of these species to present and future environmental scenarios in the Black Sea and their potential to generate blooms under these conditions.

1.6 Thesis outline

This thesis consists of six chapters. The general background information, thesis motivation and the research aims are presented in this chapter. Chapter 2 provides information regarding sampling and experimental design and sampling locations. Because the methodology used in each chapter is different, detailed descriptions of each methodological procedure covering sample processing, experimental

settings and statistical analyses are presented in each of the relevant results chapters (Chapter 3, 4 and 5).

Chapter 3 explores the differences in dinocyst assemblages relative to their environmental settings using traditional microscope identification techniques to enhance the existing Pontocaspian dinocyst database with a specific focus on some of the less well-studied areas. In addition, this chapter includes statistical analyses designed to test the viability of using dinocysts as proxies for physico-chemical and anthropogenic perturbations such as shipping activities in the Black and Caspian Seas.

Chapter 4 presents the first study of morphological and phylogenetic characterization of the Black Sea *Gymnodinium aureolum*, a red-tide dinoflagellate species, isolated from recent sediments. In addition, this chapter contains the results of a case study exploring the potential of this species to generate blooms under different temperature and salinity conditions.

Chapter 5 explores the distribution and diversity of microeukaryotic communities from coastal Black Sea water column samples using the Ribosomal Internal Transcribed Spacer (ITS, eDNA) gene amplicon sequencing focused on dinoflagellates. Statistical tests were applied to investigate the influence of environmental factors on dinoflagellate diversity, discussing the importance of the environment and geographical distance in affecting dinoflagellate community composition.

Chapter 6 provides a summary of the key findings of this thesis included in Chapters 3, 4 and 5 addressing the research aims and the specific research goals. In addition, this Chapter will outline several remaining questions, which impact our understanding of the dinoflagellate community in the Pontocaspian region.

CHAPTER 2

METHODOLOGY

In this chapter, the sampling strategy and the sampling sites are described. Although some of the samples are used for different analyses, because the methodologies used in each results chapter are different, detailed descriptions of each procedure including sample processing, experimental design and statistical analyses are presented in the relevant results chapters (Chapter 3, 4 and 5). This chapter begins with a description of the sampling strategy for the project as a whole followed by discussing the limitations faced by this project of working in such a region as geopolitically complex as the Pontocaspian. A description of the sampling sites used in this thesis is then presented. The last section of this chapter focuses on the external data that are incorporated into the results chapters.

2.1 Sampling strategy

This PhD was part of a larger EU funded ITN project, the PRIDE programme (section 1.1.1). PRIDE combined different natural science PhDs in order to assess changes in biodiversity over time in the Pontocaspian region. Consequently, sampling campaigns were designed and organized to support widely different scientific endeavours including benthic and plankton ecology, geochemistry and sedimentology. Sites of interest to more than one PhD project were targeted and often samples were shared among different PhD projects. One consequence of this multidisciplinary approach is that no individual project's sampling design was implemented without consideration of the scientific objectives of others and compromises were made by every project in order to support the collaborative science of PRIDE as a whole.

Another restriction on the sampling strategy of all PRIDE's field- and lab-based PhD projects was the political situation. The Pontocaspian region encompasses eleven countries with eleven different languages. International relationships between these countries vary from excellent to armed conflict! This geopolitical situation hinders international cooperation and as a consequence, sampling

expeditions at sea, across national boundaries was just not possible. The result of this is that most of the sampling undertaken during the PRIDE project occurred along short transects close to the coast, within one country's national waters. Sometimes these sampling expeditions were carried out on a scientific vessel. At others, PRIDE PhD students used small rubber boats to collect near-shore samples. As a result of these geopolitical restrictions, opportunities to collect new samples were quite limited and it was not always possible to sample the areas of the Pontocaspian region of most interest or where existing data were largely absent. For example, coastal areas of the Peninsula of Crimea, the coast of Abkhazia (eastern Black Sea), Dagestan (west Caspian Sea) and Turkmenistan (eastern Caspian Sea) were not accessible due to international conflicts and local political situations.

Despite the sampling restrictions, this project does extend the dinoflagellate distribution and diversity data available in the Pontocaspian region. In addition, the areas studied in this project were targeted because previously they had been poorly studied in terms of modern dinocyst assemblages (section 1.4.1, Fig. 1.9). Consequently, the dinocyst data presented in this thesis from these areas contribute to the Pontocaspian dinocyst database filling in some gaps of the fragmented existing dataset. Moreover, the analyses presented here test environmental and anthropogenic controls on the dinoflagellate community. To achieve this, the sampling strategy targeted both stages of the life cycle, resting stage (dinocyst) and motile stage (planktonic cells) along environmental gradients. Table 2.1 summarizes information concerning sampling stations studied in this project including coordinates, date of the sampling, area of the Pontocaspian region sampled, type of samples obtained and type of analyses carried out.

Table 2.1: Information of all the field stations sampled for sediment and water samples used in this thesis.

Station	Latitude	Longitude	Date	Area	Samples Type	Analyses	Chapter
BS87	43.36750	28.81597	23/05/2017	NW Black Sea	Bottom grab	Dinocysts	Chapter 3
BS102	43.36500	29.00333	23/05/2017	NW Black Sea	Bottom grab	Dinocysts	Chapter 3
BS96	43.14306	28.02778	21/05/2017	NW Black Sea	Bottom grab	Dinocysts	Chapter 3
BS98	43.16889	28.16500	21/05/2017	NW Black Sea	Bottom grab	Dinocysts	Chapter 3
BS85	43.16688	28.31803	22/05/2017	NW Black Sea	Bottom grab	Dinocysts	Chapter 3
BS99	43.16667	28.50139	22/05/2017	NW Black Sea	Bottom grab	Dinocysts	Chapter 3
BS100	43.16667	28.66556	22/05/2017	NW Black Sea	Bottom grab	Dinocysts	Chapter 3
BS95	42.51083	27.82000	21/05/2017	NW Black Sea	Bottom grab	Dinocysts	Chapter 3

BS84	42.52540	27.97720	21/05/2017	NW Black Sea	Bottom grab	Dinocysts	Chapter 3
BS97	42.50111	28.16583	20/05/2017	NW Black Sea	Bottom grab	Dinocysts	Chapter 3
BS92	42.50111	28.26389	20/05/2017	NW Black Sea	Bottom grab	Dinocysts	Chapter 3
NoC63	44.54671	50.26207	20/06/2017	NE Caspian Sea	Bottom grab	Dinocysts	Chapter 3
NoC62	44.56421	50.26018	20/06/2017	NE Caspian Sea	Bottom grab	Dinocysts	Chapter 3
NoC38	44.56675	50.25877	16/06/2017	NE Caspian Sea	Bottom grab	Dinocysts	Chapter 3
NoC39	44.62352	50.27237	16/06/2017	NE Caspian Sea	Bottom grab	Dinocysts	Chapter 3
NoC41	44.72741	50.22056	16/06/2017	NE Caspian Sea	Bottom grab	Dinocysts	Chapter 3
NoC42	44.72741	50.22056	20/06/2017	NE Caspian Sea	Bottom grab	Dinocysts	Chapter 3
NoC61	44.61348	50.12604	16/06/2017	NE Caspian Sea	Bottom grab	Dinocysts	Chapter 3
MC51	43.75634	50.75752	18/06/2017	ME Caspian Sea	Bottom grab	Dinocysts	Chapter 3
MC52	43.74141	50.67633	18/06/2017	ME Caspian Sea	Bottom grab	Dinocysts	Chapter 3
MC53	43.72398	50.57025	18/06/2017	ME Caspian Sea	Bottom grab	Dinocysts	Chapter 3
MC54	43.69947	50.46807	18/06/2017	ME Caspian Sea	Bottom grab	Dinocysts	Chapter 3
MC55	43.59659	50.27940	18/06/2017	ME Caspian Sea	Bottom grab	Dinocysts	Chapter 3
Pa1	42.09290	41.70799	04/10/2016	Paliastomi Lagoon	Plankton net	Phytoplankton	Chapter 5
Pa2	42.12253	41.73241	05/10/2016	Paliastomi Lagoon	Plankton net	Phytoplankton	Chapter 5
Pa3	42.13602	41.75308	05/10/2016	Paliastomi Lagoon	Plankton net	Phytoplankton	Chapter 5
BS1	44.13531	28.77950	22/10/2015	NW Black Sea	Niskin bottle	Phytoplankton	Chapter 5
BS2	44.08161	29.03878	22/10/2015	NW Black Sea	Niskin bottle + Bottom grab	Phytoplankton + Cultures	Chapter 5 and 4
BS3	44.54053	28.92303	21/10/2015	NW Black Sea	Plankton net	Phytoplankton	Chapter 5
RS4	44.69424	28.99504	19/10/2015	Razim Lake	Plankton net	Phytoplankton	Chapter 5
RS5	44.57275	28.89589	19/10/2015	Razim Lake	Plankton net	Phytoplankton	Chapter 5
RS6	44.57325	28.89297	19/10/2015	Razim Lake	Plankton net	Phytoplankton	Chapter 5
RS7	44.59665	28.91318	19/10/2015	Razim Lake	Plankton net	Phytoplankton	Chapter 5
RS8	44.53832	28.77836	20/10/2015	Razim Lake	Plankton net	Phytoplankton	Chapter 5
RS9	44.63470	28.88791	20/10/2015	Razim Lake	Plankton net	Phytoplankton	Chapter 5
RS10	44.62530	28.87271	20/10/2015	Razim Lake	Plankton net	Phytoplankton	Chapter 5
Dn1	46.61224	32.05193	17/05/2016	Dnieper Estuary	Plankton net	Phytoplankton	Chapter 5
Dn2	46.69558	31.92818	17/05/2016	Dnieper Estuary	Plankton net	Phytoplankton	Chapter 5
Dn3	46.71717	31.93040	16/05/2016	Dnieper Estuary	Plankton net	Phytoplankton	Chapter 5
Dn4	46.73659	31.92412	16/05/2016	Dnieper Estuary	Plankton net	Phytoplankton	Chapter 5
Dn5	46.48799	32.17974	15/05/2016	Dnieper Estuary	Plankton net	Phytoplankton	Chapter 5
Dn6	46.54662	32.14368	15/05/2016	Dnieper Estuary	Plankton net	Phytoplankton	Chapter 5
Dn7	46.60198	32.10252	15/05/2016	Dnieper Estuary	Plankton net	Phytoplankton	Chapter 5
Dn8	46.58537	32.60165	15/05/2016	Dnieper Estuary	Plankton net	Phytoplankton	Chapter 5

2.1.1 Dinoflagellate cysts

Dinocysts have been widely studied in the Pontocaspian region, however, the available data are fragmented and there are still poorly studied areas, particularly those with low salinity conditions (section 1.4.1, Fig. 1.9). The sampling strategy was designed to target these areas where possible and to obtain samples along salinity gradients. In addition, in order to test the effect of anthropogenic perturbations on dinocyst assemblages caused by shipping activities transects from sites with high-density marine traffic adjacent to important commercial ports in both the Black and the Caspian seas were sampled.

2.1.1.1 Sampling sites

The Western Black Sea (WBS) was selected for dinocyst sampling due to the presence of discharges by rivers, major coastal cities and important ports and shipping routes. The Bulgarian coast was sampled along three transects offshore (Fig. 3.1; detailed maps of each sampling sites are provided in the relevant result Chapters 3, 4 and 5); two of them from major population centres and two close to the most important seaports in the Black Sea. As a control, a third transect offshore adjacent to a small village with no industrial or shipping activities was also sampled.

The sampling strategy designed for the north and the middle sub-basins of the Caspian Sea mirrors the sampling design used in the Black Sea, thus a sampling campaign was carried out along the coast of Kazakhstan. This area is characterized by steep salinity gradients and it is affected by a variety of anthropogenic activities including shipping. Two transects offshore the main Kazakhs cities on the Caspian Sea, Bautino (north sub-basin) and Aktau (middle sub-basin and the largest port of Kazakhstan) were sampled.

These areas have previously been poorly studied in terms of modern dinocyst assemblages (section 1.4.1, Fig. 1.9). These samples and the analyses carried out on them, therefore, contribute to filling some of the gaps in the fragmented data available in the Pontocaspian region. Samples from these sites also underpin the analyses undertaken in Chapter 3, which evaluates the influence of anthropogenic

impacts on dinocyst assemblages and distribution. Consequently, a detailed description of the area and the sampling is presented in the section Material and methods in Chapter 3, Fig 3.1.

2.1.2 Planktonic dinoflagellates

Biological sampling and transport require permits. Because these permits are issued by different national authorities for each of the areas sampled, it was not possible to carry out the sampling campaigns at the same time of the year. As a result, seasonal variability was not tested in this study (Table 2.1), and how seasonality affects dinoflagellate composition and abundance is not discussed here (see Material and methods and Discussion in Chapter 5). Finally, the collection, preservation and transportation of water samples were constrained by each country's legislation, the materials available *in situ* and the remote access of the sampling locations. Consequently, water samples were collected using two different methods (see Material and methods in Chapter 5; Table 2.1). These limitations are taken into account during the discussion of this study to avoid misinterpretation of the results presented here.

2.1.2.1 Sampling sites

Water samples of the water column (see details in Material and methods in Chapter 5) were collected in order to obtain dinoflagellate motile cells from a wide range of different habitats. Four different coastal habitats were selected for the analyses carried out in Chapter 5 (Fig. 2.1, detailed maps of each site are presented in Chapter 5, Fig. 5.1).

1. The Razim-Sinoe Lake complex is located south of the Danube Delta, on the Romanian coast of the Black Sea. This lake complex is connected to the Black Sea and it is characterized by shallow waters with a. maximum depth of 2.7 m and a salinity range from 0 to ~6 psu varying with river discharge and currents from the Black Sea that introduce more saline water into the lake complex (Vadineanu et al., 1997; Breţcan et al., 2009 and references therein).
2. The Dnieper Estuary is located on the northwestern coast of the Black Sea in Ukraine and it is the sea's largest estuary, with a surface area of 1006.3 km^2. The estuary is connected to the Black Sea through the Kinbourn strait (Margvelashvily et al., 1999). The estuary is a shallow

(average depth 4.4 m), stratified environment with a salinity ranging from 0 to ~11 psu (Monte et al., 2006; Heling and Bezhenar, 2009).

3. The Paliastomi Lagoon is a coastal lagoon located in the Eastern Black Sea, in Georgia. This lagoon is connected to the Black Sea through an artificial channel and is a shallow (maximum depth 3.2 m) environment where most of the freshwater is provided by the river Pichora to the east. The salinity of the lagoon rages from 0 near the river mouth to ~12 psu near the channel connecting it to the Black Sea depending on the winds (Dassenakis et al., 2006).
4. Open Black Sea samples were obtained from the North-West Black Sea offshore Constanta port, Romania (see section 1.3.1 and material and methods in Chapter 5 for more details on the area).

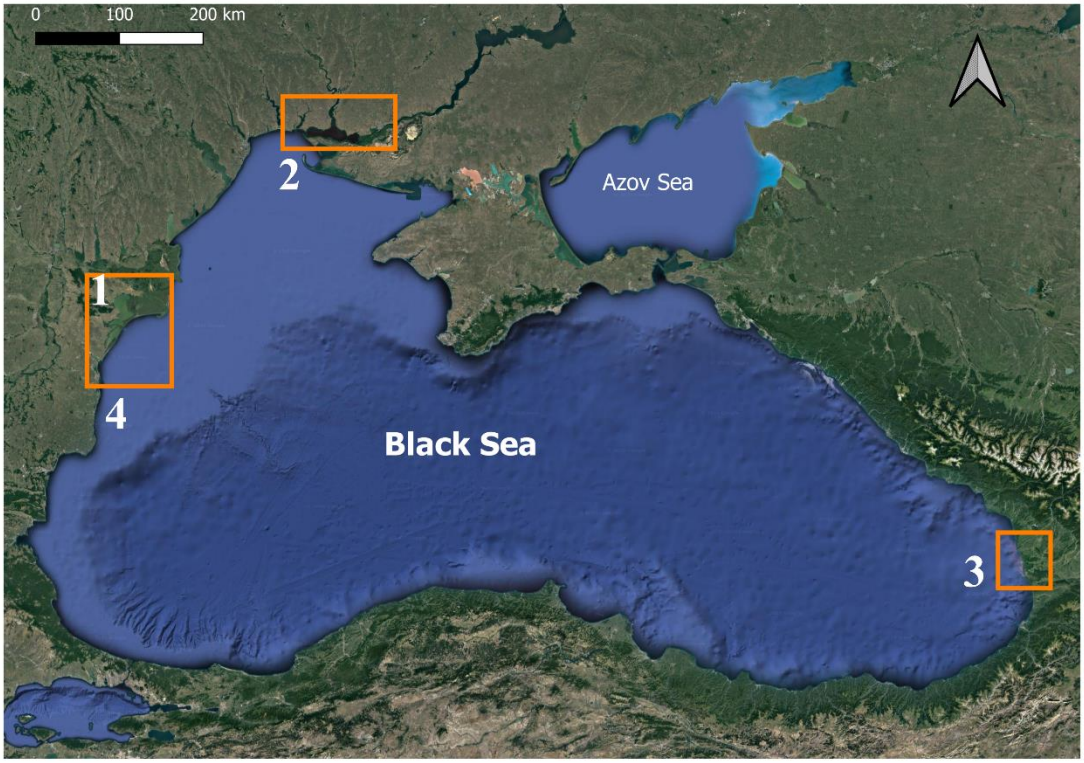

Fig. 2.1: Map showing the different sampling areas studied in Chapter 5. 1: Razim-Sinoe Lake complex, 2: Dnieper estuary, 3: Paliastomi Lagoon and 4: Open Black Sea offshore Constanta.

2.2 Additional sources of data

2.2.1 Dinocyst data from the South Caspian Sea sub-basin

The Caspian Sea can be divided into three clearly environmentally differentiated sub-basins (north, middle and south sub-basins, section 1.3.2). To investigate how these different environmental settings affect the dinocyst assemblages surface sediment samples from the north and the middle sub-basins were collected (see Material and methods in Chapter 3). However, during the time that PRIDE was being carried out, no sea expedition to the south sub-basin could be organized because of the lack of permits for biological sampling. In order to have a more complete picture of the different Caspian Sea environments, a dinocyst dataset published by Leroy et al. (2018) based on samples obtained offshore Iran and processed following the same methodology as is used in this project was included in our analyses (see Material and methods in Chapter 3).

2.2.2 Environmental and shipping variables for the Black and Caspian seas

In order to test the relationship between dinocyst assemblages and a range of environmental and shipping variables, a dataset of spatially explicit environmental (temperature, salinity, chlorophyll-*a* and turbidity) variables were extracted and calculated from the Bio-ORACLE dataset (www.bio-oracle.org; Tyberghein et al., 2012; see Material and methods in Chapter 3).

To investigate the effect of shipping activities on dinocyst assemblages, a set of shipping variables (distance to harbour, as linear distance from the closest harbours in meters multiplied for their size, defined with the number of vessel route raster cells contained in a circular buffer of 20 km radius centred in the harbour main dock; distance to shipping routes and the annual number of vessels passing per sampling location), were estimated for both the Black and the Caspian Seas. Detailed methods for obtaining these variables are explained in the Material and methods section of Chapter 3 and the supplementary data (Table S3.1).

2.2.3 Monthly and weekly sea surface temperature and chlorophyll-a for the Black Sea

To explore the distribution and diversity patterns of dinoflagellates present in the water column in the Black Sea a set of spatially explicit environmental (weekly and monthly surface temperature and chlorophyll-*a*) variables were extracted from the NASA Earth Observations dataset (https://neo.sci.gsfc.nasa.gov/) and calculated for our sampling locations at the time of sampling (see Material and methods in Chapter 5).

CHAPTER 3

DINOFLAGELLATE CYST ASSEMBLAGES AS INDICATORS OF ENVIRONMENTAL CONDITIONS AND SHIPPING ACTIVITIES IN COASTAL AREAS OF THE BLACK AND CASPIAN SEAS

T*his chapter will address the* ***specific research goals 1 and 3*** *outlined in Section 1.5.1 and 1.5.2 respectively.*

1. *Describe the differences in dinocyst assemblages relative to their environmental settings complementing previous studies and enhancing data from areas previously less studied in order to enrich the Pontocaspian dinocyst database;*
3. *Test dinocyst assemblages as proxies for physico-chemical and shipping variables of the Black and Caspian Seas*

The environmental and shipping variables included in this chapter analyses were compiled and extracted by Matteo Lattuada, an ESR from PRIDE.

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3.1 Introduction

The two main water bodies of the Pontocaspian region, the Black and the Caspian Seas (Fig. 3.1a), are ancient basins known for showing a unique regime in environmental parameters such as salinity and temperature and being the host of a unique aquatic biota (Zenkevitch, 1963; Dumont, 2000; Karpinsky et al., 2005; Vidal et al., 2010). However, over the last century, their biodiversity has been increasingly affected by multiple anthropogenic pressures (Lattuada et al., 2019) e.g. metal pollution (de Mora et al.,

2004), oil pollution (Tolosa et al., 2004) and eutrophication (Yunev et al., 2007; Mirzajani et al., 2010). Consequently, the overall ecosystem health of both basins has deteriorated (Dumont, 1995; Zonn, 2005), and endemic communities have become regionally extinct and replaced by introduced taxa (Moncheva & Kamburska, 2002; Karpinsky et al., 2005; Bologa & Sava, 2012; Zarbaliyeva et al., 2016; Mammadov et al., 2016).

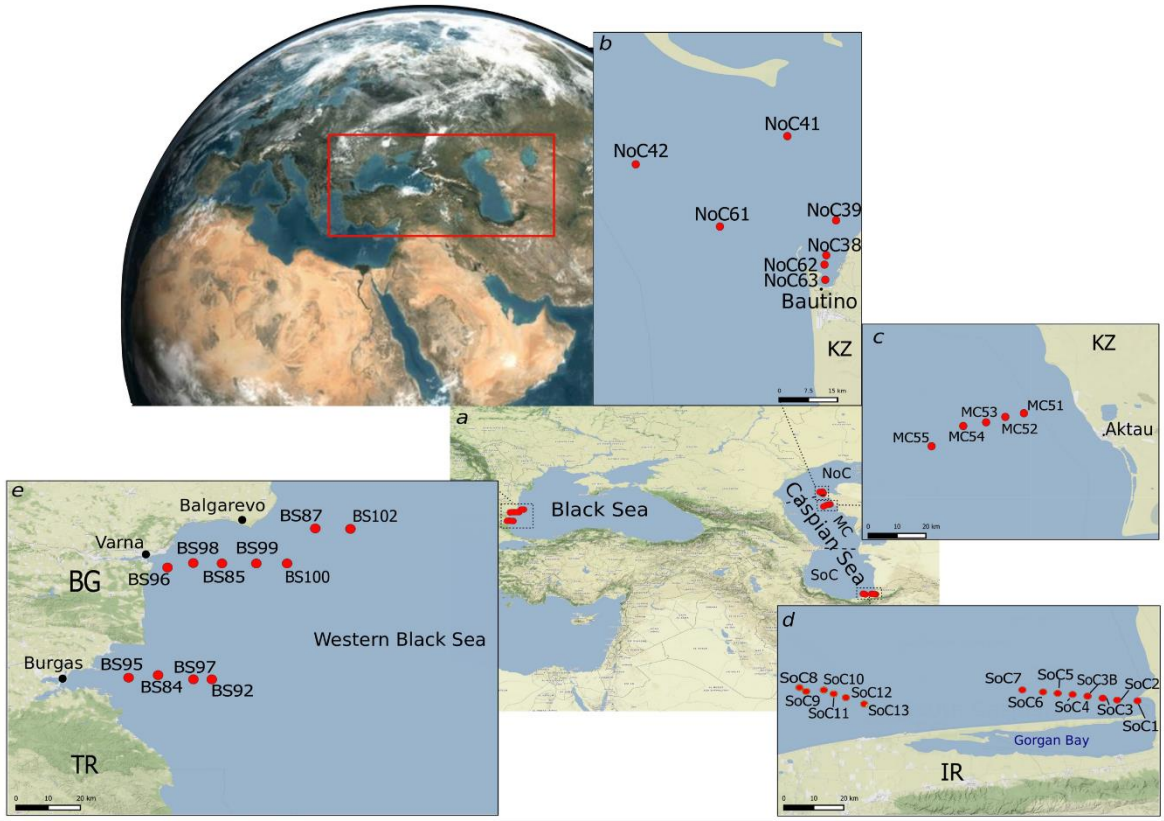

Fig 3.1: Maps of the study areas. Red dots indicate sampling stations. 1a: The Pontocaspian region formed by the two basins of the Black and Caspian seas. 1b: Location of the stations along the transect in the north sub-basin of the Caspian Sea. 1c: Location of the stations along the transect in the middle Caspian sub-basin. 1d: Location of the stations sampled in the south sub-basin of the Caspian Sea in red. 1e: Location of the stations on the Western Black Sea.

Dinoflagellates (division Pyrrhophyta, class Dinophyceae) are one of the most important groups of phytoplankton in marine and freshwater ecosystems, with more than 2000 species and more than 200 genera (Gómez, 2012). Ecologically, they play a major role in aquatic food webs and are the main taxa responsible for Harmful Algal Blooms or HABs (Hallegraeff, 1993, 2010). Shifts in spatial and temporal distribution, composition and abundance of phytoplankton communities are mostly driven by changes in environmental factors such as temperature, salinity, nutrient availability, irradiance and

oxygen concentration (Mikaelyan, 1997; Staehr and Sand-jensen, 2006; Suikkanen et al., 2006; Liu et al., 2015; Baek et al., 2019). On top of the environmental parameters, different pollution sources such as NOx and POx input (Chislock et al., 2013; Zhou et al., 2018) and industrial pollution (Furness and Rainbow, 1990; Arunakumar and Zhang, 2008) are driving shifts in phytoplankton communities. A particularly important source of industrial pollutants and disturbances affecting aquatic environments are maritime activities. Shipping activities can impact phytoplankton species by increasing chemical pollution, through the release of antifouling paint and oil compounds (Ng and Song, 2010) and by local sediment resuspension (Talley, 2016) which affects dinoflagellate assemblages and species physiology in the water column (Devilla et al., 2005; Bao et al., 2011; Lafabrie et al., 2013).

As a part of their life cycle, some species of dinoflagellate produce resting cysts as a result of sexual reproduction (i.e. dinocysts). Dinocysts can be transported and deposited on the basin floor, like silt particles, where they form a component of the sedimentary accumulation (Anderson et al., 1985). Dinocysts play an important role in the biology and ecology of the species allowing dinoflagellates to endure stressful conditions and re-inoculating the water column once favourable conditions have been restored (Bravo and Figueroa, 2014). Dinocysts, therefore, provide a reservoir of dinoflagellate diversity and a potent tool for studying long-term, spatial changes in the physico-chemical conditions of the upper water column and the depositional environment (Rochon et al., 1999; Marret and Zonneveld, 2003; Pospelova et al., 2008; Zonneveld et al., 2013; Penaud et al., 2018). Modern and fossil shifts in dinocyst diversity and taxa dominance have been suggested to be accurate proxies of anthropogenic eutrophication (Dale, 2001b; Pospelova et al., 2005; Price et al., 2017) and industrial pollution (Dale, 2001b; Liu et al., 2012; Lu et al., 2017).

Many studies in the Pontocaspian region have used dinocysts as proxies to describe natural environmental changes in the water column in past (Mudie et al., 2001; Marret et al., 2009; van der Meer et al., 2008; Leroy et al., 2007 and 2013) and modern (Marret et al., 2004; Mudie et al., 2017) times. Rapid changes in environmental parameters such as salinity, temperature and nutrient availability are thought to be the main drivers of phytoplankton species composition in coastal areas (Aubrey et al., 1996; Mudie et al, 2001 and 2017; Marret et al., 2004; Leroy et al., 2007 and 2013; van der Meer et al.,

2008; Bagheri et al., 2012).). However, except for a few studies in the Marmara Sea (Balkis et al., 2016; Mudie et al., 2017), to our knowledge, only a few studies have tried to use dinocysts to describe anthropogenic drivers in the region (Marret et al., 2009; Bradley et al., 2012).

In the present study, we statistically evaluate the relationship between dinocyst assemblages and a range of environmental and shipping variables. We examine the data from seven transects across three ecologically different coastal areas to:

1) describe the differences in dinocyst assemblages relative to their environmental settings and;
2) test dinocyst assemblages as proxies for physico-chemical and shipping variables of the Black and Caspian Seas

3.2. Material and methods

3.2.1 Study areas and sediment collection

In the Western Black Sea (WBS), samples were collected along transects that are likely to be influenced by a variety of different anthropogenic activities including shipping. Two of the transects were located offshore from major population centres, Burgas and Varna, and one offshore from Balgarevo, a small village on the northern coastal side of Bulgaria (Fig. 3.1e). The transects consist of five (BS96, BS98, BS85, BS99, BS100), four (BS95, BS84, BS97, BS92) and two (BS87, BS102) sampling stations respectively (Fig. 3.1e) collected during the MN 167 expedition between 10 and 24 May 2017 on board of the *Mare Nigrum*. Varna is the largest city on the Bulgarian Black Sea coast with 472,120 habitants followed by Burgas with 202,766 habitants in 2017 and 2016, respectively (National statistical institute of Bulgaria). Burgas is the largest harbour in Bulgaria. Varna is also a major seaport on the Black Sea and hosts the headquarters of the Bulgarian Navy. Both Burgas and Varna, are important industrial, transport, cultural and tourist centres. By contrast, Balgarevo is a rural area where tourism, agriculture, fishing and craftsmanship are the most important sources of income. The annual surface water temperature across this Western Black Sea area ranges between ~5 to 25 °C; surface water salinity is ~14 to 18 psu (Dineva, 2011).

The Caspian Sea is divided into three different sub-basins: a northern, shallow sub-basin (average and maximum depth 6 and 20 m respectively), a middle sub-basin (average 190 m) and a southern sub-basin (up to 1,025 m; Kosarev, 2005; Fendereski et al., 2014). In this paper, we identify these three sub-basins as NoC, MC and SoC, respectively. The transects selected for this study cover coastal areas of the three sub-basins of the Caspian Sea. Two transects offshore from Bautino and Aktau in Kazakhstan were sampled. The transects consist of seven (NoC63, NoC62, NoC38, NoC39, NoC41, NoC42, NoC61) and five (MC51, MC52, MC53, MC54, MC55) stations, respectively (Fig. 3.1b and 3.1c). Samples were collected between 16 and 20 June 2017 on board of the research vessel "Elen" from the Kazakhstan Agency of Applied Ecology (KAPE). Bautino is a small town with a harbour and has one of the largest fishing industries in Kazakhstan. There is a fishing port and seal fishing base, a fish canning plant and a ship repair facility (Timirkhanov et al., 2010). Aktau, on the other hand, is the largest Kazak city on the Caspian Sea coast. It houses the main Kazak seaport which has recently expanded (completion achieved in the summer of 2015) to accommodate larger quantities and more diverse cargo types (Satubaldina, 2015). A third transect covering 13 stations was performed offshore the Gorgan Bay (Fig. 3.1d), SE Caspian Sea, Iran in February 2014 and was published by Leroy et al. (2018). The North sub-basin of the Caspian Sea presents an estuarine-like environmental setting with salinity changes from 0 psu near the Volga delta to 10 psu in the far south and an annual surface temperature variation from freezing temperatures to 26 °C in summer. The middle and southern sub-basins experience a variation in surface water salinity of ~11-12 psu and ~12-13 psu respectively, while temperature varies from 3-25 °C and 7-30 °C, respectively (Zonn et al., 2010).

Sediment sampling for dinocysts, grain size and loss of ignition (LOI_{550}) was carried out at 35 stations along the coast of Bulgaria (Fig. 3.1e), and the coast of Kazakhstan (Fig. 3.1b and 3.1c), using a van Veen grab with a grabbing area of 0.11 m^2. Once onboard, around 200-300 g for dinocyst and sedimentology analyses of undisturbed sediments were collected by scooping the top (1-2 cm) layer with a stainless-steel spatula. Sediments for dinocyst and sedimentology analyses were kept in resealable plastic bags in darkness to prevent germination and at 5°C until further analyses. Sediment samples from the south sub-basin were collected and analysed as described in Leroy et al. (2018).

3.2.2 Sedimentology

Sediments collected for grain size and LOI_{550} analysis were dried for 24 h at 50 °C. Dried sediments were then broken down into their constituent grains using a pestle and a mortar and sieved at 2 mm. Approximately 4 g of the < 2 mm sieved fraction were burnt at 550 °C for 4 h (LOI_{550}) in a muffle furnace. Organic content was calculated by weight loss on ignition, according to Heiri et al. (2001). Many of the samples were rich in shells. Consequently, to measure the detrital grain size, carbonates were dissolved by adding 30 mL 1N HCl to the furnaced sample in a 50mL centrifuge tube. Samples were then mixed for 2 hours using a Fisherbrand multi-purpose tube rotator. The supernatant fluid was separated by centrifugation and pipetted off. Samples were rinsed with water, centrifuged, and the supernatant fluid was removed. Station BS100 contained high salt content. This sample was rinsed with 30 mL of Mili-Qwater, mixed for 1 h before the supernatant fluid was removed. This process was repeated five times prior to grain size analysis. A Malvern Mastersizer 3000 was used to analyse the grain size and the data were grouped into three categories: clay ≤ 0.4 μm < silt ≤ 63 μm < sand. The methodology for measuring grain size and loss of ignition (LOI_{550}) for the Black Sea and the north and middle Caspian sub-basin sites mirrors that used for the South Caspian Basin transect samples as detailed in Leroy et al. (2018).

3.2.3 Analyses of dinocysts

Approximately 2.0-3.5 mL of wet sediment samples were treated for palynological analysis following the method described in Marret et al. (2009). Initial processing of samples involved deflocculating of the sediment by sodium pyrophosphate. Samples were then treated with cold hydrochloric acid, first 10% to evaluate the reaction and then 35% to remove all carbonates, and cold hydrofluoric acid (32%), to remove all the silicates, and HCl again. Samples were then treated with sodium polytungstate (SPT) prepared to a density of 2.3-2.35 g/cm^3 to remove mineral residues. The residual organic fraction was then rinsed through 125 and 10 μm mesh sieves and mounted on slides in glycerol. Samples were washed twice with Mili-Q water between each step. *Lycopodium* tablets were added at the beginning of the process for concentration estimates, which are provided in number of dinocysts per mL of wet sediment (cyst mL^{-1}). Dinocysts were counted with a light microscope (Olympus BX41) at 400 x

magnification. A minimum of 200 dinocysts were counted at each station. Dinocyst identification was based on Matsuoka and Fukuyo (2000), Marret et al. (2004), Zonneveld and Pospelova, (2015) and Mudie et al. (2017). When species-level identification was not possible, e.g., specific cyst features were invisible due to folded cyst, identification was performed at genus level. Round brown cysts include all spherical brown dinocysts without spines or ornaments as in Zonneveld and Pospelova (2015). Dinocyst analyses for the stations located along the Gorgan Bay, Iran, South Caspian basin, were carried out as described in Leroy et al. (2018). Dinocyst counts from Leroy et al. (2018) were included together with the counts for the western Black Sea and the North and Middle Caspian sub-basins to perform the analyses described below.

3.2.4 Environmental and shipping variables

Spatially explicit environmental (temperature, salinity, chlorophyll-*a* and turbidity) variables were calculated for the Caspian Sea. For the Black Sea, we used the same environmental variables extracted from the Bio-ORACLE dataset, which contains multiple spatially explicit environmental variables at a resolution of 5 ArcMin (www.bio-oracle.org; Tyberghein et al., 2012). The Caspian Sea variables were resampled to match the Bio-ORACLE dataset. Shipping variables (distance to harbour, as linear distance from the closest harbours in meters multiplied for their size, defined with the number of vessel route raster cells contained in a circular buffer of 20 km radius centred in the harbour main dock; distance to shipping routes and the annual number of vessels passing per sampling location), were estimated for both the Black and the Caspian Seas. Finally, we extracted these variables at each of the sample locations (Table S3.1). Detailed methods to obtain the variables are explained in the Supplementary Data (Table S3.1).

3.2.5 Statistical analysis

Sampling sites were arranged in a hierarchical cluster using 1) the relative abundance of each taxon (%), to determine dinocyst assemblage zones (Leroy et al. 2018); and 2) photic zone physicochemical variables (salinity, temperature, chlorophyll-*a* and turbidity), to determine ecological zones along the study area (Fendereski et al. 2014). Clusters are joined based on the Bray-Curtis dissimilarity using

CONISS method and Euclidean distance respectively, between all stations. In addition, in order to determine the independence of the shipping variables (log-transformed distance to harbour times harbour size, distance to shipping routes and the annual number of vessels per sampling location) from the environmental variables (temperature, salinity, chlorophyll-*a* and turbidity) a correlation matrix analyses was performed with all the variables contained in the Table S1. The ratio between heterotrophic and autotrophic dinocysts (H:A), commonly used as an indicator for eutrophication and industrial pollution trends in coastal environments (Saetre et al., 1997; Sangiorgi and Donders, 2004; Liu et al., 2012), and degree of dinocyst diversity, using the Shannon-Wiener's index (H′, Shannon, 1948), were calculated using the R software (The R Foundation for Statistical Computing, 2018). Nonmetric multidimensional scaling (NMDS) analyses were performed to quantify the relative abundance of dinocysts in relation to environmental (temperature, salinity, chlorophyll-*a*, turbidity, grain size and organic matter), and shipping variables by permutation *p*-value based test (999 permutations). Multivariate analyses are based on a dissimilarity matrix of dinocyst relative abundance square-root transformed data (Hellinger transformation). Data transformation is made to decrease the variation between rare and dominant species (Legendre and Gallagher, 2001). The same analyses were performed with dinocyst concentration data. Results are not shown as no additional information was provided. In order to establish any significant relationship between the sediment profile and the total concentration of dinocysts (cyst mL^{-1}), H' and H:A correlation matrix analyses were performed with all the variables included in the Table 2. Statistical analyses were performed by R 3.5.0 (The R Foundation for Statistical Computing, 2018) with package "vegan" (Oksanen et al., 2018).

3.3 Results

3.3.1 Environmental setting of the study areas

To determine independent ecological zones in our study areas, hierarchical cluster analysis was performed on the sampling sites using the surface maximum, minimum, mean and range of temperature, salinity, chlorophyll-*a*; and turbidity as variables (see section 3.2.4 and Table S3.1). Fig. 2 shows the result of the hierarchical cluster analysis as a dendrogram. Three major clusters can be identified: a cluster with all the sampling stations located in the Western coast of the Black Sea (WBS); a second

cluster where the sampling sites located in the North and Middle Caspian Sea sub-basins (NoC and MC) fall together; and a final cluster grouping all the sites sampled offshore Iran in the south sub-basin of the Caspian Sea (SoC). Correlation analyses show that shipping variables use in this study are independent of the environmental variables (Table S3.2).

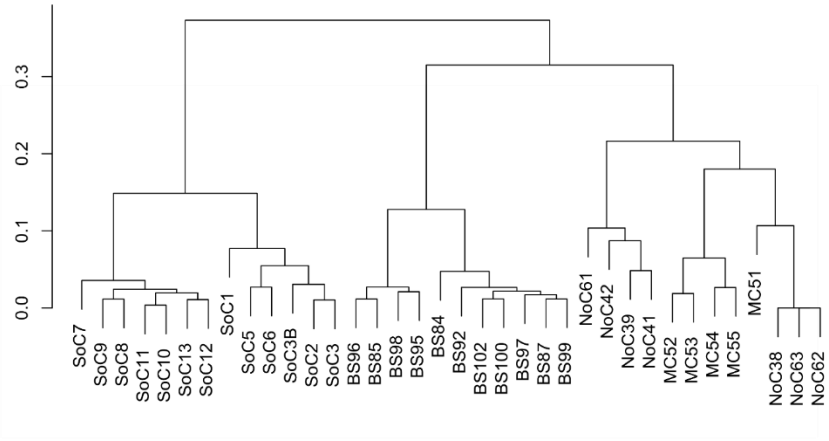

Fig. 3.2: Hierarchical cluster analyses performed to calculate Euclidean distance with the environmental variables including maximum, minimum, mean and range of salinity, temperature and chlorophyll-*a*, as well as mean turbidity for the period from 2011 to 2015. Detailed information on how these variables are calculated is presented in Table S3.1 in Appendix A.

3.3.2 Sedimentology

Sampling locations with abundant fine (< 63 µm) sediments were targeted along the study transects to ensure a sufficient and comparable abundance of dinocysts. Most of the samples were dominated by material ranging from 44.3 to 97.0% silt measured at stations BS100 and SoC3b, respectively. The highest percentage of sand (44%) was measured at the station SoC2 and the lowest (0%) was measured at several stations located offshore Iran (Table 3.1). The percentage of clay in the studied sediments ranged between 1.0 and 47.5% measured at stations SoC2 and BS100, respectively. The organic matter content (LOI_{550}) ranged between 3.1 and 16.5% (sampling stations BS96 and MC55 respectively; Table 3.2). A significant ($p < 0.05$) positive correlation between the percentage of organic matter and dinocyst

concentration was found in the study area. In addition, organic matter content positively correlated with clay ($p < 0.001$) and depth ($p < 0.01$, Fig. 3.3).

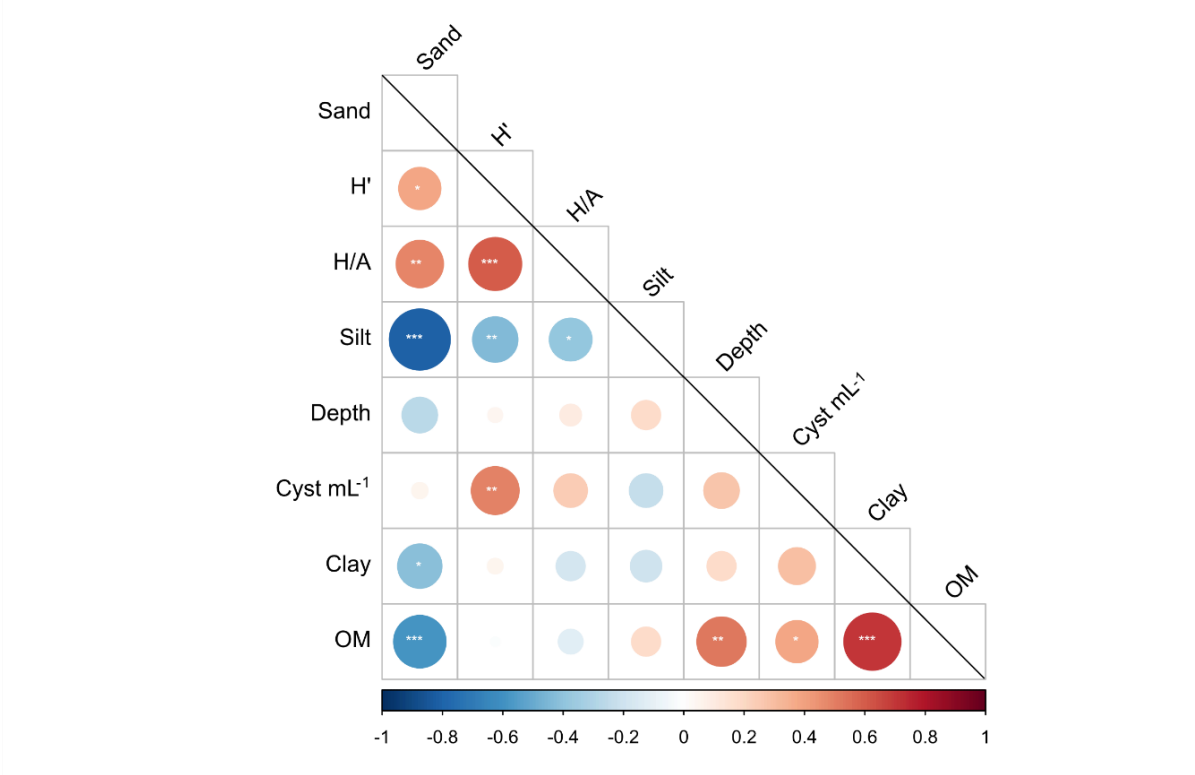

Fig. 3.3: Correlation analyses matrix between grain size, LOI_{550}, depth and dinocyst abundance, H' and H:A for all 36 sampling stations. The size of the dots represent the r-value of each correlation pair and blue and red tones indicate negative and positive correlation respectively. Significant correlations are indicated by *, ** and *** for *p*-values $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively.

Table 3.1. Depth, dinoflagellate cyst concentration, Shannon–Wiener's diversity index (H'), heterotrophs/autotrophs ratio (H:A), grain size and Loss of ignition (LOI_{550}) percentage for sampling stations.

Station	Depth (m)	Cyst mL^{-1}	H'	H:A	Sand (%)	Silt (%)	Clay (%)	LOI_{550} (%)
BS87	90.3	30669	1.81	0.24	24.79	65.36	9.85	7.55
BS102	94.4	35537	1.79	0.16	12.56	73.25	14.20	9.60
BS96	20.7	5052	1.79	0.51	27.15	69.94	2.92	3.11
BS98	74.8	6086	1.96	0.84	35.50	61.18	3.32	3.11
BS85	36.7	6809	2.1	0.71	20.28	73.61	6.12	3.77
BS99	45.6	29567	1.52	0.35	26.74	62.74	10.53	8.50
BS100	77.3	24344	1.63	0.21	8.19	44.34	47.47	16.23
BS95	35	20912	1.94	0.42	10.68	83.78	5.56	6.81
BS84	23.7	11378	1.9	0.96	26.45	70.32	3.24	5.75
BS97	44.3	20550	1.86	0.61	8.90	78.53	12.58	10.85
BS92	79.1	21615	1.75	0.35	10.57	76.90	12.53	11.29
NoC63	91.5	8641	1.34	0.31	7.88	78.25	13.88	6.32
NoC62	7.3	4644	1.43	0.43	15.77	75.75	8.47	4.53
NoC38	7.6	11233	1.37	0.4	10.54	77.28	12.19	8.19
NoC39	8	12681	1.44	0.43	14.70	74.66	10.64	7.59
NoC41	8.5	9340	1.29	1.24	41.61	57.20	1.19	3.83
NoC42	7.3	5154	1.28	0.94	33.61	57.51	8.88	4.35
NoC61	6	4905	1.31	0.6	13.60	78.85	7.55	3.96
MC51	14.4	13532	1.41	0.59	1.74	82.54	15.72	8.10
MC52	61.8	28869	1.42	0.48	1.98	83.88	14.14	8.92
MC53	101	20684	1.5	0.84	3.96	79.52	16.52	8.86
MC54	142	45318	1.44	0.76	23.42	69.16	7.43	5.61
MC55	249	21866	1.45	1.23	0.43	87.63	11.94	16.45
SoC1	2	7349	0.94	0.01	0	90.31	9.69	7.25
SoC2	5	1774	0.67	0	44	55.18	0.82	3.75
SoC3	7	4101	0.85	0.04	14.15	81.84	4	5
SoC3B	9	5864	0.65	0	0.11	96.71	3.4	6.25
SoC5	10	8715	0.83	0.02	0.1	93.63	6.26	7.25
SoC6	12	8145	0.82	0.02	0	79.33	20.67	7.25
SoC7	13	8764	0.87	0.01	0	88.41	11.59	8.5
SoC13	25	5954	0.74	0.04	0	81.64	15.78	9.25
SoC12	46	7505	1.07	0.04	0	89.52	10.48	9.5
SoC11	92	4288	0.91	0.05	0	87.57	12.43	9.75
SoC10	148	5480	0.87	0.02	0	88.8	11.2	10
SoC9	200	3605	0.87	0.06	3.11	91.85	5.04	8.75
SoC8	221	2343	1.01	0.14	0	84.69	15.31	8.75

3.3.3 Dinocyst abundance and species assemblages

All samples were rich in dinocysts with absolute abundances (cyst mL^{-1} of wet sediment) varying between 1774 (SoC2, 5 m) and 45318 (MC54, 150 m) cysts per mL of wet sediment. A total of 36 dinoflagellate cyst morphotypes were identified from 36 surface sediment samples, of which 25 taxa were classified as autotrophic and 11 as heterotrophic. Among the 36 dinoflagellates taxa identified, cysts of five potentially harmful or toxic taxa were observed (*Alexandrium*, cysts of *Gymnodinium*, a genus of which *G. catenatum* belongs, cysts of *Gonyaulax spinifera complex* for which some strains are considered harmful, *Lingulodinium polyedra*, and *Protoceratium reticulatum*). Dinocyst assemblages from the stations located in the Black Sea were largely dominated by *L. machaerophorum* and Round brown cysts with values ranging from 25.7 to 60% and from 8.2 to 31.6% respectively, followed by *Spiniferites* and *O. centrocarpum*. Additionally, stations located in the Caspian Sea were dominated by *L. machaerophorum, Impagidinium caspienense* and Round brown cysts with a maximum relative abundance of 80.8, 62.8 and 52.1% respectively. A decrease of Round brown cysts in the stations located in the south sub-basin is observed. The highest number of cyst taxa was 21, recorded at BS87 in the transect offshore of Varna port on the west coast of the Black Sea and the lowest number of cyst taxa recorded was three at the SoC2 station located along the offshore Gorgan transect. Cysts of *L. machaerophorum* were recorded at all the stations in both basins, while cysts of Round brown cysts were found in all stations except in SoC2 in the Gorgan transect. The H:A ranged from 0 to 1.24 calculated for the stations SoC2 and SoC3B, and station NoC41, respectively. Total autotrophic cyst concentrations were higher than the heterotrophic cyst concentrations at all stations except for NoC41 and MC55, located in the North and the Middle sub-basins of the Caspian Sea respectively. In the study

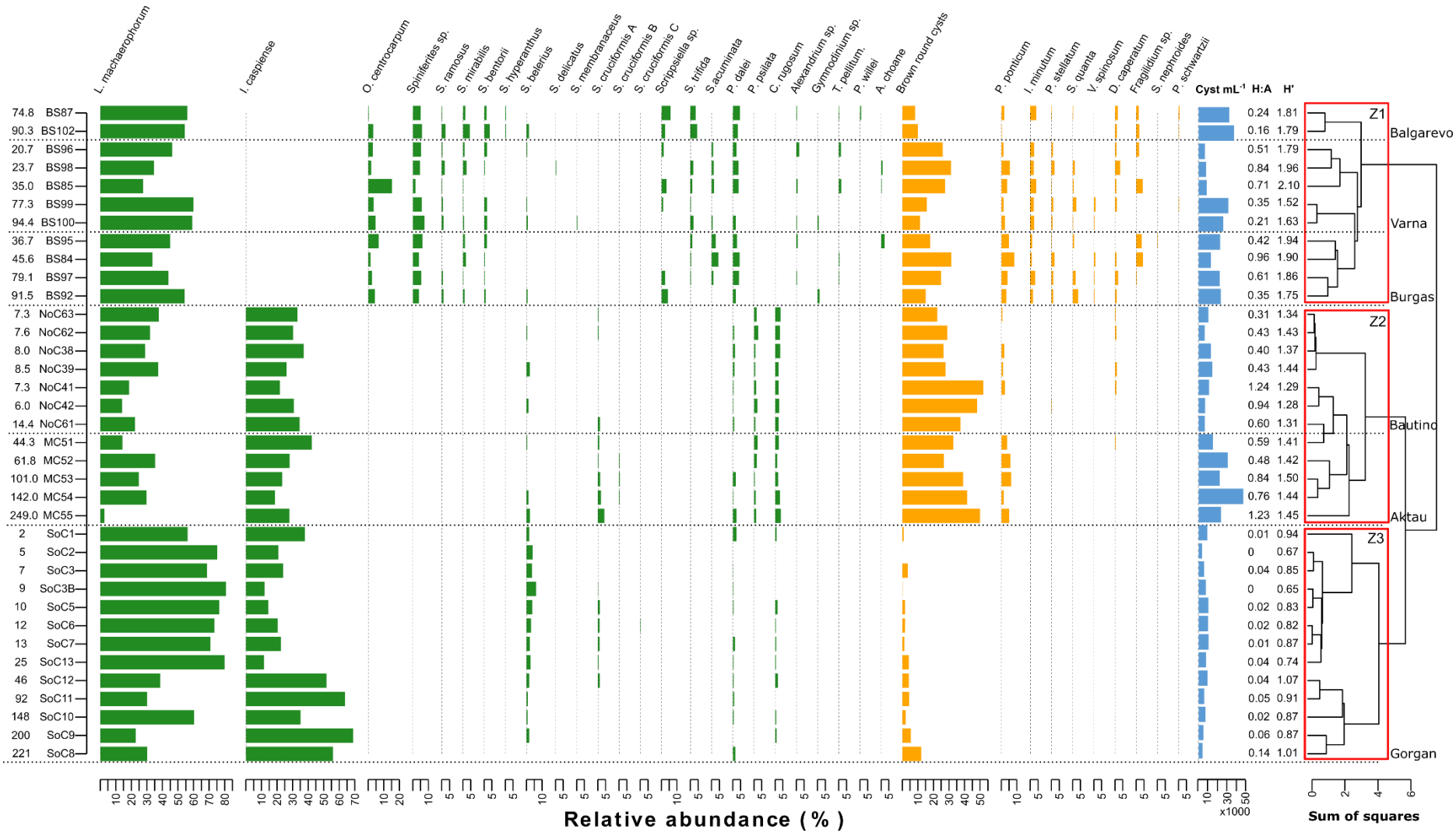

Fig. 3.4: Relative abundance (%) of dinocyst taxa at each station. Autotroph taxa are represented in green and heterotrophs taxa in orange. In addition, the heterotrophs/autotrophs ratio (H:A), the Shannon–Wiener index (H') and the dinocyst concentration per mL of wet sediment are calculated for each station. Hierarchical cluster analysis was performed with the dinocyst matrix to calculate dissimilarity (Bray-Curtis) using CONISS method among the stations according to the species assemblages and abundances. Station's bottom depth is shown in meters. Dashed lines indicate perpendicular transects from the coast named after the main coastal city.

areas, the Shannon Weiner diversity index (H') varied between 0.65 and 2.10 calculated for the stations SoC3B and BS85, respectively (Table 3.1, Fig. 3.4).

Based on the cyst composition and abundance at these sites, a dissimilarity analysis was conducted. The result shows that the dinocysts at the thirty-six locations can be classified into three major groups (Fig. 3.4) following the clustering of the ecological zonation (Fig. 3.2). The Z1 cluster groups all the WBS stations (Fig. 3.4). Three small sub-clusters are differentiated in this area. Stations BS87 and BS102, which are located outside of main port routes and main coastal population nucleus fall together in a separate sub-group distinct from the stations located in the transects adjacent to the two main ports, Varna and Burgas. The deepest stations in the transect from Varna port (BS99 and BS100) also sub-cluster together within the Z1 cluster (Fig. 3.4) and the stations from the transect performed from Burgas port (Fig. 3.1e). Cluster Z2 groups stations sampled in the north and the middle Caspian Sea sub-basins. Stations located inside Bautino Bay (NoC63, NoC62, NoC38 and NoC39; Fig. 3.1b) form a separate subgroup, while the stations located outside form a different sub-cluster in Z2 with the stations located along the transect from Aktau port (Fig. 3.1c, Fig. 3.4). Cluster Z3 groups the stations located in the south Caspian sub-basin (Fig. 3.1d). The two distinct Z3 sub-clusters relate to depth and the shift in species dominance from *L. machaerophorum* to *I. caspienense* in the deeper stations.

3.3.4 Dinocyst preservation

Dinocyst preservation was good to excellent in most samples and none of the taxa recorded showed signs of degradation, including oxidation sensitive taxa such as Round brown cysts. We are therefore confident that our results are not significantly influenced by degradation. Dinocyst distribution and concentration can be strongly influenced by sediment transport and the hydrology of the area (Milzer et al., 2019) as dinocysts act like silt particles while sinking in the water column (Lewis, 1988). Our results show no significant correlation between dinocyst abundance and sediment grain size in the area studied (Fig. 3.3). This, in addition to the low abundance of reworked dinocysts (data not shown), as indicated by the fact that cell content was still present in most of the cysts, suggests that the taxa

recorded in the studied stations represent local productivity of the upper water layers and that input of long-distance transported dinocysts did not affect the species composition.

3.3.5 Multivariate analyses

Nonmetric Multidimensional Scaling (NMDS) analyses were performed with all the variables compiled in Table S1, grain size and organic matter content for dinocysts relative abundance data (Fig. 3.5). The NMDS results (Fig. 3.5) reveal the same sampling site groupings inside the ecological zones as those from the cluster analyses (Fig. 3.2). Permutation analyses were performed with all variables but Fig. 3.5 shows only those variables which are statically significant ($p < 0.05$; Table S3.3). Most dinocyst assemblages in the study area are defined by temperature, salinity and chlorophyll-a concentration. *Lingulodinium machaerophorum* is significantly influenced by temperature (minimum, maximum and mean) while the chlorophyll-*a* range, defines the relative abundance of the heterotrophic taxa Round brown cysts (Fig. 3.5). Salinity is the main contributor defining dinocyst assemblages in the WBS. From our results, it seems that the distance to the harbour may influence the species assemblages in the North and Middle Caspian basins. Distance to vessel routes, minimum chl-*a* concentration and percentage of silt are significant controls on the dinocyst assemblages in the South Caspian basin. In addition, silt significantly influences the relative abundance of *Spiniferites belerius*. Percentage of sand and temperature range are significantly correlated to the relative abundance of heterotrophic dinocyst *Peridinium ponticum* (Fig. 3.5).

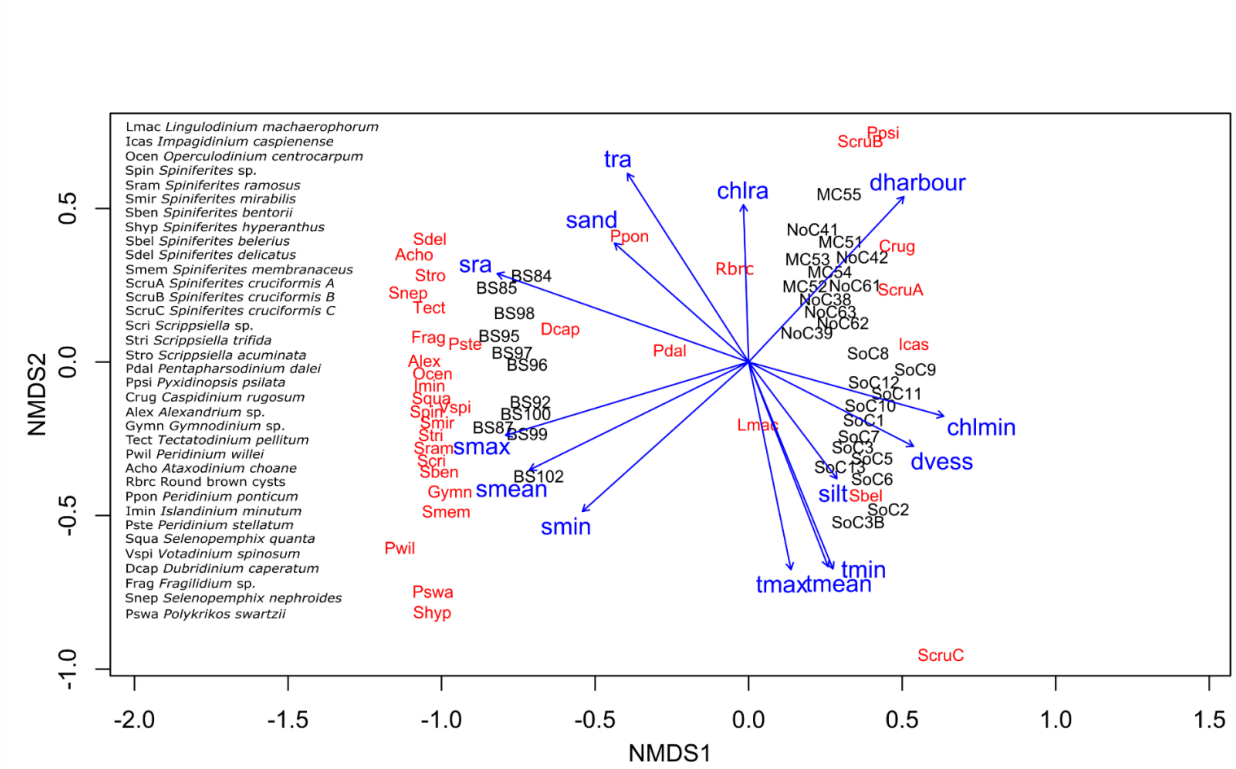

Fig. 3.5: Triplot based on non-metric multidimensional scaling (NMDS) of dinocyst relative abundance data for 36 sampling stations and a set of explanatory variables (Table S1), grain size and LOI_{550}. Distances between sampling sites (black labels) on the ordination plot reflect dissimilarity in dinocyst (red labels) composition using Bray-Curtis coefficients. The average position of dinocysts in ordination reflects their dominance in geographical space. A variable is statically significant if $p < 0.05$. Significant variable axes are in blue. The angle and length of vector loadings indicate the direction and strength of associations of variables with the dinocyst composition in sampling sites, respectively. Dinocyst taxa abbreviations are listed on the left side of the plot with their scientific name.

3.4 Discussion

3.4.1 Dinocysts abundance

All dinocyst morphotypes observed in this study have been reported before for the Black and the Caspian Seas (Mudie et al., 2017). Dinocyst concentration in surface sediments depends on complex relationships between abiotic (depositional environment, hydrology of the area and seasonality) and biotic (dinoflagellate abundance in the water column, nutrient availability) factors (Dale, 1979; Zonneveld et al., 2013; Ellegaard et al., 2017). Comparing dinocyst abundance data across different studies is, in certain cases, impossible due to the different dinocyst concentration units used in the literature, e.g. g^{-1} dry sediment and mL^{-1} wet sediment. The methodology used to analyse the sediments collected in the Western Black Sea (WBS), the North and Middle Caspian sub-basins mirrors the

methodology followed by Leroy et al. (2018); hence, the dinocysts' concentration differences between our study and Leroy et al. (2018) are likely to be environmentally driven rather than a product of the methodology. The range of dinocyst abundance compiled in this paper (Table 3.1) is similar to the values (mL^{-1} wet sediment) reported previously in similar areas of the Caspian Sea (Mudie et al. 2017). Unfortunately, to our knowledge, no published data on modern dinocyst abundance from the WBS in mL^{-1} wet sediment have been made. Dinocyst concentrations show high variability and no clear trend among sampling sites. This reflects the environmental heterogeneity and complexity of the dinocyst production and deposition. Nevertheless, from our results, it can be observed that the South Caspian stations (SoC) have lower dinocyst concentrations than the sites studied in the WBS, the North (NoC) and Middle Caspian (MC) sub-basins (Table 3.1). The processes controlling the differences in dinocyst abundance among the different basins analysed are likely to have been driven by three major causes: 1) the encystment rate of the species present in the water column, which is controlled by the environmental conditions (unfavourable conditions for vegetative growth) and the species genetics (Sgrosso et al., 2001; Kremp et al., 2009; Figueroa et al., 2011); 2) relative abundance of the cyst forming species in the phytoplankton community and 3) the sedimentation rate, which directly affects the deposition of particles on the basin floor and might therefore enhance the accumulation of dinocysts in areas with high sedimentation rates. For instance, due to the lack of major rivers draining into the South Caspian basin, the sedimentation rate for Gorgan Bay is estimated between 1.4-5.1 mm yr^{-1} (Karbassi and Amirnezhad, 2004; Amini et al., 2012; Leroy et al., 2013); while sedimentation rates for the North Caspian basin can reach 20-50 mm yr^{-1} in the Volga Delta (Overeem et al., 2003). This can directly influence the dinocyst abundance of such areas.

3.4.2 Species assemblages

Despite the environmental heterogeneity of the sampling sites (Fig. 3.2), the dominant taxa observed across all the study areas is largely homogeneous (Fig. 3.4). Protoperidinoids represented by Round brown cysts morphotype, and Gonyaulacoids identified as *L. machaerophorum*, dominate dinocyst assemblages in all the locations included in this study (Fig. 3.1). In addition, *I. caspienense* was dominant in all the Caspian Sea stations (Fig. 3.3). This agrees with previous studies carried out

throughout the Pontocaspian region (Marret et al., 2004; Aydin et al., 2015; Mudie et al., 2017). These most dominant taxa are known for showing a wide range of temperature and salinity tolerance. For example, *L. machaerophorum* and *I. caspienense* are autotrophic taxa with ubiquitous distribution (Mudie et al. 2017), which can tolerate temperatures from nearly the freezing point to above 30° C (24°C for *I. caspienense*) and salinities from ~5 to 35 psu (18 psu for *I. caspienense*). Round brown cysts is a heterotrophic taxon tolerating temperatures ranging from 0 to 30 °C and salinities between ~3 and 35 psu (Zonneveld et al., 2013). Species with wide tolerances of temperature and salinity are likely to dominate dinocyst assemblages in highly dynamic environments like the Pontocaspian region that experience rapid fluctuations in surface temperature and salinity (Bakan and Buyukgungor, 2000; Aladin and Plotnikov, 2004). In our surveys, the potentially harmful dinocyst morphotypes (e.g., *L. machaerophorum* was the most dominant morphotype followed by *O. centrocarpum* and morphotypes of the genera *Spiniferites* Fig. 3.4 see Table 3.2 for motile affinities) comprised 45% of the total morphotypes observed. These taxa are associated with HABs events in coastal areas of temperate regions (Howard et al., 2009).

Table 3.2: List of dinocyst taxa observed in this study listed in Fig. 3.4 with their motile form affinity and acronyms used in Fig. 3.5. * sensu Wall and Dale, 1966. ** sensu Mudie et al., 2017.

Abbrev.	Cyst-defined name	Motile-defined name
Lmac	*Lingulodinium machaerophorum*	*Lingulodinium polyedra*
Icas	*Impagidinium caspienense*	*Gonyaulax baltica*
Ocen	*Operculodinium centrocarpum**	*Protoceratium reticulatum*
Spin	*Spiniferites* sp.	*Gonyaulax* complex
Sram	*Spiniferites ramosus*	*Gonyaulax scrippsae, G. spinifera* complex
Smir	*Spiniferites mirabilis*	*Gonyaulax spinifera* complex
Sben	*Spiniferites bentorii*	*Gonyaulax digitale, G. spinifera* complex
Shyp	*Spiniferites hyperanthus*	*Gonyaulax spinifera* complex
Sbel	*Spiniferites belerius*	*Gonyaulax scrippsae, G. spinifera* complex
Sdel	*Spiniferites delicatus*	*Gonyaulax spinifera* complex
Smem	*Spiniferites membranaceus*	*Gonyaulax spinifera* complex
ScruA	*Spiniferites cruciformis* morphotype A**	unknown
ScruB	*Spiniferites cruciformis* morphotype B**	unknown
ScruC	*Spiniferites cruciformis* morphotype C**	unknown
Scri	Cyst of *Scrippsiella* sp.	*Scrippsiella* sp.
Stri	Cyst of *Scrippsiella trifida*	*Scrippsiella trifida*
Stro	Cyst of *Scrippsiella acuminata*	*Scrippsiella acuminata*
Pdal	Cyst of *Pentapharsodinium dalei*	*Pentapharsodinium dalei*
Ppsi	*Pyxidinopsis psilata*	unknown
Crug	*Caspidinium rugosum*	unknown
Alex	Cyst of *Alexandrium* sp.	*Alexandrium* sp.
Gymn	Cyst of *Gymnodinium* sp.**	*Gymnodinium* sp.
Tect	*Tectatodinium pellitum*	*Gonyaulax spinifera* complex
Pwil	Cyst of *Peridinium willei***	*Peridinium willei*
Acho	*Ataxodinium choane*	*Gonyaulax spinifera* complex
Rbrc	*Round brown cysts*	*?Protoperidinium* sp.
Ppon	*Peridinium ponticum*	*Protoperidinium cf. divergens*
Imin	Cyst of *Islandinium minutum*	*Islandinium minutum*
Pste	Cyst of *Protoperidinium stellatum*	*Protoperidinium stellatum*
Squa	*Selenopemphix quanta*	*Protoperidinium conicum*
Vspi	*Votadinium spinosum*	*Protoperidinium claudicans*
Dcap	*Dubridinium caperatum*	*Preperidinium meunieri*
Frag	Cyst of *Fragilidium* sp.	*Fragilidium* sp.
Snep	*Selenopemphix nephroides*	*Protoperidinium subinerme*
Pswa	Cyst of *Polykrikos schwartzii*	*Polykrikos schwartzii*

3.4.3 Dinocysts response to biotic and abiotic parameters

The term ecoregion, a homogenous area with similar biogeophysical and environmental characteristics (Bailey, 1996), was applied to the Caspian Sea by Fendereski et al. (2014). In that work, the biological significance of these ecoregions was tested using distribution and biomass data for 25 species including zooplankton, fish and benthic invertebrates. Fendereski et al. (2014)'s results show species assemblages for these groups correspond with the ecoregion division for the Caspian Sea. The same pattern can be observed in our results for Hierarchical cluster analyses based on environmental variables and for dinocyst assemblages, which clearly group the sampling locations in three clusters corresponding to two ecoregions in the Caspian Sea (No+MC and SoC) and one in the western Black Sea (Fig. 3.2 and Fig. 3.4, respectively). This suggests that dinocyst assemblages are defined mostly by the environmental variables in the study area.

Nonmetric multidimensional scaling (NMDS) analyses were carried out to test for any groupings and dissimilarities within the sampling locations. The results show three major groups, WBS, NoMC and SoC basins. Figure 3.5 confirms our observations of homogeneity within each of the areas analysed. These results confirm that environmental parameters such as temperature, salinity and chlorophyll-*a* concentration are commonly the main environmental drivers defining the dinocyst distribution and species assemblages across the areas included in this study (Fig. 3.5). Similar trends have been reported for studies around the world identifying surface temperature, salinity and chlorophyll-*a* as the main factors driving dinocysts distribution and assemblages (Rochon et al., 1999; Pospelova et al., 2004 and 2008). Round brown cysts are the most abundant heterotrophic taxa present in our samples (Fig. 3.4). According to our analyses (Fig. 3.5), their relative abundance is controlled, by the range of chlorophyll-*a* concentrations. Heterotrophic taxa such as Round brown cysts thrive in coastal areas with high-nutrient waters derived from rivers or upwelling (Harland et al., 2006; García-Moreiras et al., 2018). Primary production in these areas is commonly dominated by diatoms, which are the main prey for motile forms of Round brown cysts (Milzer et al., 2013). This is also supported by the high abundance of Round brown cysts in all the locations except the stations sampled in the South Caspian basin (Fig. 3.4) where minimum chlorophyll-*a* concentrations are found as a significant driver of the species

assemblages (Fig. 3.5). Our results show that the relative abundance of *L. machaerophorum* is strongly affected by temperature (Fig. 3.4). *Lingulodinium machaerophorum* is the cyst of *L. polyedrum*, a warm-water species known for producing blooms in late summer, which may lead to massive cyst production (Figueroa and Bravo, 2005; Wang et al., 2007). Moreover, the dominance of *L. machaerophorum* in the Caspian Sea was documented as a response mainly to the increasing sea surface temperature during the Anthropocene (Leroy et al., 2013).

3.4.4 Dinocysts response to trophic state and shipping activities

3.4.4.1 Trophic state

Dinoflagellate resting cysts respond to both, environmental and anthropogenic perturbations (Sætre et al., 1997; Pospelova and Kim, 2010; Lu et al., 2017). For instance, abundance and diversity of dinocysts may initially increase with nutrient enrichment and decline once eutrophication of the water body is reached, while percentages of heterotrophic and mixotrophic taxa may increase with nutrient enrichment (Dale, 2000; Pospelova and Kim, 2010). Our results (Fig. 3.4, Table 3.1) do not show this trend in dinocyst abundance and diversity since the highest dinocyst richness and H' were observed in the Western Black Sea stations (BS102 and BS85, respectively), while the highest dinocyst concentration was measured in the Middle Caspian (MC54) and the lowest H' index was recorded for the stations located in the South Caspian basin (Fig. 3.4, Table 3.1). These results can be explained by the brackish salinity of the Caspian Sea, which strongly controls the low diversity and productivity of the system (Karpinsky, 2005). Diversity of cyst-forming dinoflagellates decreases in a decreasing salinity gradient often found in estuaries and enclosed seas (Dale, 1996; Pospelova et al., 2004). Nevertheless, dinocyst diversity seems not to be a good proxy for eutrophication in the Pontocaspian region, where assemblages analysed from the WBS, the NoC and MC comprise a high number of heterotrophic taxa, which is likely to be related to nutrient-rich conditions in the water, availability of prey organisms such as diatoms, and the presence of a well-mixed water column. Dominant cyst taxa recorded in this study are commonly found in eutrophic environments with some of them (e.g. Round brown cysts and *L. machaerophorum*) being commonly used as eutrophication proxies in coastal areas around the world (Sangiorgi and Donders, 2004; Dale, 2009; Zonneveld et al., 2012). This supports the

findings of Gomoiu (1992), Dumont (1998), Moncheva et al. (2001) and Aladin and Plotnikov (2004) who identified eutrophication as one of the main anthropogenic pressures in local areas of the WBS and the NoC. The H:A ratio (Fig.3.4, Table 3.1) is commonly used as an indicator of eutrophication. It also mirrors this pattern where the lowest values occur at stations where heterotrophic taxa are absent. This is particularly apparent in the SoC where low nutrient enrichment has been reported (NASA, 2017).

3.4.4.2 Shipping activities

Nonmetric multidimensional scaling (NMDS) analyses (Fig. 3.5) identified "distance to the harbour", calculated in this study as the linear distance of the sum of the two closest harbours in meters multiplied by the harbor size, is defined as the number of vessel routes within a circular buffer of 20 km radius centred on the harbour main dock. The reason for including harbour size is that a larger harbour may have a higher impact on sediment distribution and resuspension than in a small harbour because of the larger number of vessels operating nearby, which might influence the dinocyst assemblages. The variable assumes that each navigation route has an impact that decays with distance, as a significant variable correlating with dinocyst assemblages in the NoC and MC areas. Aktau is the main commercial port of Kazakhstan (Satubaldina, 2015). In addition, the Northern Caspian sub-basin displays a high density of maritime traffic due to the oil and gas exploitation (AIS Marine Traffic, 2016) while the distance to vessel routes is found to significantly correlates with dinocyst assemblages in the SoC. This may be explained by the lack of important ports in Gorgan Bay, isolating the sampling location of the SoC from the main shipping routes in the region. Species assemblages analysed from the WBS show no relationship with any of the shipping variables included in this study despite proximity to the two main ports of Bulgaria. This may be because the sampling locations may not encompass enough variation in relation to distance from shipping lanes in an area of such a prolific ship activity and because of the depth of the stations sampled in the WBS. Disturbances caused by shipping activities can be more

important in shallow areas with high maritime traffic such as the North Caspian Sea where its impact may affect the whole aquatic community from the surface to the bottom (Talley, 2016).

3.5 Conclusions

The distribution and diversity of dinocyst assemblages confirmed previous studies that they are controlled by temperature, salinity and by primary productivity. Dinocyst concentrations are lowest in the South Caspian sub-basin, where low chlorophyll-*a* values are observed due to the lack of major river discharge and land-originated nutrient input. Heterotrophic dinocyst presence is related to rich-nutrient input that favours the presence of available prey as observed in the WBS and the NoC. Temperature has been confirmed to be the main driver defining the presence and relative abundance of the dominant autotrophic taxa, *L. machaerophorum*. High cyst abundance of potentially toxic species may initiate a population of motile cells to the extent to which they eventually form a bloom. This highlights the importance of improving our knowledge of local dinoflagellate cyst banks to assess these events. Dinocysts are a significant component of the organic particles preserved in the Pontocaspian sediments and can be used to reconstruct accurately the environmental conditions of the upper water column. To our knowledge, this study assessed for the first time the use of dinocysts as proxy for shipping activities, across different ecological areas of the Black and the Caspian seas. Shipping activities significantly correlated with dinocyst assemblages locally in the North and Middle Caspian Sea, while the dinocyst community reflects perturbations related to nutrient load in the Western Black Sea and the North Caspian Sea. These results suggest that dinocysts can record anthropogenic disturbances, including shipping perturbations and further studies in the Pontocaspian region should consider exploring this approach in the future.

CHAPTER 4

EFFECT OF TEMPERATURE AND SALINITY ON THE GROWTH AND CELL SIZE OF THE FIRST CULTURES OF *GYMNODINIUM AUREOLUM* (DINOPHYCEAE) FROM THE BLACK SEA

*This chapter will address the **specific research goal 6** outlined in Section 1.5.2*

6. *To test the effects of temperature and salinity on the growth of newly established dinoflagellate cultures obtaining novel understanding in the adaptation and acclimatization ability of these species to present and future environmental scenarios in the Black Sea and their potential to generate blooms under these conditions.*

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4.1 Introduction

Algal blooms are natural or human-induced phenomena that may cause human health, millions of dollars in losses and ecological disasters worldwide (Hallegraeff, 1993; Anderson et al., 2012). The periodicity, intensity and geographical occurrence of such events have been suggested to increase worldwide in the last few decades (Anderson, 2009; Hallegraeff, 2010; Fu et al., 2012; Wells et al., 2015; Glober, 2020). It is an indication of general phytoplankton assemblages shifting towards harmful and bloom-forming species. Algal blooms are driven by environmental physicochemical conditions such as light, temperature and salinity, as well as stratification, nutrient availability and interspecific interactions such as competition and grazing (Paerl, 1997; Smayda, 1997; Juhl et al., 2000; Glibert et al., 2005; Heisler et al., 2008; Hallegraeff, 2010; Peacock et al., 2014; Wells et al., 2015). Alterations

in these parameters due to large scale perturbations such as global warming, ocean acidification and pollution have the potential to strongly alters the growth, distribution and dominance of phytoplanktonic organisms (Sellner et al., 2003; Fu et al., 2012). Therefore, it is of key importance to understand how the main environmental parameters affect the ecophysiology of harmful algae populations to properly assess the risk of algal blooms under changing environmental conditions.

The Black Sea is a eutrophic semi-enclosed sea connected to the Marmara Sea through the Bosporus Strait. Due to the hydrogeography of the region, large freshwater influx and narrow opening to the Mediterranean Sea through the Bosporus Strait, the Black Sea behaves as a typical estuarine basin, including rapid changes in salinity and temperature in surface waters (Stanev, 2005). The average values of temperature and salinity in the Black Sea are from ≈ 11 to 16 °C and from ≈ 10 to 21 psu respectively (Zaitsev et al., 2001). In the last century, the Black Sea has suffered increasing environmental pressure from human activities. Among the most serious outcomes of such activities is the increasing eutrophication state of the Black Sea basin due to nutrient discharge from rivers, mostly the Danube River (Bakan and Buyukgungor, 2000). Increasing eutrophication favours the growth and spatial expansion of microalgae species and may lead to shifts in the species dominance (Mikaelyan et al., 2015). Many of these species, identified as potential HAB, have been commonly reported in high abundances on the Black Sea coast in the last decades such as *Akashiwo sanguinea*, *Prorocentrum lima*, *Scrippsiella acuminata*, *Gymnodinium aureolum* as well as species of *Alexandrium* and *Dinophysis* (Velikova et al., 2005; Morton et al., 2009; Eker-Develi et al., 2012; Mikaelyan et al., 2015).

The dinoflagellate *Gymnodinium aureolum* (Hulburt) G. Hansen (syn: *Gyrodinium aureolum* Hulburt) is an unarmoured red-tide dinoflagellate species. Red-tide species such as *G. aureolum* are known for producing large blooms that may generate water discolouration, anoxia and foam production impeding commercial and recreational activities in the affected areas (Wardiatno et al., 2004; Zingone et al., 2006; Aissaoui et al., 2012). *G. aureolum* presents a global distribution and blooms have been reported worldwide including Chesapeake Bay, USA, (Tang et al., 2008), the coast of Korea (Jeong et al., 2010), Catalonian coast (Reñé et al., 2015) and the coast of South Africa (Botes et al., 2003) among other locations. Nevertheless, some of *G. aureolum* records have been under debate due to the complex

taxonomical history of this species. It was first reported as *Gyrodinium aureolum* by Hulburt (1957) from the coast of Massachusetts, USA. Hansen et al. (2000), after a detailed examination of previously reported isolates of *Gyrodinium aureolum*, concluded that many of these reports were, in reality, observations of *Karenia mikimotoi* (Miyake & Kominami ex Oda) (G. Hansen and Moestrup) = *Gymnodinium mikimotoi* (Miyake & Kominami ex Oda). In addition, after study of the cellular ultrastructure, SEM microscopy, pigment analyses and a new molecular phylogeny based on partial large subunit (LSU) rDNA sequences, of cells isolated from Rhode Island, USA, *Gyrodinium aureolum* was renamed *Gymnodinium aureolum* (Hulburt) G. Hansen (Hansen et al., 2000).

Gymnodinium aureolum was first reported in the Black Sea in 2002 and has been suggested as non-indigenous species likely introduced by shipping activities such as ballast water (Terenko, 2005). Like many dinoflagellate species, *Gymnodinium aureolum* can produce, during its life cycle, resting cysts with an organic wall (dinocysts) to overcome stressful conditions and inoculate the water column once the environmental conditions are favourable again (Tang et al., 2008). This mechanism allows many planktonic species to disperse in ballast water tanks. Since its first record, *G. aureolum* has been identified in both water and surface sediments in high abundances, reaching cell densities of 66,600 cells L^{-1}, along the coast of the Black Sea (Terenko, 2005 and 2007; Boicenco, 2010; Dzhembekova et al., 2018). *G. aureolum* can be classified as a euryhaline species, which is confirmed by observation of the species in marine environments (Hansen et al., 2000; Jeong et al., 2010b) as well as brackish locations such as the Baltic Sea (Hällfors, 2004) and the Caspian Sea (Lewis et al., 2018). Despite its global distribution and its ecological importance, only a few physiological studies have been conducted on how natural populations of *G. aureolum* respond to different biological and physicochemical parameters since the year 2000 (Jeong et al., 2010b; Yoo et al., 2010; Tang et al., 2008; Aissaoui et al., 2012). Thus, there are still uncertainties on how changing environmental conditions may affect the distribution and growth performance of this species.

In Autumn 2015, a research cruise from Constantza, Romania to the western coast of the Black Sea, with the research vessel *Mare Nigrum* with a focus on the examination of surface sediment and plankton samples. During this cruise, surface sediment samples were collected aiming to obtain living

dinoflagellate resting cysts (dinocysts) to establish new cultures of the current Black Sea dinoflagellate populations. This presents for the first time the opportunity to carry out a morphological and phylogenetic study of the Black Sea *Gymnodinium aureolum* population. In addition, this enables a detailed examination of the effects of temperature and salinity on this species growth providing new data on the capability of the species for adaption and acclimatization and its potential to perform blooms under present and future climatic scenarios in the Black Sea.

4.2 Material and methods

4.2.1 Sample collection, cell isolation and cultures

Surface sediment samples were collected using a Van Veen grab on the West coast of the Black Sea in October 2015 (Fig. 4.1). Samples were maintained at 5 °C and in the dark until further analyses. Five-gram samples of sediment were manually homogenised, using a stainless-steel spatula, and re-suspended using a vortex for two minutes in 50 mL of sterile artificial seawater (ASW) at 15 psu. The resulting slurry mixture was immediately passed through 120-20 µm nylon sieves. Afterwards, the residue fraction retained at 20 µm was recovered using 20 mL of f/10 medium without silica (Guillard, 1973), prepared at 15 psu from sterile ASW, and vortexed for one minute. The recovered fraction was then split between sterile Petri dishes containing each 2 mL of the residue mixture. The plates were placed in a light incubator (Model 230, LMS Ltd, UK) equipped with white fluorescent tubes at 15.0 ± 1.0 °C with a 14:10 h light:dark photoperiod and photon flux density of 80-100 µmol photons m^{-2} s^{-1}. The plates were checked for germinated cells using an inverted microscope (Olympus IX53) every second day. Non-axenic clonal cultures of *G. aureolum* were established by micro-pipetting single germinated cell from surface sediment samples under the inverted microscope to a 96-well polystyrene cell culture plate containing 200 µL of fresh sterile f/2 medium without silica (f/2-Si) adjusted at 15 psu. Successfully isolated cells were steadily moved up to different small Petri dishes until placed in 65-mL polystyrene tissue culture flasks. These cultures were kept under previously described conditions until analysis. The stock culture was kept in the exponential growth phase by transferring into fresh f/2-Si medium bi-weekly. The identification of *Gymnodinium aureolum* was first made with light microscopy (Olympus IX53) and thereafter, a sub-sample of the isolate Ga13A_1 from a culture

growing at exponential phase was processed for Scanning Electron Microscopy (Quanta 400, UK) detailed observation following the method described by Botes et al. (2002). Isolate identification was further confirmed with molecular characterization as detailed below.

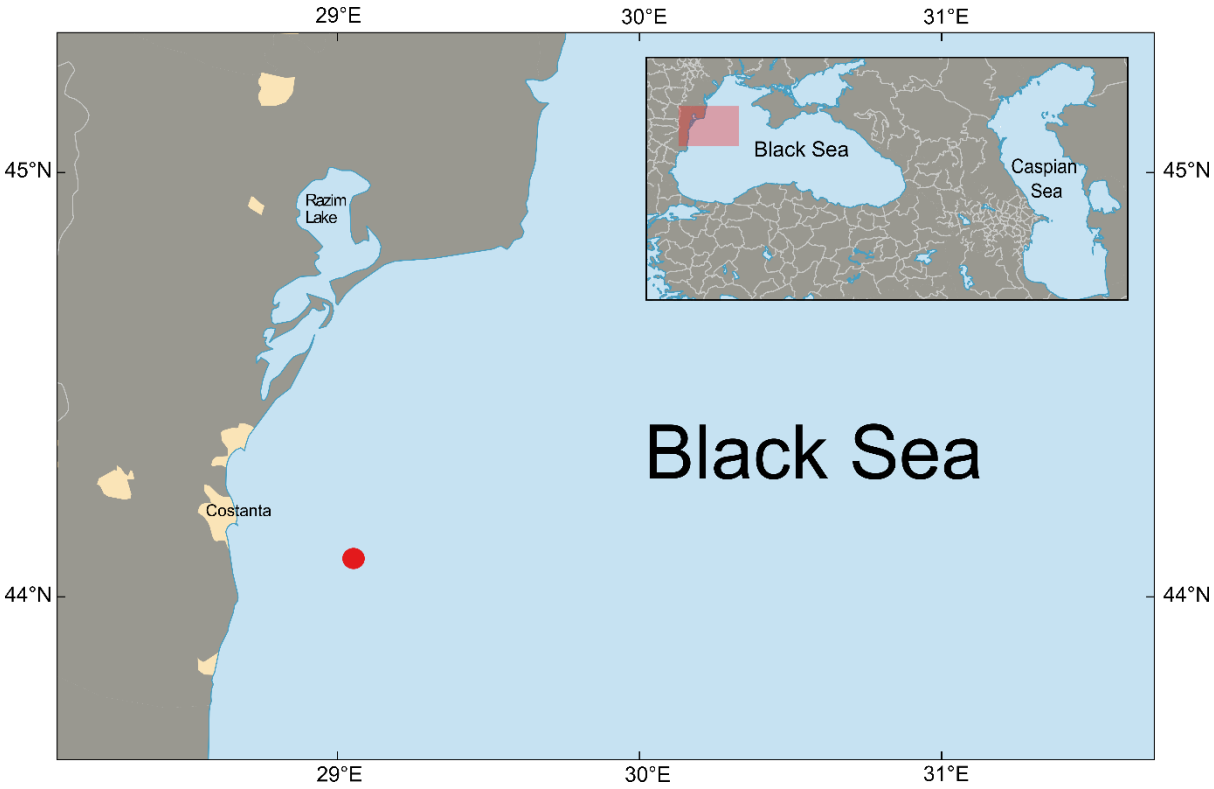


Fig. 4.1: Map of the study area. The red dot indicates the sampling station from where the cultures used in this study were isolated

4.2.2 Molecular identification and phylogenetic analysis

DNA was extracted from 50 mL of an experimental culture sampled during the exponential phase from isolates Ga13A_1 and Ga13A_3. Samples were centrifugated at 4,000 rpm for 15 min to concentrate the cells. Cell pellets were moved into 2 mL Eppendorf tubes and centrifuged again at 10,000 rpm for 10 min. The resulting pellets were processed using a DNeasy Plant MiniKit (Qiagen), following the instructions of the manufacturer to obtain total genomic DNA. The extracted DNA was immediately frozen at -20 °C. Amplification of an LSU rDNA gene fragment about 1,400 bases long was carried out with the primers D1R (Scholin et al., 1994) and 28-1483R (Daugbjerg et al., 2000) and the extracted DNA. PCR conditions were one initial cycle of denaturation at 95 °C for 5 min followed by 40 cycles each consisting of 1 min of denaturation at 95 °C, 1 min of annealing at 55 °C and 2 min of extension

at 72 °C. The cycle was completed by a final extension step at 72 °C for 7 min (Tang et al., 2007). PCR products were sequenced by Source BioScience Limited (Cambridge, UK). Forward and reverse sequences were assembled and trimmed in BioEdit v7.2 (Hall, 1999) and in order to identify previously published G. aureolum sequences a BLAST search was carried out in GenBank using the resultant sequences. Sequences of *G. aureolum* obtained from Genbank (Table 4.1) and sequences obtained from our Black Sea isolates were aligned together with additional sequences used by Jeong et al. (2010b) and unidentified *Gymnodinium* species from the Mediterranean Sea (Reñé et al., 2015) using CLUSTAL v1.2.2 (Sievers et al., 2011) using the R package APE v5.4. Alignments were manually checked in BioEdit v7.2 and limited in length to the sequences obtained from the Black Sea strains in a final alignment of about 550 pb. Phylogenetic relationships were determined using the RAxML (Randomized Axelerated Maximum Likelihood) v8.0 program (Stamatakis, 2014) with general time reversible (GTR) model as the substitution model. Bootstrap values were calculated using 1000 replicates and the consensus tree was prepared with the R software (The R Foundation for Statistical Computing 2018).

Table 4.1: Sequences used in phylogenetic analyses, their GenBank accession numbers and collection area.

Taxon	GenBank N°	Area
Akashiwo sanguinea	DQ156229	Singapore
Akashiwo sanguinea	AF260397	Denmark
Alexandrium minutum	AF318221	France
Gymnodinium aureolum	AY464687	False Bay, South Africa
Gymnodinium aureolum	FJ024697	Gulf of Naples, Italy
Gymnodinium aureolum	KJ508392	Bay of Biscay, France
Gymnodinium aureolum	KY921624	Caspian Sea
Gymnodinium aureolum	KY921616	Caspian Sea
Gymnodinium aureolum	KP790183	Catalan coast, Spain
Gymnodinium aureolum	AY263965	Port River, Australia
Gymnodinium aureolum	AF200671	Denmark
Gymnodinium aureolum	AF200670	Denmark
Gymnodinium aureolum	AY947659	New Zealand
Gymnodinium aureolum	AY947661	Wedge Point, New Zealand
Gymnodinium aureolum	AY947660	Kawau Island, New Zealand

Gymnodinium aureolum	KX035106	St. Martin River, USA
Gymnodinium aureolum	DQ917486	USA
Pelagodinium beii	DQ198075	New Jersey, USA
Gymnodinium catenatum	AF200672	Denmark
Gymnodinium fuscum	AF200676	Denmark
Gymnodinium impudicum	AF200674	Denmark
Gymnodinium litoralis	JN400081	Muga River, Spain
Gymnodinium myriopyrenoides	AB591416	Wakayama, Japan
Gymnodinium palustre	AF260382	Denmark
Gymnodinium sp. isolate AR251	KP790189	Catalan coast, Spain
Gymnodinium sp. isolate AR252	KP790190	Catalan coast, Spain
Gymnodinium venator	AY455681	Denmark
Gyrodinium dominans	AY571370	Denmark
Karenia brevis	AF200677	Denmark
Karenia mikimotoi	U92247	New Zealand
Karenia mikimotoi	AF200678	Denmark
Lepidodinium chlorophorum	AF200669	Denmark
Levanderina fissa	AY916541	Tasmania, Australia
Polykrikos kofoidii	EF192411	Tasmania, Australia
Polykrikos schwartzii	EF192408	Tasmania, Australia
Prorocentrum donghaiense	AY822610	Qingdao, China
Takayama helix	AY284950	Tasmania, Australia
Takayama pulchellum	U92254	Kawau Island, New Zealand
Woloszynskia tenuissima	AY571374	Denmark

4.2.3 Growth experiments

For the purpose to study the growth performance of Black Sea *G. aureolum* to varying temperature and salinity, the isolate Ga13A_1 was selected for meticulous growth experiments combining two temperatures (15 and 20 ± 1.0 °C) and five salinities (5, 10, 15, 20 and 25 psu) in ten experimental treatments. Cultures were gradually pre-adapted to the experimental conditions by levels of five salinity units/degrees to each experimental treatment previous inoculating the experimental cultures. Thus, GA13A_1 cultures, which were routinely kept at 15 °C and 15 psu, were moved to 10 and 20 psu at 15

°C. Cultures at 20 psu/15 °C and 10 psu/15 °C were then placed at 25 psu/15 °C and 5 psu/15 °C after adaptation. The same procedure was repeated for experimental treatments at 20 °C. Cultures were maintained at the experimental conditions at exponential phase for at least 10 generations before inoculating the experiment cultures (Sala-Pérez et al., 2016). At each experimental treatment, cultures were prepared by triplicate in 65 mL tissue flasks in f/2-Si medium at an initial cell density of approximately 200 cells mL^{-1} at photon flux density of 80–100 µmol s^{-1} m^{-2} 14:10 h light:dark photocycle. Based on the growth rate, sub-samples were obtained from each replicate in intervals of two to four days for cell counts. To ensure homogenous cell distribution, before each sub-sampling cultures were cautiously homogenized by hand.

Cell concentration was calculated by cell counting using a sedimentation chamber (Sedgewick Rafter chamber) with an inverted microscope (Olympus IX53) using Lugol's solution fixed culture sub-samples at 1% final concentration. Subject to the cell density of each culture at the moment of the sampling, the final volume extracted to estimate cell concentration ranged from 0.1 to 1 mL to ensure that the final counted cells were always >200. Cultures were maintained until the stationary phase was confirmed (defined by no increase in cell concentration in three consecutive cell counts). Growth rate (µ, day^{-1}) was estimated individually for every experimental replicate for a delimited period identified as exponential phase. Growth rate calculations of each isolate was carried out by exponential regression of cell concentration throughout time. Growth curves of cultures at each experimental treatment were obtained from mean cell concentration of the triplicate cultures (Sala-Pérez et al., 2016).

Cell size was calculated by sub-sampling replicate cultures of each experimental treatment at exponential phase with cell concentration of 2000-2500 cells mL^{-1} approximately. Sub-samples were then fixed with Lugol (1% final concentration) and cells were observed using an inverted microscope and photographed at 400x magnification with a digital camera (Digital EyeCam Plus, Brunel Microscopes Ltd, UK). Cell size was measured using the analysis tool of the EyeCam Plus software (Brunel Microscopes Ltd). Length and width were measured for a total of 50 cells from each of the experimental treatment combining 5 mL of each replicate culture at each experimental treatment.

4.2.4 Statistical analyses

Analysis of the variance (ANOVA) and Tukey's HSD post-hoc tests were carried out to test the effect of our experimental treatments on the growth rate and cell size of *G. aurealum* isolate Ga13A_1. Previous to this, Shapiro-Wilk's test and Bartlett's test were applied to test normality and homogeneity of variance assumptions respectively.

4.3 Results

4.3.1 Identification, morphologic and molecular characterization of G. aureolum

Gymnodinium aureolum was usually observed as single cells, but pairs of cells were also observed. Cells of *G. aureolum* isolate GA13A_1 and GA13A_3 from the Black Sea were generally ovoid, slightly longer than wide and lightly dorsoventrally flattened but fluctuating in cell form and size. Chloroplasts shape was elongate and irregular while colour was variable from yellow-orange to brownish. The mean cell length of all measurements (all treatments) was 25.1 ± 2.5 µm, 20.1 ± 2.6 µm for cell width and 1.26 ± 0.12 for the cell length/width (L/W) ratio (n = 400). Statistical analyses show significant differences among experimental treatments for cell size measurements (i.e. for cell length and for length/width (L/W) ratio at experimental temperatures F= 62.8, $p < 0.0001$ and F= 5.7, $p < 0.05$, respectively). In addition, cell size was significantly affected by the salinity treatments as well (i.e. cell length/width ratio F = 9.0, $p < 0.0001$) (Table 4.2). The cingulum is wide and median and shows a displacement of one to two times its width and the sulcus was narrower towards the apex. Cells presented horseshoe-like apical groove, distinctive for this species, originating at the end of the sulcus surrounding the apex counter-clockwise, and coming near without reconnecting with its origin was observed in *G. aureolum* cells (Fig. 4.2).

Table 4.2: *Gymnodinium aureolum* cell size of isolate GA13A_1 at experimental treatments (temperature and salinity) at exponential phase. Cell length (µm) and the cell length and width ratio (ratio L/W) are presented. Values are calculated using the mean of 50 cell measurements combining three replicates for each experimental treatment (± SD). Letters indicate homogenous grouping among experimental treatments (ANOVA, Tukey's HSD test, $p < 0.05$).

		Length	Ratio L/W
Temperature (°C)	Salinity (psu)		
15	10	24.6 ± 2.3^{ab}	1.31 ± 0.13^{a}
15	15	25.8 ± 2.6^{a}	1.27 ± 0.13^{a}
15	20	27.3 ± 1.8^{b}	1.31 ± 0.13^{a}
15	25	26.4 ± 2.4^{b}	1.20 ± 0.09^{b}
20	10	25.6 ± 1.7^{cd}	1.25 ± 0.12^{c}
20	15	23.0 ± 1.7^{c}	1.26 ± 0.09^{c}
20	20	24.0 ± 2.0^{d}	1.24 ± 0.12^{c}
20	25	24.6 ± 2.4^{d}	1.21 ± 0.11^{d}

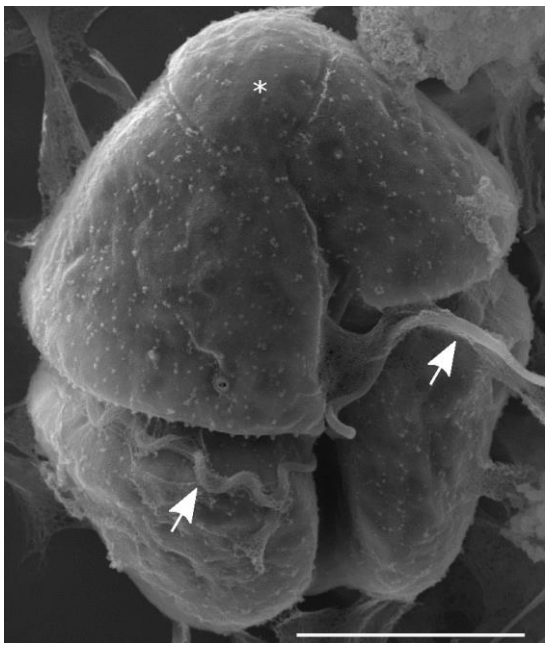


Fig. 4.2: SEM micrograph of ventral view of *Gymnodinium aureolum* isolate GA13A_1. Scale bar = 10 µm. Arrows indicate the cingulum and its flagella. * indicates the apical horseshoe-like acrobase on the apex of the cell.

Phylogenetic trees generated from the dinoflagellate sequences of LSU rDNA indicate that these dinoflagellate isolates belong to the *G. aureolum* clade. Figure 4.3 shows all the *G. aureolum* strains clustered together (100 %) and are distant from the *Karenia mikimotoi* strains that form their own cluster (98 %). Based on morphological and molecular analyses, the isolate Ga13A_1 and Ga13A_3 clearly are *Gymnodinium aureolum*.

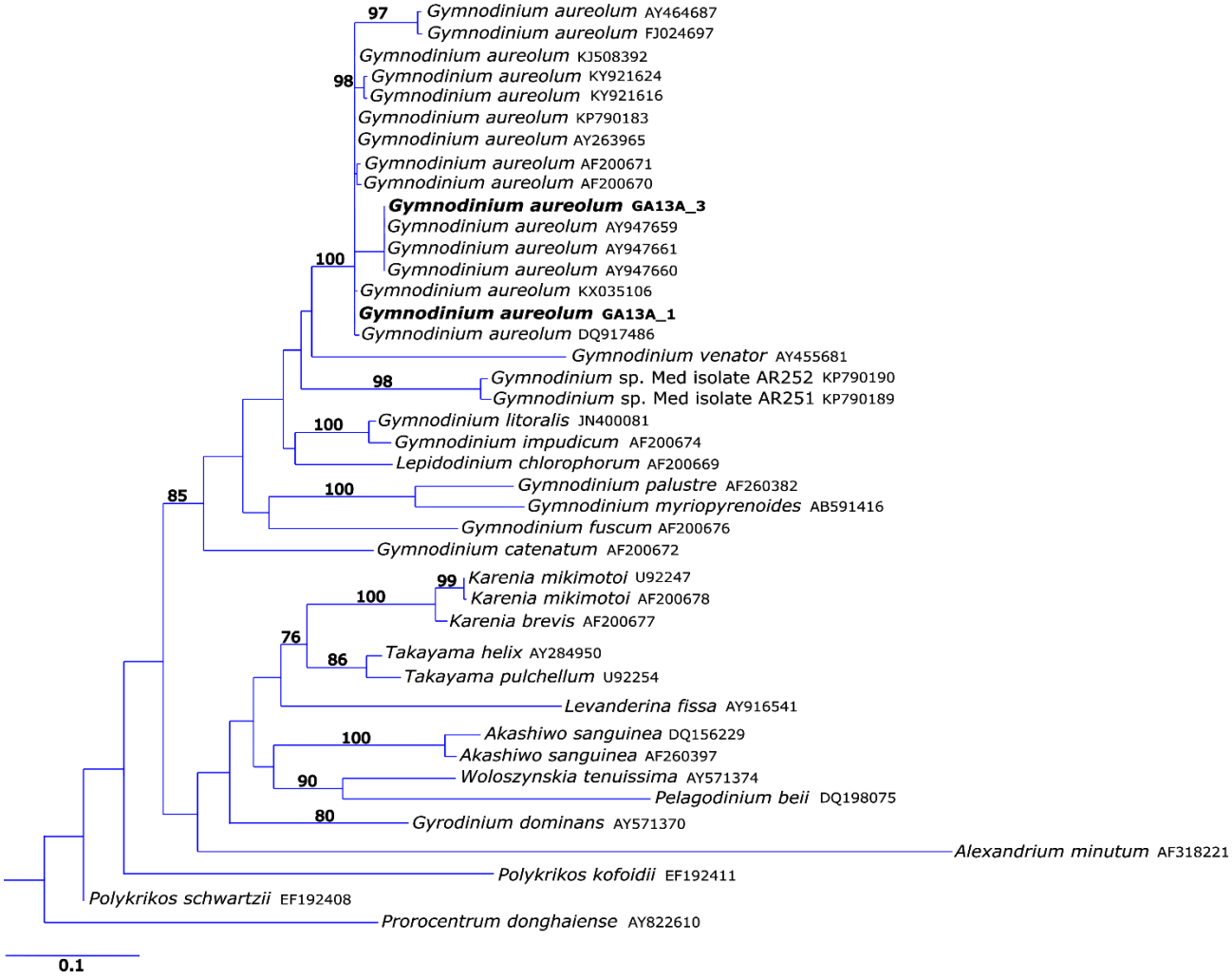


Fig. 4.3: Maximum-likelihood phylogenetic tree of compiled sequences of LSU rRNA alignments for dinoflagellates species. The bootstrap (%) values are displayed on the nodes. Only bootstrap values >75% are shown. *Prorocentrum donghaiense* sequence was used as outgroup. Organisms isolated from the Black Sea and sequences obtained in this work are highlighted in bold. GenBank accession numbers for the new Black Sea isolates GA13A_1: MW159715 and GA13A_3: MW159716.

4.3.2 Growth of Gymnodinium aureolum

Cells of *G. aureolum* isolate Ga13A_1 grew exponentially at all experimental temperatures and salinities, with the exception of the cultures kept at 5 psu, in which cell concentration quickly declined and completely vanished fifteen days after the inoculation. All cultures that performed exponential

growth reached stationary phase after 15 to 17 days of incubation and 10 to 12 days of incubation at 15 °C and at 20 °C respectively. Mucus accumulation was observed in all the cultures that reached stationary phase but not during the exponential growth phase. The maximal cell density at stationary phase ranged from 3 to 6 x 10^3 cells mL^{-1}. Cultures grown at 20 °C and 15 psu, displayed a progressive divergence from the maximum exponential growth towards linear growth before reaching the stationary phase. In addition, cultures grown at 15 °C and 25 psu displayed an adaptation stage at the initial exponential growth with almost unnoticeable increase in cell densities (Fig. 4.4).

Growth rate rose significantly with increasing temperature (Fig. 4.5) displaying the highest at 20 °C and 15 psu (0.38 ± 0.02 d^{-1}) nearly followed by the cultures incubated at 20 °C and 25 psu (0.34 ± 0.03 d^{-1}). Cultures at 15 °C and 25 psu displayed the lowest growth rate (0.23 ± 0.02 d^{-1}), while our statistical analyses have shown that growth rate was significantly altered by temperature and salinity ($p > 0.05$; Fig. 4.5).

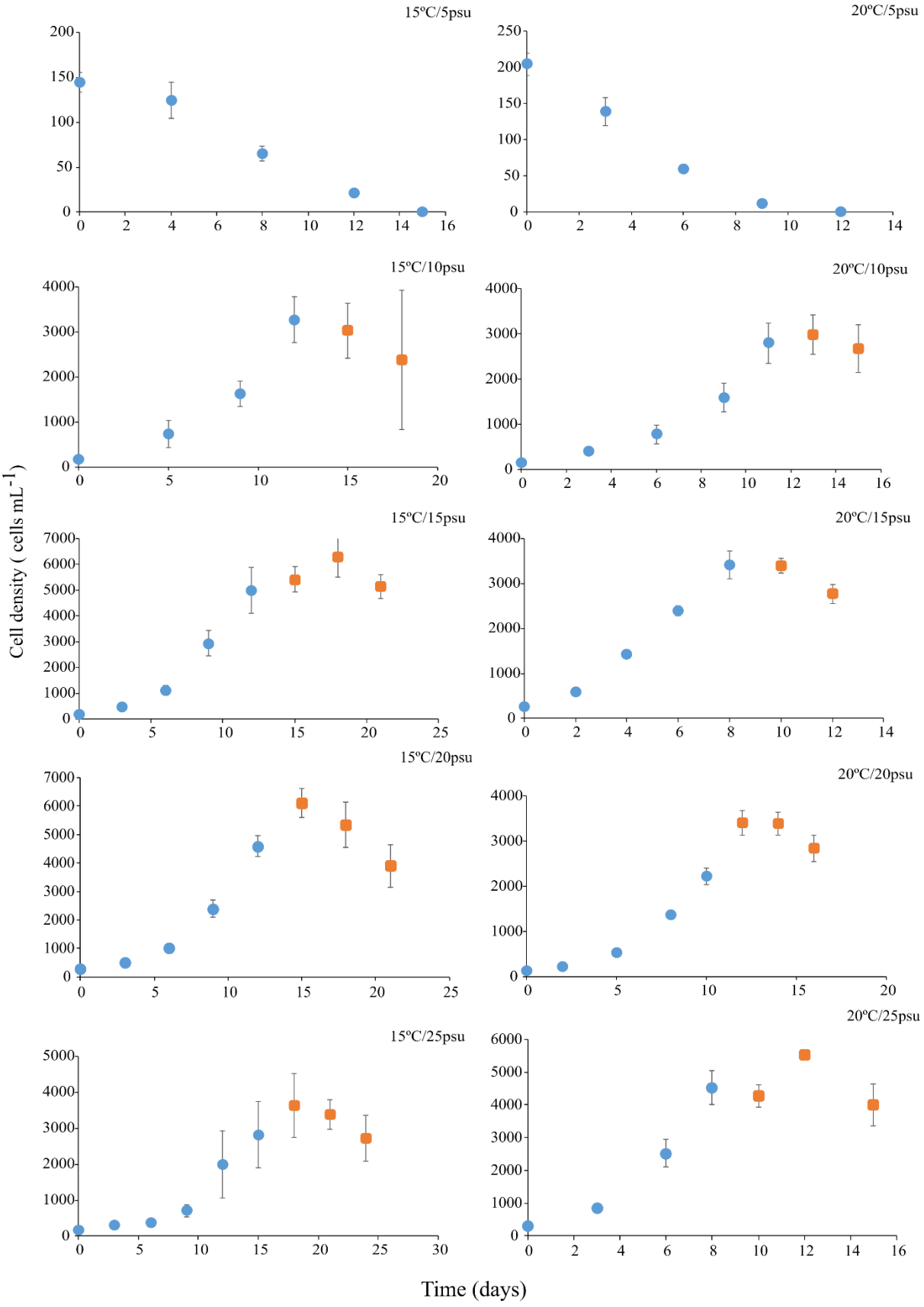


Fig. 4.4: Growth curves of *Gymnodinium aureolum* clonal isolate GA13A_1 at different experimental treatments (temperature and salinity are indicated above of each plot). Data points are calculated by mean cell concentration of three replicates (± SD). Blue dots were used for exponential growth rate calculations while orange square dots indicate stationary phase.

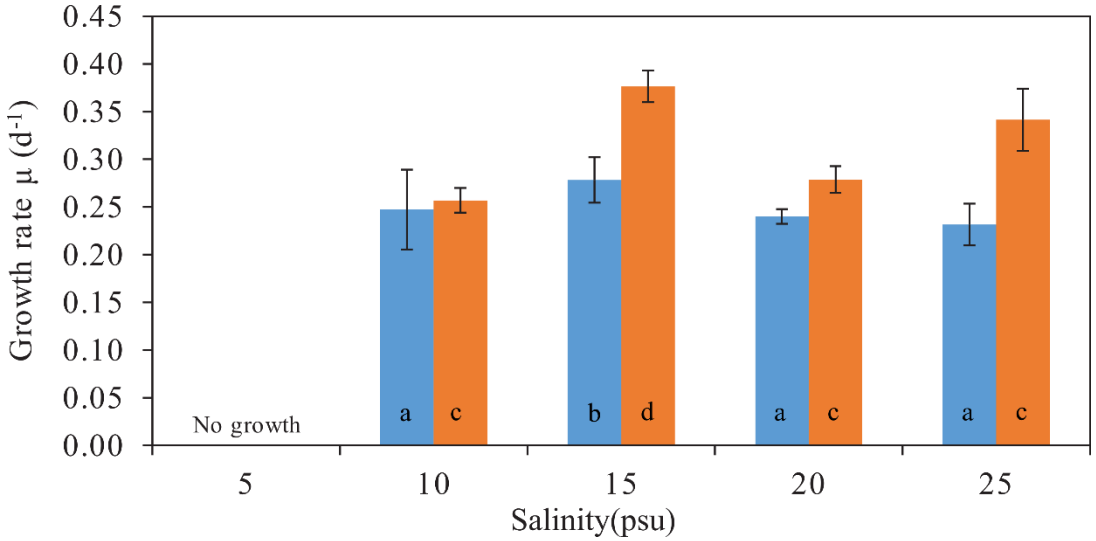


Fig. 4.5: Growth rate (μ, d^{-1}) of *Gymnodinium aureolum* (isolate GA13A_1) for each experimental treatment. Plot bars illustrate the mean of triplicates cultures (± SD). Blue bars represent experiments carried out at 15 °C while orange bars represent experiments carried out at 20 °C. Letters represent significant differences between experimental groups (ANOVA, Tukey's HSD test, $p < 0.05$).

4.4 Discussion

4.4.1 Gymnodinium aureolum isolated from the Black Sea

Several studies have documented observations of either *G. aureolum* theca or its cyst in the Black Sea (Boicenco, 2010; Dzhembekova et al., 2018). The Black Sea isolate as verified by morphological and phylogenetic analyses (Fig. 4.2 and 4.3) are the first cultures established and confirmed of *Gymnodinium aureolum* from the Black Sea waters to our knowledge. *G. aureolum* is not known to be a toxin producing dinoflagellate. Nevertheless, *Karenia mikimotoi*, which is a known toxin producer (Mitchell and Rodger, 2007; Chen et al., 2011; Li et al., 2019), presents a similar cell morphology to *G. aureolum* requiring high taxonomical expertise to accurately differentiate these species from each other. This differentiation was only resolved in the year 2000 (Daugbjerg et al., 2000; Hansen et al., 2000). Records and laboratory experiments previous to these publications are not used in this discussion due to the risk of confusion between these two different species. Therefore, only studies after the year 2000 are used for comparison and discussion with the results.

4.4.2 Morphology and phylogeny of Gymnodinium aureolum

Morphological and phylogenetic analyses identified our strain as *Gymnodinium aureolum*. Cell size range at exponential phase varied among all experimental treatments but in agreement to the size ranges published previously from Chesapeake Bay, USA (Hansen et al., 2000; Tang et al., 2008) and Korea (Jeong et al., 2010b) for exponential photosynthetic growth, as well as the cell shape being longer than wider (Table 4.2 and 4.3). However, the average cell size reported by Terenko (2005) from Odessa Bay, Ukraine (L = 42 and W= 36 µm) is slightly bigger than the cell size reported in this study, unfortunately, only average length and width, but not range were reported in that study. Moreover, average size reported by Terenko (2005) is based on field observations, which may include cells in stationary phase that would increase the cell size average (see discussion below). In addition, *G. aureolum* has been described previously by Hansen (2001), Tang et al. (2008) and Jeong et al. (2010) to present a characteristic horseshoe-like apical groove that can be clearly appreciated here in Fig. 4.2. Although cell size at all experimental treatments fits previously published cell size data, our results show significant differences in cell size between experimental treatments (Table 4.2). This might be because of differences in growth rate at each treatment. Slower cell division might result in an accumulation of intracellular products and thus a slightly larger cell. This phenomenon has been previously reported to produce differences in cell size between exponential and stationary phase (when cell divisions stop but cellular products are still being synthesized) for dinoflagellate species (Sala-Pérez et al., 2016). Our experiments show a trend of significantly smaller cells at higher temperature treatments as well as an increase in the growth rate (Table 4.2, Fig. 4.5). In addition, cultures used in this study were established from resting cysts, and consequently, despite the fact that, no cyst formation was observed in our cultures, it was not possible to confirm if the excysted cells were vegetative cells or planomeiocytes. This could have influenced the cell size range measured since at different life stage, cells such gametes and planozygotes can present different cell size and morphological features from vegetative cells (Persson et al., 2013; 2021).

Table 4.3. *Gymnodinium aureolum* cell size (length and width range) of isolate GA13A_1 from the Black Sea compared to previously published strains from Chesapeake Bay, USA (Tang et al. 2008), Pettaquamscutt River, USA (Hansen et al. 2000) and western Korea (Jeong et al. 2010).

Isolate	Length (µm)	Width (µm)
Black Sea	18.4–32.3	14.0–27.5
Tang et al. 2008	14–47	11–43
Hansen et al. 2000	18.9–38.7	13.9–32.7
Jeong et al. 2010	18.0–28.4	11.6–21.2

The sequences of LSU rDNA of the strains isolated from the Black Sea waters were the same as the isolates of *G. aureolum* from South Australia (de Salas et al., 2003), the Bay of Biscay, France (Nézan et al., 2014), the Catalan coast, Spain (Reñé et al., 2015), for the isolate GA13A_1 and the strains isolated from New Zealand waters (de Salas et al., 2005; Fig. 4.3) for the isolate GA13A_3. Black Sea strains are conspecific with other *G. aureolum* strains isolated elsewhere, which aligns with previous studies confirming *G. aureolum* as a worldwide-distributed species with constant presence in estuary areas. However, the phylogenetic analyses of the LSU sequences show intraspecific variability among the sequences of *G. aureolum* (differences of 1 to 19 bp) despite all sequences are clustered in a well-supported clade (Fig. 4.3). These differences are larger than differences in base pair number reported from Tang et al. (2008) and Jeong et al. (2010) for LSU rDNA sequences. In contrast with these authors, the molecular analyses of this study included up to seven more *G. aureolum* sequences isolated from elsewhere (Table 4.2, Fig. 4.3). These results suggest that *G. aureolum* clade can potentially be a species complex. To test this, phylogenetic analyses should be expanded using additional genes such as SSU, ITS or COI including all the possible sequences available for *G. aureolum* together with an exhaustive morphological examination of geographically distant populations' specimens to assess if these differences are defined by geographical origin as in the case of *Alexandrium tamarense* complex (Lilly et al. 2007).

4.4.3 Growth of Gymnodinium aureolum

Many studies on phytoplankton population dynamics and ecology used growth rate as a parameter to measure the effect of any environmental parameter on organism performance and adaptation to the

environment (Caperon and Meyer, 1972; Larsen and Bryant, 1998; Sathyendranath et al., 2009; Edwards et al., 2012). Growth rate, defined as changes in the number of cells or biomass over time, summarizes various physiological processes as a single output allowing inter- and intra- specific comparisons. This is of particular importance in the case of dinoflagellate cultures. Dinoflagellates are haploid organisms, thus clonal cultures, genetically identical individuals, will not produce new genotypes, which allows direct comparison of species or population performance between cultures (Haapala and Soyer, 1974; Lakeman et al., 2009). Temperature and salinity are not only two of the main parameters constraining a species distribution but also defining its potential growth. Due to the characteristics of the Black Sea, salinity is certainly the main parameter delimiting species ecology. *G. aureolum* isolated from the Black Sea shows the highest growth at 15 psu and no significant differences were found in growth rate between treatments at 10, 20 and 25 psu (Fig. 4.4), which might suggest a generally better adaptation of *G. aureolum* to Black Sea salinity than full marine salinity. In addition, *G. aureolum* shows no growth at 5 psu for any of the two experimental temperatures, but cells survived for 10-15 days. This indicates that *G.aureolum* has the ability to survive for long periods at conditions not sustaining growth, which may favour its wide distribution and its ability to spread to new environments. This ability to overcome low and brackish salinity conditions concurs with the observations that *G. aureolum* has been reported from the Baltic Sea (Hällfors, 2004) and the Caspian Sea (Lewis et al., 2018) as well as its presence in high densities from estuarine environments such as Chesapeake Bay (Tang et al. 2008).

Unfortunately, to our knowledge, there are no published studies after solving the taxonomy of *G. aureolum* (Daugbjerg et al., 2000; Hansen et al., 2000) testing growth rate of *G. aureolum* under different conditions except the work by Jeong et al. (2010b). This work carried out growth experiments with a *G. aureolum* strain isolated from the west coast of South Korea at 20 °C and 30.5 psu as experimental conditions. The growth rate of *G. aureolum* measured at these conditions was 0.101 ± 0.014 d^{-1}, although direct comparison can only be indicative due to slightly different conditions, it was significantly lower than the growth rate measured for the Black Sea isolate at 20 °C and 25 psu (0.342 ± 0.033 d^{-1} Fig. 4.5). This concurs with previous studies showing high intra-specific variability in

growth performance among dinoflagellate species (Costas, 1990; Bachvaroff et al., 2009; Sala-Pérez et al., 2016).

G. aureolum has a considerably extensive distribution, however, most of the published observations of this taxon are from temperate areas. In addition, *G. aureolum* blooms have been detected exclusively in such areas, for example, bloom in the Gulf of Tunis (Aissaoui et al., 2012), a bloom in western Korea (Jeong et al., 2010b), and a bloom in Shiwha Bay, Korea (Kang et al., 2013), which all developed in water temperatures of 10-27 °C and salinity values 13-38 psu supporting our results that *G. aureolum* is a euryhaline species with potential to form blooms in the Black Sea. In addition, accumulation of mucus was observed in cultures at stationary phase, which may be indicative of the potential of this population to cause disruptions in the Black Sea habitat in case of large blooms formation. Further studies comparing multiple strains of this species are recommended to be carried out to assess the ability of these populations to produce mucus, what factors may influence this physiological feature and its impact on the ecosystem.

The growth rate measured in this study is due to the photosynthetic activity of *G. aureolum*. However, as with many species of dinoflagellates, *G. aureolum* is proven to be a mixotrophic species, which can increase its growth rate by preying on other organisms while performing photosynthesis as demonstrated by Jeong et al. (2010b). Since in natural conditions several species of phytoplankton co-exist, it is reasonable to expect that the isolated *G. aureolum* from the Black Sea might demonstrate a higher growth rate in the natural environment when its prey is available. Although our results allow us to obtain for the first time an idea of the potential of the *G. aureolum* population in the Black Sea to grow under different environmental scenarios, it is known that dinoflagellate populations can show wide intraspecific variability in the nature for different physiological traits such as growth rate (Brand, 1981; Costas, 1990; Sala-Pérez et al., 2016). Therefore, additional clone cultures should be established to assess an average growth rate for this species in the Black Sea.

4.5 Conclusions

This work presents the first clonal cultures of *Gymnodinium aureolum* isolated from the Black Sea. Both *G. aureolum* isolates grouped in the same widespread phylogenetic clade with other *Gymnodinium aureolum* strains. The growth performance of one isolate over a range of experimental treatments combining salinity and temperature with the highest growth rates at 15 psu and 20 °C usually indicates an adaptation of the Black Sea population to the Black Sea salinity and temperate waters and therefore probably a long-established population introduced from other regional populations. This, and previous records of *G. aureolum* in both Black Sea waters and sediments, support the idea that this may be a bloom-forming population of *G. aureolum*.

CHAPTER 5

Implementation of NGS and DNA barcoding to study the biodiversity and biogeography of the Black Sea microeukaryotic communities

T*his chapter will address the* ***specific research goals 2,*** *outlined in Section 1.5.1,* ***4*** *and* ***5*** *outlined in Section 1.5.2.*

2. *to investigate the distribution and diversity patterns of dinoflagellates in different ecological settings across the Black Sea at an intermediate spatial scale using NGS and DNA barcoding methods to study dinoflagellate assemblage changes along environmental gradients.*
4. *To explore the relationship between environmental variables and dinoflagellate biogeography*
5. *To study the contribution of environmental factors and geographical distance in controlling the dinoflagellate community composition.*

Bioinformatics analyses presented in this Chapter were carried out using QIIME pipeline and the Eukaryotic ITS database developed by ***Dr Gary Baker*** *of the University of Bristol, who also assisted with running the analyses in QIIME and the interpretation of the OTUs recovered results. The methodology follows the methodology published in Winfield et al. (2018).*

This Chapter is written with the intention to submit to *Aquatic Microbial Ecology*. The version presented here presented includes the ITS sequences recovered and included in the bioinformatics analyses as supplementary material in **Appendix C** accessible by a link to the University of Bristol services for large data files (FLUFF). For the manuscript preparation, these sequences will be submitted to GenBank for public access and accession numbers will be provided in the manuscript.

5.1 Introduction

Unicellular eukaryotes, or protists, are important components of all aquatic ecosystems, playing diverse key roles as primary producers, consumers, decomposers and parasites (de Vargas et al., 2015; De Long, 2009). Moreover, protist taxa may produce harmful algal blooms (HABs), which can negatively impact ecosystems and public health. For example, dinoflagellate genera such as *Alexandrium*, *Gymnodinium* and *Lingulodinium*, can often form dense blooms in coastal areas. Toxin-producer species of these taxa can develop blooms that may produce significant impacts in the environment, including fish or even mammal mortality events and shellfish poisoning that threaten human health and produce large economic losses; for example in the U.S.A., it is estimated that HABs caused an average annual loss of US$ 75 million over the period 1987 to 2000 (Anderson, 2009). Therefore, it seems of great importance to understand how environmental variables influence changes in the diversity and biogeography of these groups.

Extensive literature is available, in which traditional taxonomical studies identify protist species based on morphological features. This requires a high level of expertise and time to be accurate and many examples of cryptic protist species or morphologically different types of the same species are documented (Montresor et al., 2003). Recently, different species, morphologically identical, of the toxic marine dinoflagellate *Ostreopsis* were recognised during a HAB event using Quantitative PCR methods that considerably facilitate the monitoring efforts of such events (Hariganeya et al., 2013). Throughout the last decades the development of new molecular technologies such as high-throughput sequencing (HTS) and DNA barcoding, named metabarcoding, has enabled to reduce the sampling processing time and to increase accuracy and resolution of microorganisms identification in biodiversity studies and for monitoring purposes, revealing up to one-third of unknown operational taxonomic units (OTUs, de Vargas et al., 2015). These methods generate massive amounts of sequences as a result; these sequences are clustered into bins called ‘Operational Taxonomic Units’ (OTUs) based upon similarity above 97 % at least, which enhanced the computational analyses to study diversity and abundance of biological communities (Nam-Phuong et al., 2016).

This study focuses on dinoflagellates, one of the most functionally important and diverse groups of microeukaryotic organisms in aquatic ecosystems and the main contributor to HABs worldwide (Granéli and Turner, 2006). Dinoflagellates are a group with highly diverse morphological, physiological and biochemical features; can present armed or naked cells and play diverse roles in the food web as phototrophs, heterotrophs, mixotrophs and endosymbionts (Taylor et al., 2008). HABs are driven by environmental physicochemical conditions such as temperature, salinity, nutrient availability and interspecific interactions (Juhl et al., 2000; Hallegraeff, 2010; Wells et al., 2015). Alterations in these parameters due to global perturbations such as global warming, ocean acidification and pollution can potentially alter the growth, distribution and dominance of these phytoplankton taxa (Fu et al., 2012). Thus, studying patterns in dinoflagellate's diversity in relation to the impact of environmental variables on the community composition seems key to understanding microeukaryotic communities.

Dinoflagellate blooms and HABs events are predicted to increase in the near future as a consequence of rising temperature, which allows species to expand their geographical range, and changes in nutrient input in coastal areas altering the size and composition of the nutrient concentration and nutrient species which consequently, has created a more favourable environment for certain HAB species (Anderson, 2009). The Black Sea presents an ideal natural laboratory to study the effects of rapid environmental changes on the biota due to its unique geological history and environmental setting. Near future scenarios, predict dryer and warmer conditions for the Black Sea, which likely will lead to higher sea surface temperature and salinity (Ludwig et al., 2010). This, together with enriched-water conditions may favour the growth and shift in dominance toward certain harmful species (Anderson, 2009; Fu et al., 2012). So far, there are only a handful of investigations in the Black Sea using molecular methods such as HTS to determine patterns in diversity and biogeography (Dzhembekova et al.,2017; 2018). Therefore, the vast majority of diversity assessments on dinoflagellates for this area has been based on traditional taxonomical studies under light microscopy. It is anticipated that the use of HTS and metabarcoding techniques will increase the baseline knowledge of dinoflagellate diversity and biogeography that would support monitoring efforts.

This study aims to investigate the distribution of microeukaryotic communities in surface waters of coastal sites of the Black Sea using the Ribosomal Internal Transcribed Spacer (ITS, eDNA) gene amplicon sequencing to (i) describe the biogeography patterns of microeukaryotic assemblages, focused on dinoflagellates, in typical coastal areas of the Black Sea; (ii) test the influence of environmental factors on dinoflagellate community structure; and (iii) study the contribution of environmental factors and geographical distance in controlling the dinoflagellate assemblages.

5.2 Material and methods

5.2.1 Study area

The Black Sea is the largest semi-enclosed sea in the world. It is connected to the Mediterranean Sea through the narrow Bosphorus Channel. The Black Sea has unusual environmental conditions such as a positive net freshwater balance and over 90 % of the basin deepwater volume consists of anoxic water. Some of Europe's largest rivers flow into it, including the Danube and the Dnieper. As a result, the Black Sea presents average values of temperature and salinity ranging from ≈ 11 to 16 °C and from ≈ 10 to 21 psu respectively (Zaitsev et al., 2008). This environmental setting offers an excellent opportunity to study biodiversity patterns along salinity gradients. For this purpose, four sampling zones along the Black Sea coast were defined according to their environmental setting: proper Black Sea (BS), Razim-Sinoe Lake complex (RS), Dnieper Estuary (Dn) and Paliastomi Lagoon (Pa) (section 2.1.2.1, Fig. 5.1).

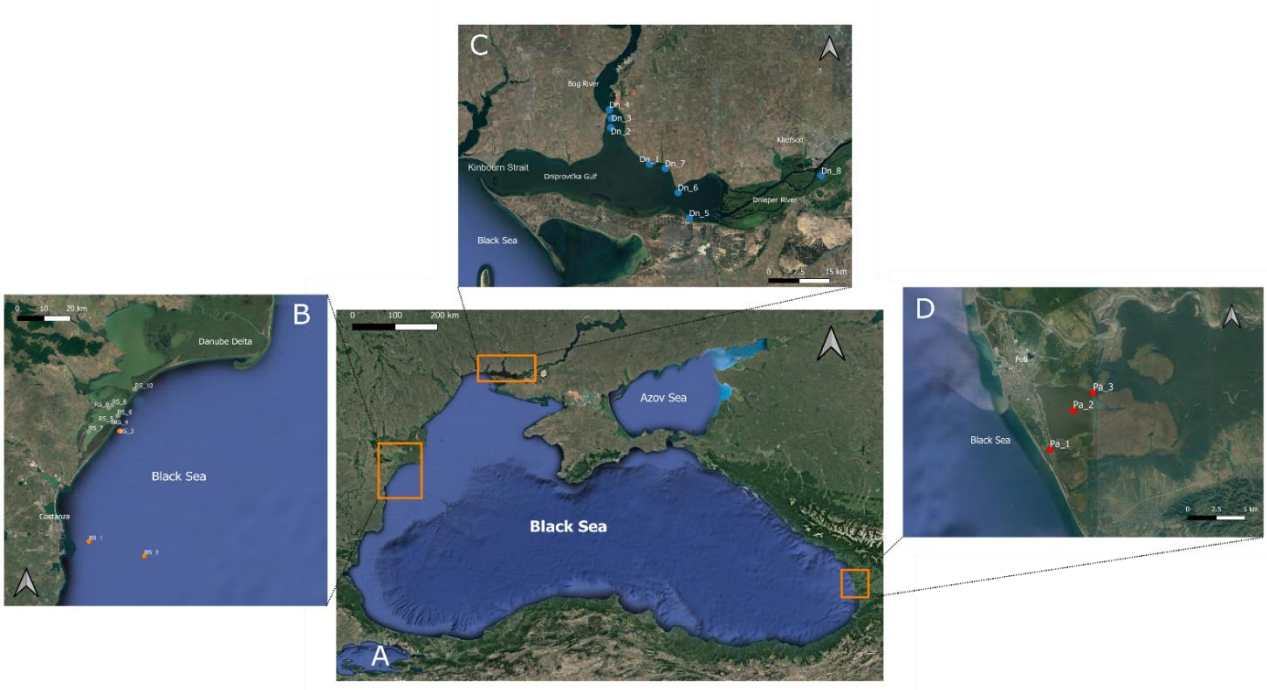


Fig. 5.1: Maps of the study areas. 1A: The Black Sea. Orange rectangles mark three sampling areas. 1B: Location of the stations sampled in the open Black Sea (BS orange dots) and Razim-Sinoe Lake complex (RS grey dots). 1C: Location of the stations sampled in the Dnieper Estuary (Dn blue dots). 1D: Location of the stations sampled in the Paliastomi Lagoon (Pa red dots).

5.2.2 Sampling collection

Twenty-eight water samples were collected from twenty-one stations across four coastal zones of the Black Sea, including nine samples from the Black Sea (BS) near Constantza port, Romania; eight samples from the Razim-Sinoe Lake complex (RS), Romania; eight samples from Dnieper Estuary (Dn), Ukraine and three samples from Paliastomi Lagoon (Pa), Georgia (Table 5.1, Fig 5.1). All stations were sampled with a 20-µm-mesh Nitex plankton net, except samples from stations BS_1 and BS_2 where plankton nets were not available and water samples were obtained at different depths by Niskin bottles. Where the water depth at stations was >3 m deep, vertical net tows were collecting samples from all areas of the water column from one meter about the bottom to the surface covering the first 20 m of the water column. At stations where the water was < 3 m deep samples were collected by horizontal tows run for two minutes at a speed no higher than 2 m/s. Net tows concentrate (at least 30 mL) were transferred to sterile conical tubes and fixed with Lugol's solution (1% final concentration) until further analyses were performed. The net was washed at each station before sampling to avoid cross-contamination between stations. 47mm diameter polycarbonate filters with 0.2 µm-pore size membrane (Millipore, USA) were used to filter through the Niskin bottle's samples (5 l). Thereafter, the filters

were fixed in ethanol > 70 % until further analyses. To avoid cross-contamination between samples, the filtration system was carefully washed with MiliQ water before filtration. As the sampling was not performed at the same time of the year, differences between sampling zones are merely descriptive. Thus, seasonality is not discussed in this study (Table 5.1).

Table 5.1: List of samples used in this study with information for the station where was sampled, latitude, longitude, date and depth of the station. Abbreviations: Paliastomi Lagoon (Pa), Black Sea (BS), Razim-Sinoe Lake complex (RS), Dnieper Estuary (Dn). Samples from the stations BS_1 and BS_2 were taken at different depth, the first number indicates the station and the second number the depth at which the sample was taken.

Sample	Station	Latitude	Longitud	Method	Date	Temperature (°C)	Salinity (psu)	Depth (m)
Pa_1	Pa_1	42.09290	41.70799	Horizontal net	04/10/2016	19.9	0.8	2.2
Pa_2	Pa_2	42.12253	41.73241	Horizontal net	05/10/2016	19.1	1.6	2.8
Pa_3	Pa_3	42.13602	41.75308	Horizontal net	05/10/2016	17.6	0.0	1.5
BS_1.10	BS_1	44.13531	28.77950	Niskin bottle	22/10/2015	18.3	18.1	10.0
BS_1.15	BS_1	44.13531	28.77950	Niskin bottle	22/10/2015	18.3	18.1	15.0
BS_1.2	BS_1	44.13531	28.77950	Niskin bottle	22/10/2015	18.3	18.1	2.0
BS_1.20	BS_1	44.13531	28.77950	Niskin bottle	22/10/2015	18.3	18.1	20.0
BS_2.0	BS_2	44.08161	29.03878	Niskin bottle	22/10/2015	16.8	16.8	0.0
BS_2.10	BS_2	44.08161	29.03878	Niskin bottle	22/10/2015	17.0	16.9	10.0
BS_2.15	BS_2	44.08161	29.03878	Niskin bottle	22/10/2015	17.0	16.9	15.0
BS_2.2	BS_2	44.08161	29.03878	Niskin bottle	22/10/2015	16.8	16.8	2.0
BS_2.5	BS_2	44.08161	29.03878	Niskin bottle	22/10/2015	16.8	16.8	5.0
BS_3	BS_3	44.54053	28.92303	Vertical net	21/10/2015	16.2	5.5	10.2
RS_4	RS_4	44.69424	28.99504	Horizontal net	19/10/2015	15.4	5.5	1.1
RS_5	RS_5	44.57275	28.89589	Horizontal net	19/10/2015	15.8	5.4	1.7
RS_6	RS_6	44.57325	28.89297	Horizontal net	19/10/2015	14.2	4.1	0.8
RS_7	RS_7	44.59665	28.91318	Horizontal net	19/10/2015	15.3	4.6	2.0
RS_8	RS_8	44.53832	28.77836	Horizontal net	20/10/2015	14.4	4.1	1.5
RS_9	RS_9	44.63470	28.88791	Horizontal net	20/10/2015	14.6	3.7	0.5
RS_10	RS_10	44.62530	28.87271	Horizontal net	20/10/2015	15.6	0.0	0.9
Dn_1	Dn_1	46.61224	32.05193	Vertical net	17/05/2016	20.3	2.1	6.0
Dn_2	Dn_2	46.69558	31.92818	Vertical net	17/05/2016	17.4	3.1	4.0
Dn_3	Dn_3	46.71717	31.93040	Vertical net	16/05/2016	18.5	3.8	5.3
Dn_4	Dn_4	46.73659	31.92412	Horizontal net	16/05/2016	19.7	3.2	2.1
Dn_5	Dn_5	46.48799	32.17974	Vertical net	15/05/2016	20.3	0.4	3.2
Dn_6	Dn_6	46.54662	32.14368	Horizontal net	15/05/2016	17.5	0.5	1.3
Dn_7	Dn_7	46.60198	32.10252	Horizontal net	15/05/2016	20.5	0.8	1.5
Dn_8	Dn_8	46.58537	32.60165	Horizontal net	15/05/2016	20.5	0.3	1.0

5.2.3 Molecular methods, sequencing and bioinformatics

Samples were processed using a DNeasy Plant MiniKit (Qiagen), following the instructions of the manufacturer to obtain genomic DNA. This DNA was immediately frozen at -20°C. DNA concentrations were measured using a Qubit fluorometer Assay (Invitrogen, USA). Amplification of the Ribosomal Internal Transcribed Spacer (ITS) was performed using primers ITS1 5'-GGTGAACCTGAGGAAGGAT-3' and ITS4 5'-TCCTCCGCTTATTGATATGC-3' (White et al., 1990; Gardes et al. 1991). PCR reaction was performed on 25–100 ng of DNA as follows, denaturalization step at 94 °C for 3 minutes followed by 35 cycles of 94 °C for 30 seconds, 30 seconds at 47 °C and 45 seconds at 72 °C, ending with a 72 °C extension step for 7 minutes. PCR products obtained ranging from 500 to 600 bp. All PCR products were visualized on a 1% agarose gel containing RedGel Stain. PCR products were submitted to the Genomic facilities of the University of Bristol, UK, for library preparation and quantification. Ultimately, the ITS libraries were submitted to an Illumina MiSeq sequencing in a paired-end 2 x 300bp sequence read run. ITS sequences were de-multiplexed and quality trimmed to PHRED 20 using the packages "ShortRead" and "DADA2" in R software (Morgan et al., 2009; Callahan et al. 2016) followed by data analysis using the Quantitative Insights into Microbial Ecology (QIIME, v1.9) pipeline (qiime.org; Caporaso et al., 2010). Analyses in QIIME were performed as detailed by Winfield et al. (2018).

5.2.4 Environmental variables

Surface water temperature and salinity were recorded *in situ* while sampling using a multimeter. In addition, a set of spatially explicit environmental (weekly and monthly surface temperature and chlorophyll-*a*) variables were extracted from the NASA Earth Observations dataset, which contains multiple spatially explicit environmental variables at a resolution of 0.1 degrees (https://neo.sci.gsfc.nasa.gov/). Variables were extracted for the sampling stations and the weekly and monthly means of each station by the time of the sampling using R statistical software (The R Foundation for Statistical Computing, 2018; Table S5.1).

5.2.5 Statistical analyses

All statistical analyses were carried out using operational taxonomic units (OTUs) classified as dinoflagellate at genus level. The α- and β-diversity, species richness, Shannon diversity index (H′), Simpson's and inverse Simpson's index were calculated using the R package 'vegan' v.2.3 (Oksanen et al., 2017). The relative abundance of microeukaryotic organisms at the phylum and genus (for dinoflagellates) level were calculated. Non-metric multidimensional scaling (NMDS) analyses were carried out on the Bray-Curtis dissimilarity matrix using function 'metaMDS'. Thereafter, in order to describe the differences in biodiversity between and within the sampling zones analysis of similarity (ANOSIM) was applied on Bray-Curtis dissimilarity matrix. Then the contribution of the geographical distance on the dinoflagellate β-diversity between samples was tested using Mantle test analyses, which allows the estimation of the relative influence of different variables on the dinoflagellate community composition. These analyses were applied to test statistically the effect of environmental variables and geographical distance on the microeukaryotic and dinoflagellate assemblages (Diniz-Filho et al., 2013).

To study potential interactions between dinoflagellate α-diversity and environmental factors Spearman's rank correlation tests were carried out. Then, the environmental variables were displayed in a CCA (canonical correspondence analysis) plot together with the samples analysed using the function 'envfit' of the vegan package. The relative contribution of each environmental factor compiled in this study was tested by Permutational multivariate analysis of variance (PERMANOVA), using the function "adonis", and multiple regression on dissimilarity matrices (MRM) models, using the function "MRM", (Goslee and Urban, 2007). In addition, a Principal Coordinate Analysis (PCoA) on the Bray-Curtis dissimilarity matrix of the dinoflagellate community composition was implemented connecting all stations. Geographical distance and compiled environmental variables were used in a partial redundancy analysis (dbRDA). The dbRDA test separates the total dissimilarity of the set of variables into portions that reveal the contribution of only environmental factors. All statistical analyses were executed using R statistical software (The R Foundation for Statistical Computing, 2018).

5.3 Results

5.3.1 Community composition at Phyla and Dinoflagellate genera

Ciliophora, Chlorophyta, Dinoflagellata and Rhodophyta were the most dominant taxa along all sampling zones (Fig. 5.2A). Particularly, Ciliophora (13.1-85.9 %) were the most abundant taxon in the Black Sea (BS), Dnieper Estuary (Dn) and Paliastomi Lagoon (Pa) zones but were less abundant in the Razim-Sinoe Lake complex (RS). In contrast, Chlorophyta (69.2 %) were the most abundant taxon in the RS. Moreover, OTUs identified as Dinoflagellata (0.03-12.6 %), Rhodophyta (0.03-14.2 %) and Streptophyta (1.67^{-5}-13.6 %) were found dominating as well in the sampling areas. For each specific sample (Fig. 5.2B), Ciliophora were normally the dominating taxa in most of them except for samples BS_1.10, RS_6, RS_7, RS_8 and RS_9. Chlorophyta were the most abundant taxa for the samples BS_1.10, BS_2.2, BS_3, RS_4, RS_6, RS_7, RS_8 and RS_10. Dinoflagellata were detected in all samples with high relative abundance above 10 % in samples BS_1.10, BS_1.2, BS_2.0, BS_2.10, RS_6, Dn_1 and Dn_3; while Rhodophyta were dominant taxa in four samples in the Dnieper sampling zone (Dn_5, Dn_6, Dn_7 and Dn_8). Sample RS_9 was dominated by Streptophyta with a relative abundance of 93.5 %.

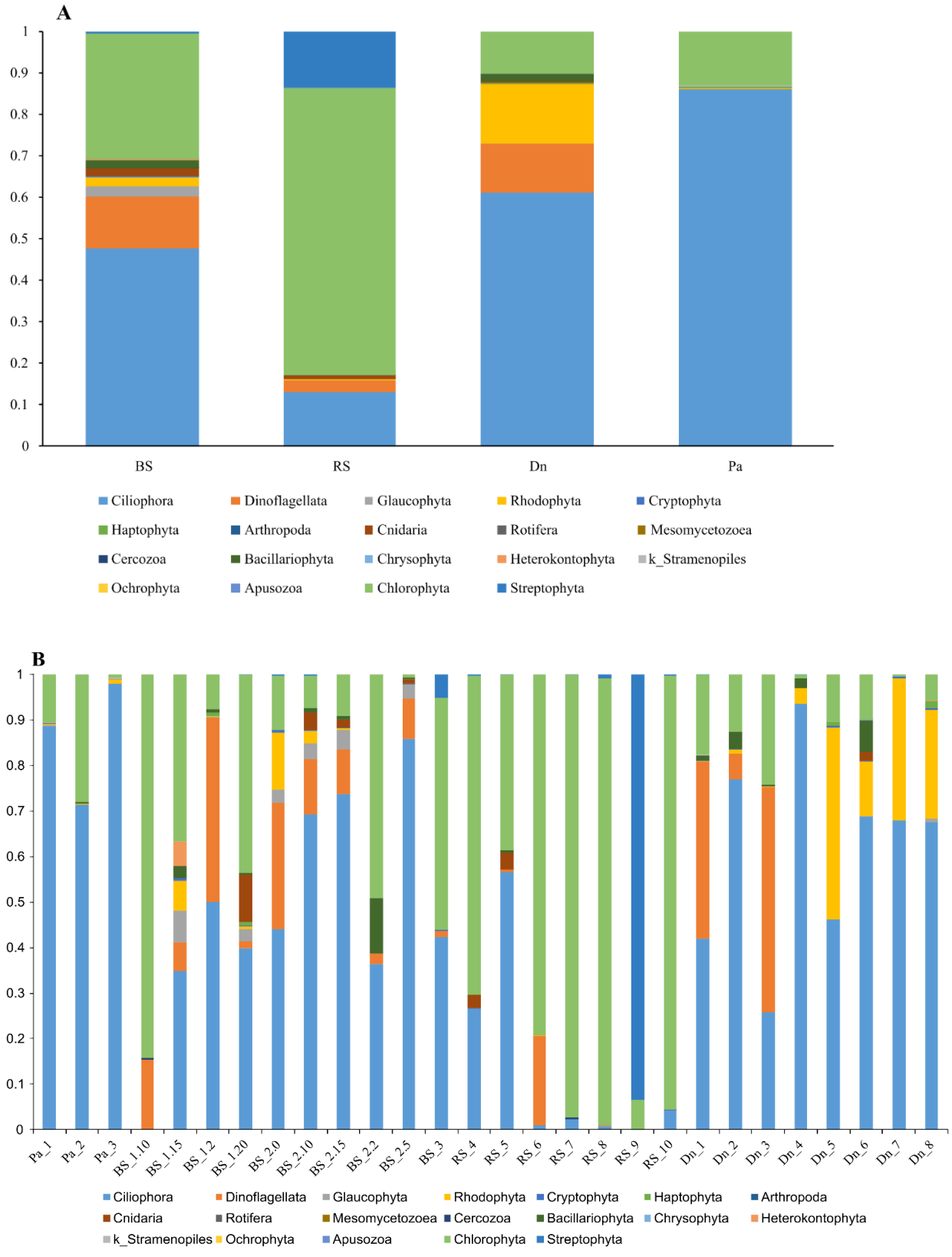
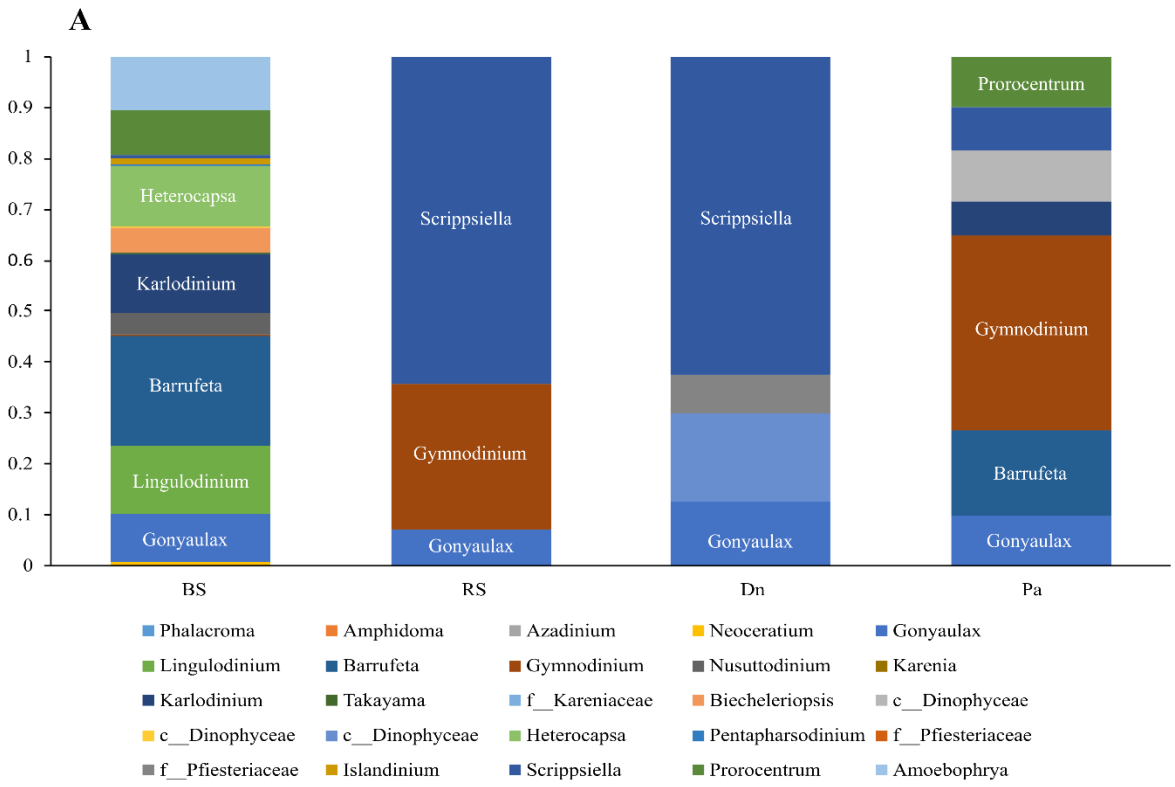


Fig. 5.2: Relative abundance of microeukaryotic taxa in the sampling areas, Black Sea (BS), Razim-Sinoe Lake complex (RS), Dnieper Estuary (Dn) and Paliastomi Lagoon (Pa) at phylum level (A) and in the specific samples at phylum level (B). Y-axis represent relative abundance. OTUs that could not be identified to phylum level were identified to kingdom (k_).

Dinoflagellate dominance at genus level shifts from one sampling zone to another (Fig. 5.3A). *Scrippsiella* dominated RS and Dn zones with relative abundance of 64.3 and 62.6 % respectively. *Gymnodinium* was the most abundant taxon (38.2 %) in Pa sampling zone and the second most abundant (28.6 %) in RS. Dominance in BS sampling zone was shared by *Barrufeta* (21.5 %), *Lingulodinium* (13.4%), *Heterocapsa* (11.9 %) and *Karlodinium* (11.5 %). In addition, other genera of harmful blooms forming species such as *Gonyaulax* (7.2-12.5 %) and *Prorocentrum* (8.8 % in Pa) were found frequently. According to sampling stations (Fig. 5.3B), *Scrippsiella* dominated most stations in RS and Dn excepting stations RS_4 and RS_8, dominated by *Gymnodinium*, and station Dn_8 where the most abundant taxon was *Gonyaulax. Scrippsiella* also dominated two of the stations (Pa_1 and Pa_2) sampled in the Paliastomi coastal lagoon with relative abundance of 50 and 64.7 % respectively. *Lingulodinium* was the most common taxon at stations BS_1.15 (54.5 %) and BS_2.0 (58.6 %), while *Barrufeta* dominated Pa_1 (50 %), BS_1.15 (45.1 %), BS_2.10 (48.5 %), BS_2.15 (47.2 %) and BS_2.5 (45.4 %). Moreover, sequences classified as *Karlodinium*, *Amoebophrya*, *Gonyaulax* and *Prorocentrum* were frequently found with relative abundances up to 52.6, 43.2, 71.3 and 50 % respectively.



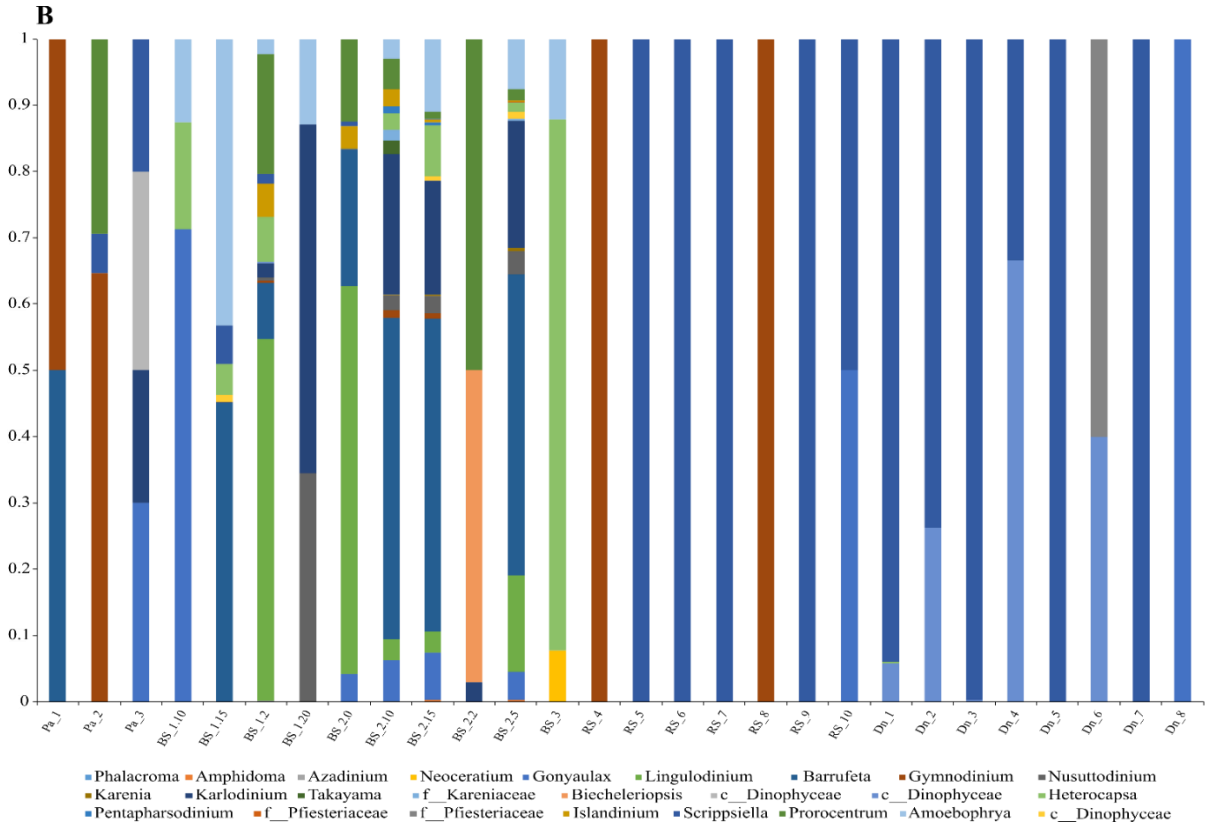


Fig. 5.3: Relative abundance of dinoflagellate taxa in the sampling areas, Black Sea (BS), Razim-Sinoe Lake complex (RS), Dnieper Estuary (Dn) and Paliastomi Lagoon (Pa) at genus level (A) and in the specific samples at genus level (B). Y-axis represent relative abundance. OTUs that could not be identified to genus level were identified to family (f_) or class (c_). Taxa mentioned in the text are indicated in panel A for clarification.

5.3.2 Relationships between dinoflagellate α-diversity and environmental factors

The highest number of observed taxa (richness) and alpha-diversity indexes were found at the BS sampling zone where 23 dinoflagellate genera were recorded, while the lower was at RS (Table 5.2). Both samples, BS_2.10 and BS_2.15 show the highest dinoflagellate genera recorded (16) however, the highest α-diversity (Simpson, 0.89 and H', 2.45) was measured at sample BS_2.10. The lowest taxon richness was one measured at nine stations in RS and Dn (Table 5.2). Spearkman's correlation analyses show sea surface salinity at the moment of sampling (sss.s) significantly positively correlated to taxon richness and α-diversity indexes ($p < 0.001$, Table 5.3). In addition, mean weekly chlorophyll-*a* concentration was found negatively correlated to taxon richness and inverse Simpson's index ($p < 0.05$, Table 5.3).

Table 5.2: Dinoflagellate richness and α-diversity indexes calculated in the samples analysed (left panel) and in the sampling zones, Black Sea (BS), Razim-Sinoe Lake complex (RS), Dnieper Estuary (Dn) and Paliastomi Lagoon (Pa; right panel).

Sample	Richness	Simpson	Inv.Simpson	H'
Pa_1	2	0.50	2.00	0.69
Pa_2	3	0.60	2.53	1.00
Pa_3	4	0.75	3.96	1.38
BS_1.10	3	0.61	2.56	1.02
BS_1.15	6	0.73	3.70	1.47
BS_1.2	13	0.85	6.70	2.17
BS_1.20	3	0.64	2.80	1.06
BS_2.0	9	0.77	4.39	1.68
BS_2.10	16	0.89	9.01	2.45
BS_2.15	16	0.88	8.28	2.38
BS_2.2	3	0.59	2.45	0.96
BS_2.5	15	0.87	7.78	2.31
BS_3	3	0.57	2.32	0.96
RS_4	1	0.00	1.00	0.00
RS_5	1	0.00	1.00	0.00
RS_6	1	0.00	1.00	0.00
RS_7	1	0.00	1.00	0.00
RS_8	1	0.00	1.00	0.00
RS_9	1	0.00	1.00	0.00
RS_10	2	0.50	2.00	0.69
Dn_1	5	0.39	1.65	0.74
Dn_2	2	0.47	1.88	0.66
Dn_3	4	0.15	1.17	0.34
Dn_4	2	0.49	1.94	0.68
Dn_5	1	0.00	1.00	0.00
Dn_6	2	0.49	1.98	0.69
Dn_7	1	0.00	1.00	0.00
Dn_8	1	0.00	1.00	0.00

Zone	Richness	Simpson	Inv.Simpson	H'
Pa	7	0.84	6.37	1.90
BS	23	0.92	12.11	2.68
RS	3	0.61	2.57	1.01
Dn	7	0.71	3.47	1.37

Table 5.3: Spearman's rank correlation between dinoflagellate α-diversity and environmental variables used in this study, sea surface salinity at the sampling moment (sss.s), sea surface temperature at sampling moment (sst.s), weekly average sea surface temperature of the week of sampling (sst.w), monthly average sea surface temperature of the month of sampling (sst.m), weekly average sea surface chlorophyll-*a* concentration of the week of sampling (chla.w), monthly average sea surface chlorophyll-*a* of the month of sampling (chla.m) with 9999 permutations. Significant correlations are indicated by *, ** and *** for *p*-values $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively.

Variables	Richness	Simpson	Inv.Simpson	H'
sss.s	0.68***	0.64***	0.69***	0.72***
sst.s	0.006	0.14	-0.013	0.073
sst.w	0.077	0.27	0.13	0.19
sst.m	0.024	0.35	0.12	0.23
chla.w	-0.4*	-0.3	-0.41*	-0.36
chla.m	0.11	0.27	0.14	0.23

5.3.3 Environmental and spatial factors affecting dinoflagellate β-diversity

The Non-metric multidimensional scaling analyses show that our samples cluster roughly along the NMDS axis 2 (Fig. 5.4), suggesting an even distribution of high and low ranks in dinoflagellate community composition at genus level within and between sampling zones across distinct coastal zones. The ANOSIM analysis statistically supported that significant differences in the dinoflagellate community composition are greater within sampling areas than between sampling areas (Global R = 0.35, $p < 0.001$, Table S5.2). Geographic distance between the stations was used as a variable to test if differences in dinoflagellate composition among stations were affected by the distance between the stations. Mantle tests (Table 5.4) shows no significant correlation between geographical distance and dinoflagellate community composition among samples, while a significant correlation ($\rho = 0.232$, $p < 0.01$) was found between dinoflagellate β-diversity and sea surface salinity (sss.s). Results from the Mantel test indicates that salinity is the main contributor in shaping the distribution of dinoflagellates in the Black Sea among the variables analysed here (Table 5.4).

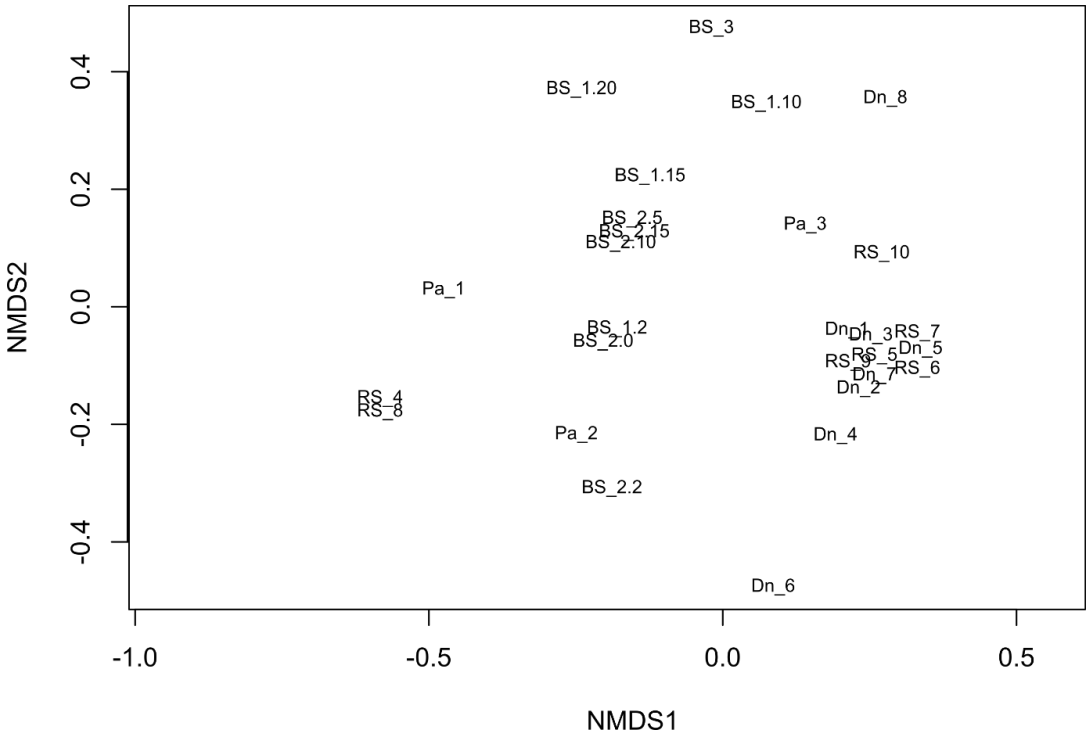


Fig. 5.4: Non-metric multidimensional scaling (NMDS) plot based on Bray-Curtis dissimilarity of the dinoflagellate community composition (β-diversity). Distances between sampling sites (black labels) on the ordination plot reflect dissimilarity. Stress = 0.091.

Table 5.4: Mantle test results for correlations between environmental factors, including geographical distance, (Euclidean distance) and Bray-Curtis dissimilarity of the dinoflagellate community composition (β-diversity) with 9999 permutations. Geographical distance between sampling stations (G.distance), sea surface salinity at the sampling moment (sss.s), sea surface temperature at sampling moment (sst.s), weekly average sea surface temperature of the week of sampling (sst.w), monthly average sea surface temperature of the month of sampling (sst.m), weekly average sea surface chlorophyll-a concentration of the week of sampling (chla.w), monthly average sea surface chlorophyll-a of the month of sampling (chla.m). Numbers in bold indicate significant *p*-values ($p < 0.05$).

Variables	**ρ**	***p*-value**
G.distance	0.131	0.063
sss.s	0.232	**0.002**
sst.s	0.027	0.326
sst.w	0.02	0.572
sst.m	0.036	0.319
chla.w	0.047	0.176
chla.m	0.039	0.287

The CCA analysis shows that environmental factors significantly affected ($p < 0.05$) the structure of the dinoflagellate community (Fig. 5.5). Sea surface salinity is a significant driver of the dinoflagellate community composition ($p < 0.001$). Moreover, PERMANOVA and MRM analyses identify each environmental variable contribution in structuring the community composition (Table 5.5). Both analyses found sea surface salinity as the strongest variable affecting dinoflagellate β-diversity ($p < 0.005$). In addition, PERMANOVA analyses showed the mean weekly sea surface temperature (sst.w) as a significant contributor ($p < 0.05$, Table 5.5) to dinoflagellate diversity.

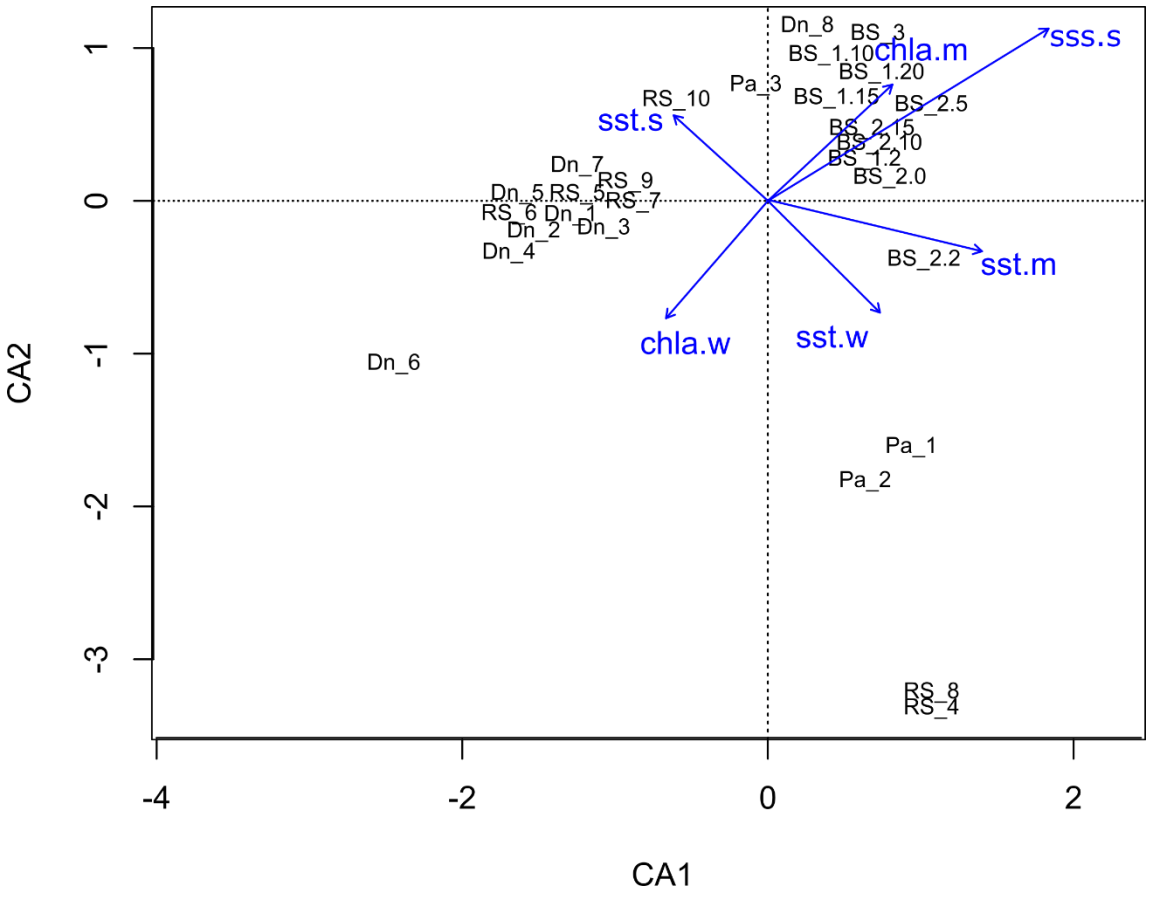


Fig. 5.5: Correspondence canonical analysis (CCA) biplot based on dinoflagellate communities (β-diversity) and environmental parameters, sea surface salinity at the sampling moment (sss.s), sea surface temperature at the sampling moment (sst.s), weekly average sea surface temperature of the week of sampling (sst.w), monthly average sea surface temperature of the month of sampling (sst.m), weekly average sea surface chlorophyll-a concentration of the week of sampling (chla.w), monthly average sea surface chlorophyll-a of the month of sampling (chla.m). Distances between samples (black labels) on the ordination plot reflect dissimilarity in the dinoflagellate community based on Bray-Curtis coefficients. The angle and length of vector loadings (blue arrows) indicate the direction and strength of associations of variables with the dinoflagellate composition in samples, respectively.

Table 5.5: PERMANOVA and multiple regressions on dissimilarity matrix (MRM) model results applied on Bray-Curtis dissimilarity of dinoflagellate community composition (β-diversity) with 9999 permutations. Sea surface salinity at the sampling moment (sss.s), sea surface temperature at sampling moment (sst.s), weekly average sea surface temperature of the week of sampling (sst.w), monthly average sea surface temperature of the month of sampling (sst.m), weekly average sea surface chlorophyll-a concentration of the week of sampling (chla.w), monthly average sea surface chlorophyll-a of the month of sampling (chla.m). Numbers in bold indicate significant *p*-values ($p < 0.05$).

	MRM		**PERMANOVA**	
Variables	R^2	*p*-value	R^2	*p*-value
sss.s	0.252	**<0.001**	0.213	**<0.001**
sst.s	0.022	0.757	0.01	0.924
sst.w	-0.085	0.341	0.083	**0.019**
sst.m	0.048	0.604	0.034	0.315
chla.w	0.048	0.611	0.031	0.374
chla.m	-0.082	0.508	0.013	0.864

CCA results are supported by the dbRDA analysis (Fig. 5.6) showing that the environmental variables are significant in driving the dinoflagellate β-diversity at genus level ($p < 0.01$). The dbRDA ordination describes the relationship between the environmental variable and the taxonomic dissimilarity between samples, showing that over 65 % of the variability is due to the first axis, which can be linked to the surface salinity. The dbRDA plot represents the variables as arrows according to their sum of squares values explaining the differences in dinoflagellate community composition among stations. Axis 1 explains 65.7 % of the variability in β-diversity of dinoflagellates ($p < 0.005$). Analysis of the variance (ANOVA, Table S5.3) shows that sea surface salinity and monthly mean sea surface temperature are significant variables (p=0.001 and p=0.040 respectively) driving the dbRDA clusters (dinoflagellate β-diversity).

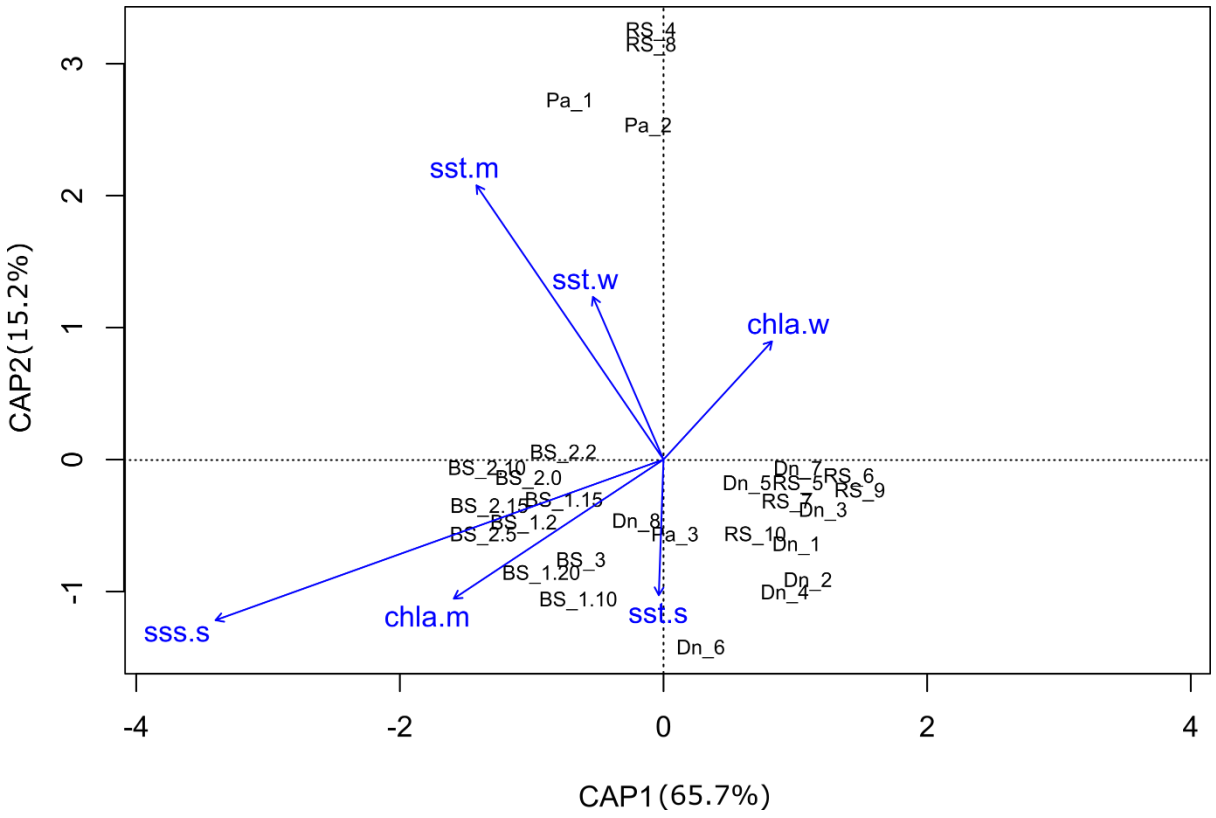


Fig. 5.6: Distance-based redundancy analysis (dbRDA) biplot based on dinoflagellate communities (β-diversity) and environmental parameters, sea surface salinity at the sampling moment (sss.s), sea surface temperature at sampling moment (sst.s), weekly average sea surface temperature of the week of sampling (sst.w), monthly average sea surface temperature of the month of sampling (sst.m), weekly average sea surface chlorophyll-a concentration of the week of sampling (chla.w), monthly average sea surface chlorophyll-a of the month of sampling (chla.m). Distances between samples (black labels) on the ordination plot reflect dissimilarity in the dinoflagellate community based on Bray-Curtis coefficients. The angle and length of vector loadings (blue arrows) indicate the direction and strength of associations of variables with the dinoflagellate composition in samples, respectively.

5.4 Discussion

The primary aim of this work was to study distribution and diversity patterns of dinoflagellates using Next Generation Sequencing (NGS) and DNA barcoding to recover more accurate OTU sequences and to reveal unrecognized taxa diversity from the Black Sea and identify environmental factors driving them. Only a few studies (see section 1.4.2) address biogeography and diversity of microeukaryotic and dinoflagellate communities in the Black Sea using NGS. With the results obtained from the Illumina MiSeq platform combined with ITS barcode, we gained an overview of the protist and dinoflagellate distribution and community structure across different coastal habitats of the Black Sea. Beyond their many advantages such as the capacity to process a large number of samples in a short time and detecting taxonomical challenging groups like parasites or symbionts (Massana et al., 2015), the routine application of metabarcoding for planktonic monitoring and diversity studies requires overcoming some limitations. The main limitation involves the incompleteness of reference sequence databases against which comparisons can be made to monitor diversity changes through time. Despite considerable barcoding efforts, reference sequences are still very rare for some dinoflagellate taxa, for instance, the most abundant (≈ 500 sequences) in GenBank are for the genus *Alexandrium* while for other genera only one sequence is available. Sequencing efforts for dinoflagellates are highly biased by genera (Sze et al., 2018). It is crucial to increase the number of sequences available for different barcodes and species to support accurate diversity and monitoring studies and as well as testing, which factors affect shifts in the microbial community. This work significantly contributes to this aim.

5.4.1 Correlations between dinoflagellate α-diversity and environmental variables

Our results show differences in taxon richness and α-diversity within and between sampling zones (Table 5.2). Dinoflagellate richness and α-diversity in this area were significantly correlated with sea surface salinity at the moment of sampling (sss.s., Table 5.3) inferring that, among the limited set of environmental parameters tested here, salinity has the most profound impact on dinoflagellate diversity (Table S5.1). An expanded set including other key parameters such as nutrient content in the water (e.g dissolved inorganic and organic carbon) might reflect better the complexity of the system driving dinoflagellate. Salinity is a key parameter to define dinoflagellate community composition (Quinlan

and Phlips, 2007), as well as to trigger changes in species dominance and to drive species outbreaks. This has been demonstrated by Dhib et al. (2013) with *Prorocentrum micans* in a Mediterranean coastal lagoon characterised by season changes of salinity. In addition, strong correlations between salinity and dinoflagellate species richness and α-diversity have been well documented worldwide along with salinity gradients as well as for the Black Sea (Mudie et al., 2017 and references therein). The highest dinoflagellate richness and α-diversity were found in the samples with the higher salinity close to marine values. It is well known that brackish environment such as estuarine and riverine habitats are physiological stressful habitats due to rapid changes in salinity that only euryhaline species previously adapted or with a wide ecological range can tolerate (Whitfield et al., 2012). This has been observed for phytoplankton communities in both, natural and experimental conditions, where brackish conditions tend to present lower diversity than fresh or marine conditions (Huang et al., 2004; Flöder and Burns, 2004). It has been found that the Caspian Sea wich has a salinity range from 0 to 13 psu, has lower dinoflagellate diversity than the Black Sea (Mudie et al., 2017).Unfortunately, sampling areas included in this work were not sampled at the same time of the year due to project limitations as described in Chapter 2 (Table 2.1). Undoubtedly, seasonality affects dinoflagellate taxa abundance and community composition, therefore, differences in taxa richness and α-diversity between sampling areas cannot be attributed exclusively to the environmental parameters tested here.

5.4.2 Microeukaryotic and dinoflagellate distribution in the Black Sea

The microeukaryotic assemblages described in our study were mostly dominated by Ciliophora, Chlorophyta and Dinoflagellata (Fig. 5.2A) that are also dominant in other brackish and eutrophic seas such as the Baltic Sea (Gasinaite et al., 2005; Spilling, 2007) and estuarine areas (Wang et al., 2015). Similar studies carried out along salinity gradients for instance in the East China Sea have identified the abundance of Ciliophora as an indicator of low salinity conditions, which may favour its growth (Zhang et al., 2018). However, from our results, we cannot infer the same conclusion. Ciliophora seems to dominate assemblages despite the salinity regime of the area in both estuaries and the Black Sea assemblages. The trophic status of the Black Sea may bring an explanation since eutrophication is one of the main stressors affecting the biota of the region (Kideys, 2002). This aligns with the findings of

Wang et al. (2014) where a significant positive correlation was found between Ciliaphora and Chlorophyta and high concentrations of NH_4-N and PO_4-P in the South China Sea. High nutrient concentrations are directly favourable, until a certain threshold, for the growth of photosynthetic and mixotrophic (e.g. ciliates and dinoflagellates) microeukaryotic organisms and indirectly the growth of heterotrophic taxa by making their prey more readily available (Pospelova and Kim, 2010; Zhang et al., 2018). In addition, a recent study by Dzhembekova et al. (2018) assessing phytoplankton diversity, using DNA barcoding from accumulated cysts in recent sediments, across the Black Sea also found Chlorophytes and Dinoflagellates among the dominant groups supporting the hypothesis that these groups dominate the microeukaryotic communities in the Black Sea and can be tracked back in recent years in the sediment records.

Our Illumina MiSeq sequencing found 25 OTUs identified as dinoflagellate at genus level (Fig 5.2B), including two genera (*Barrufeta* and *Biecheleriopsis*) of free-living dinoflagellates and one genus of parasitic dinoflagellates *(Amoebophrya*) that have not been previously been observed in the Black Sea. Species of this parasitic genus are challenging to detect using light microscopy, and have not been reported previously from planktonic samples from the Black Sea. The species belonging to the genus *Amoebophrya* are obligate parasites and can occur in marine environments from the water column to seafloor sediments (Guillou et al., 2008). Species of *Amoebophyra* are defined as key players in the decline and termination of HABs' dynamics (Choi et al., 2017). These species can be present in low abundance, however, the population could rapidly grow and negatively impact the abundance of its host, thus affecting the functioning of the ecosystem (Chambouvet et al., 2008). This taxon was only found in stations sampled in the open Black Sea (BS). Being obligate parasites, their distribution is limited to the natural range of their hosts, which are all marine species (Sarkar, 2018). Both *Barrufeta* and *Biecheleriopsis* are small genera of marine dinoflagellates with only two and one species described respectively. Species of these genera have been described as bloom-forming species recently. Sampedro et al. (2011) described a new species of *Barrufeta* (*B. bravensis*) as a bloom-forming species from the Mediterranean coast of North-West Spain inside semi-enclosed bays. Expansion of dinoflagellate species from the Mediterranean Sea into the Black Sea due to both natural spread and human-driven

activities, together with rising temperature, may lead to increasing HABs events in the Black Sea in the near future.

Lingulodinium is a dinoflagellate genus with only one described species, *L. polyedrum* (Lewis, 1988). It is a common bloom-forming species of temperate areas as well as a proven toxin-producing species (Lewis and Hallet, 1997). Our findings align with previous studies where it has been reported abundantly in both planktonic and sediment samples, across the Black Sea but not present in coastal lagoons and estuarine areas such the Razim-Sinoe Lake complex or the Dnieper Estuary (Gómez and Boicenco, 2004 and references therein; Mertens et al., 2009; Krakhmalnyi et al., 2018). Therefore, we infer that salinity is the main driver limiting the distribution of *Lingulodinium,* which was also concluded by Mertens et al. (2009) using *Lingulodinium* cysts processes as salinity proxy (Fig 5.3B, 5.5 and 5.6). *Phalacroma* species are mostly heterotrophic dinoflagellates found in both marine and freshwater environments (Marret et al., 2013). From our analyses (Fig. 5.3B) we infer that their distribution is linked to areas where nutrient loads are entering the Black Sea such as the Dnieper estuary or the Danube Delta. Enriched and partially stratified waters found in these areas favour the growth of fast-growing microalgae such as diatoms or small-sized dinoflagellates that serve as food for heterotrophic dinoflagellates including *Phalacroma* species (Caroppo et al., 1999; Dale, 2009). Autotrophic dinoflagellates distributed in estuary-like areas of our study align with the findings in similar areas such as the Patapsco River estuary, (USA), Croatian Adriatic coast and Suez Canal. In these settings, shallow enriched estuary waters, small to intermediate-sized dinoflagellate dominate the community; *Gymnodinium* and *Scrippsiella* (Fig 5.3B) species are among the most abundant, indicating chemically disturbed waters by nutrient loads, which also contribute to the low phytoplankton diversity (Sellner et al., 2001; Gaballa, 2014; Ninčević-Gladan et al., 2015). Taxa dominance and community structure vary according to the sampling season as a result of the changing environmental conditions present at that moment e.g. temperature, light, salinity, nutrient concentrations and availability as well as stratification. In coastal areas such as the locations studied in this work, most of these environmental parameters are directly influenced by river discharge, which is strongly dependent on the season. Therefore, the results presented here can only be descriptive and indicative of the environmental

dynamics of each area at the moment of sampling. No direct comparison can be drawn between sampling zones regarding the taxa dominance and community composition.

5.4.3 Dinoflagellate β-diversity response to environmental and spatial factors

Many earlier studies have shown not only the influence of environmental parameters in the biogeography of planktonic protists (Spilling, 2007; Zhang et al., 2018) but also the influence of spatial factors due to site-specific variations related to coastal hydrodynamics (Spilling, 2007; Gong et al., 2015; Chen et al., 2017; Sun et al., 2020). Studies demonstrated that both environmental factors and geographical distance play important roles in driving community structure at different scales (Horner-Devine et al., 2004; Martiny et al., 2006; Balzano et al., 2015).

Dispersal limitation due to geographical distance and physical barriers has recently been proven as an important factor for microbial biogeography in seawater sediments (Bik et al., 2012) and coastal lagoons (Lepère et al., 2013) at different spatial scales. The ANOSIM test shows that there are differences between habitats, but dissimilarities are evenly distributed between and within groups ($r = 0.3506$, $p < 0.001$). ANOSIM tests, that discriminate sites (or times) from each other by similarities between samples within an area, are consistently higher than similarities between samples at different areas where R values closer to 0 indicate an even distribution of dissimilarities (Clarke and Warwick, 2001). In addition, Mantle test results show no significant correlation between geographical distance and dinoflagellates β-diversity. This suggests that the exchange of dinoflagellate between sample stations is not limited by distance but by environmental factors. Many species of dinoflagellate make resting cysts, allowing them to overcome unfavourable environmental conditions over seasons, even decades, as well as facilitate their dispersal since cysts, once entered in the water column, behave as silt particles which allow them to be transported across large areas and over hundreds of kilometres (Anderson, 1985). This feature may limit the impact of the geographical distance in the distribution of dinoflagellates compared with other microeukaryotic groups with lower dispersal rates, e.g. species of *Symbiodinium* have been documented to overcome hundreds of kilometres, distances much larger than dispersal of other marine microeukaryotic organisms (Pettay and LaJeunesse, 2013). This approach can only be indicative given the limitations of the sampling strategy where seasonality could not be

controlled. Sampling at different seasons would vary the community composition and therefore influence the statistical analyses testing the influence of the geographical distance versus the environmental parameters. I, therefore, assume that in this study area environmental parameters, particularly salinity, are the main drivers shaping the dinoflagellates community structure and biogeography, however confirmation of this would need to be carried out including a larger set of psysico-chemical variables as well as adequate sampling strategy to control the seasonality.

Our analyses show sea surface salinity as the main driver shaping dinoflagellate β-diversity among the limited set of variables including here. Salinity is known as an important factor that may alter diversity, distribution and is also a stronger driver than other environmental factors affecting the microeukaryotic community composition worldwide (Sunagawa et al., 2015). Despite the ability of dinoflagellates to be transported long distances and the assumed exchange of taxa between stations and habitats, local communities appear to exhibit a specific salinity-related signature. This is likely to be because exchange between communities as well as salinity changes are occurring fast enough to prevent taxa from adapting to the local salinity to gain dominance and replace the local community. Salinity is one of the main stressors limiting dinoflagellate distribution but also cell growth and in some cases, if maintained, causes cellular death (Hernando et al., 2015; Nche-Fambo et al., 2015; Skarlato et al., 2018). This aligns with the results of a large-scale meta-analysis that found salinity is one of the major determinants across different habitats (Lozupone and Knight, 2007).

Our dbRDA and PERMANOVA analyses indicate that surface sea temperature is the second most important variable affecting biogeography and β-diversity of dinoflagellates after salinity (Fig. 5.6, Table 5.5) among the variables tested. Despite the proven influence of temperature on cell growth and community structure (Kim et al., 2004; D'Costa et al., 2008; Halac et al., 2013; Sala-Pérez et al., 2016) the temperature measured in the different habitats does not seems to be limiting the distribution of dinoflagellate taxa. This may be explained by the homogenization of temperature during the blooming season along with coastal habitats of the Black Sea where, despite the river influence, no wide vertical changes in temperature were measured at the sampling locations (Table S5.1). However, the

temperature is affected directly by the seasonality so this statistical relationship and the assumption presented here would need to be confirmed by including samples obtained at the same time of the year.

5.5 Conclusions

Here, we provide new insights into the structure and biogeography of protist communities by using the NGS and DNA barcoding data set of ITS. Despite the stringent thresholds such as limited reference sequences available, we have recovered a high diversity of dinoflagellates taxa, 25 OTUs, including taxa reported for the first time in the region, demonstrating the utility of this approach for monitoring and diversity studies, complementing traditional taxonomical studies in the region. Additional studies sampling wider ranges of organism size and depth, as well as poorly studied habitats, are likely to detect more taxa, both undetected and recorded. The OTUs obtained in this study contribute to the constant expanding of DNA sequence databases, which will enable as more studies are carried out more precise matches for DNA barcodes in this region and allow further comparison with global studies.

Dinoflagellate communities in estuarine-like habitats require adaptation to wide salinity gradients. Black Sea dinoflagellates are mainly derived from seawater with only a few taxa from freshwater origins. Dinoflagellate community composition differed significantly between the different stations (Fig. 5.2B) and no taxon dominated stations in our study area. This can be a result of the moment of sampling since different zones were sampled at different seasons for geopolitical reasons. The results suggest that none of the dinoflagellate taxa identified here show the ability to overcome the different habitats studied. Salinity rather than geographical distance or temperature seems to be the main factor controlling dinoflagellate community structure and biogeography in the Black Sea. This would differentiate our study area from similar semi-enclosed areas where geographical distance was identified as the main factor. These results allow us to hypothesize that the diversity of planktonic dinoflagellate is mainly dependent on niche-based processes (environmental factors limiting the distribution of taxa) instance of on neutral processes, (dissimilarity increase with geographical distance due to limited dispersion). This would need to be confirmed with further analysis including a larger set of environmental parameters as well as seasonal samples for each zone.

CHAPTER 6

SYNTHESIS AND CONCLUSIONS

In this chapter, the findings of this study are synthesized and the two main aims and the specific research goals outlined in section 1.5 are addressed. Finally, several key questions that remain unanswered are outlined identifying the future work necessary for increasing our understanding of dinoflagellates and the factors that impact them.

6.1 Biodiversity and biogeographic patterns of dinoflagellate assemblages in the Black and Caspian seas

Dinoflagellate assemblages in the Pontocaspian region are dominated by taxa with ubiquitous distribution. These taxa tolerate wide ecological ranges, which enable them to thrive in highly dynamic environments such as coastal areas of the Black and the Caspian seas, where rapid shifts in temperature and salinity are found (see section 1.3).

6.1.1 Describing the differences in dinocyst assemblages relative to their environmental settings in order to enrich the Pontocaspian dinocyst database

Dinocysts are commonly used as proxies to describe surface environmental conditions in the Pontocaspian region; however, the available records of modern dinocyst are fragmented (section 1.2.2). One of the objectives of this thesis is to complement the Pontocaspian dinocyst dataset by generating new data from poorly studied areas, such as areas with low salinity (section 1.4.1, Fig 1.9). To achieve this, the sampling strategy targeted areas of the eastern Caspian Sea, including the north and middle sub-basins, and part of the western Black Sea (see section 2.1.1). Chapter 3 contains new dinocyst assemblage data as well as dinocyst abundance data for these areas filling some of the spatial data gaps identified in the literature (section 3.3, Fig. 3.4).

Results discussed in Chapter 3 demonstrate that *L. machaerophorum* is the dominant dinoflagellate taxa across all Pontocaspian assemblages including areas with salinity as low as ≤ 5 psu, such as the Northeast Caspian Sea (see section 3.4.2, Fig. 3.4). These results confirm the ubiquitous distribution and the wide ecological range of this taxon. However, assemblages in the north and middle Caspian sub-basins showed co-dominance with *I. caspienense*. Although this taxon is only found in the Caspian Sea today, fossil records have identified this species in Holocene sediments from the Black Sea during periods when Black Sea surface conditions were similar to the current Caspian Sea (Mertens et al., 2017 and references therein). Heterotrophic dinoflagellate cysts represented by Round brown cysts morphotype are present in high abundances in dinocyst assemblages from all the samples collected from the Black and Caspian seas during this study except the South Caspian Sea, where they are almost absent, this is discussed below as a result of the environmental characteristic of that area (section 3.4.2, Fig. 3.4). Dinocyst abundance measured here agrees with abundance range data previously published for other areas of the Black and the Caspian seas (Mudie et al., 2017). As previously published, dinocyst diversity is higher in the samples analysed from the Black Sea than the Caspian Sea. This is a result of the salinity conditions in the Caspian Sea today. The salinity range of ~0-13 psu (section 1.3.2), limits the biodiversity and productivity of the Caspian system because fewer taxa are able to exist under these conditions (see section 3.4.2).

6.1.2 Investigating dinoflagellate assemblages across the Black Sea using NGS and DNA barcoding methods.

While dinocyst assemblages represent accumulation over a period of up to a few years, phytoplankton assemblages respond to the precise environmental conditions they experience on a much shorter time-scale, usually a few weeks. Methods that increase the speed and volume of sample processing and produce accurate identification of taxa such as DNA barcoding are therefore being used worldwide to assess phytoplankton diversity and biogeography (section 1.4.2).

In Chapter 5 of this thesis, the first dataset of dinoflagellate OTUs (see section 5.1 and 5.3.1) using Ribosomal Internal Transcribed Spacer (ITS, eDNA) gene amplicon sequencing on Black Seawater samples from different coastal environments at an intermediate spatial scale are presented. On the basis

of these data, the microeukaryotic community in the water column at phyla and genus level is described. Dinoflagellates, Ciliophora and Chlorophyta were found to be the dominant phyla of the microeukaryotic community in all the Black Sea study areas. This finding is in agreement with studies performed in other brackish and estuarine areas such as the Baltic Sea (Gasinaite et al., 2005; Spilling, 2007; section 5.4.2). At dinoflagellate genus level, the dominance of taxa was clearly defined by the area. For example, *Scrippsiella* and *Gymnodinium* dominate assemblages in the estuarine Razim-Sinoe Lake complex, Dnieper Estuary and Paliastomi Lagoon, while taxa associated with marine conditions dominate the assemblages in the open Black Sea (e.g. *Barrufeta* and *Lingulodinium*, section 5.4.2; Fig. 5.3A).

The results included in Chapter 5 reveal taxa not previously described from the Black Sea. Specifically, two genera (*Barrufeta* and *Biecheleriopsis*) of free-living dinoflagellates and one genus of parasitic dinoflagellates *(Amoebophrya*) were detected using NGS and DNA barcoding methods. This approach is therefore demonstrably a successful method for detecting previously invisible or hard to detect taxa to complement diversity studies using traditional microscopy identification, including taxa that are dominating dinoflagellate assemblages such as *Barrufeta* (Fig. 5.3A). In addition, this approach has great potential in future coastal monitoring programs for the region. *Barrufeta* and *Biecheleriopsis* are small genera of marine dinoflagellates identified as bloom-forming taxa in coastal areas of the Mediterranean Sea. For example, *Barrufeta bravensis* was identified in 2011 during a bloom event in the Western Mediterranean (Sampedro et al., 2011). These molecular methods can also be potent tools for the detection and monitoring of new species arriving in the Black Sea from adjacent water bodies as a result of human-mediated activities such as shipping or by natural spread pose a challenge for monitoring using traditional methods alone, which require taxonomical expertise to detect those taxa. Algal bloom events are increasing in frequency and intensity due to climate change and anthropogenic impacts such as eutrophication (see section 1.2.1). The speed and efficiency of monitoring will need to increase to keep pace with challenges like these. Methods like those used in this thesis can support monitoring and address the limitations of traditional microscopy through both the detection of taxa of interest and processing large volumes of samples in a relatively short time. For example, investigating the arrival of new species or non-previously recorded taxa of interest, in the Black Sea by monitoring

areas nearby to main ports and shipping routes, which are known to be one of the main vectors for the introduction of these taxa.

6.2 Environmental and anthropogenic factors affecting the biodiversity and biogeography of dinoflagellates

A vast literature is available describing the impact of environmental factors such as temperature, salinity and nutrient availability on phytoplankton communities' composition on species distribution (see section 5.1). In addition, there are a number of anthropogenic factors that can influence biodiversity and affect taxa dominance and distribution. Analyses presented in this thesis show that salinity and temperature are the main factors affecting dinoflagellate and dinocyst assemblages and taxa distribution. However, anthropogenic perturbations such as impacts caused by shipping activities or nutrient loads affect dinoflagellate communities as well. This work has presented the first results showing a significant correlation between shipping activities variables and dinocyst assemblages in the Pontocaspian region (Fig. 3.5). These results can support future work investigating the impact of maritime activities on microeukaryotic communities.

6.2.1 Testing dinocyst assemblages as proxies for physico-chemical and shipping variables of the Black and Caspian seas

Dinocysts have been used commonly as indicators for surface water conditions and for anthropogenic disturbances such as heavy metal pollution and eutrophication (see section 1.2.2). Maritime traffic is the main transport activity worldwide and its impact has been demonstrated at many trophic levels (see section 3.1). This work includes the first attempt in the Pontocaspian region to test statistically dinoflagellate cysts as a proxy for shipping activities as well as to extend further our knowledge of the environmental factors that impact the distribution and diversity of the group.

Nonmetric multidimensional scaling (NMDS) analyses included in Chapter 3 confirm that the main factors affecting dinocyst taxa relative abundance and their distribution in the Pontocaspian region are sea surface temperature, salinity and chlorophyll-*a* (Fig. 3.5). This is in agreement with previous studies in the region and elsewhere confirming these environmental parameters as the main drivers of

dinoflagellates assemblage composition and distribution (see section 3.4.3). In addition, some taxa are significantly correlated with certain physico-chemical parameters indicating their potential as proxies for specific environmental conditions. For example, the abundance of the cyst morphotype identified as Round brown cysts in this thesis are significantly positively correlated with chlorophyll-*a* concentration (Fig. 3.5). Heterotrophic dinoflagellates and their abundance and distribution are limited by the presence of their prey. Areas with nutrient input from land, such as the western Black Sea or the north Caspian Sea, favour the presence of phytoplankton on which heterotrophic dinoflagellate feed. Primary producers such as diatoms thrive in coastal areas with high-nutrient input, which in some areas like the north Caspian Sea or the western Black Sea can be related to eutrophication from human activities.

Another example is the dominant dinocyst taxon, *L. machaerophorum*. This species has a wide ecological range that results in its ubiquitous distribution. However, large blooms of this species are associated with warm water temperatures that typically occur at the end of summer (see section 3.4.3). The analyses included in Chapter 3 (Fig. 3.5) show that the relative abundance and distribution of *L. machaerophorum* is significantly positively correlated with temperature, confirming this taxon as a good proxy of surface water temperatures. In addition, analyses in Chapter 3 show a significant correlation between shipping variables and dinocyst assemblages in the Caspian Sea. Specifically, distance to the harbour and distance to vessel routes significantly positively correlate with dinocyst diversity in the North and Middle Caspian sub-basins and the South Caspian Sea respectively (see section 3.4.4.2 and Fig. 3.5). This suggests that shipping activities can affect dinocyst assemblages, however, further studies will be needed to further define these variables and their effect on dinoflagellate cysts as well as in the planktonic and benthic dinoflagellates.

6.2.2 Relationships between environmental variables and dinoflagellate biogeography in the Black Sea

Highly dynamic environments such as estuaries and coastal lagoons are characterized by rapid changes in environmental parameters such as salinity and temperature; replicating conditions that are commonly present across the Pontocaspian region (see section 1.3). As a result, local dinoflagellate communities present a specific salinity-related signature and the exchange of organisms between habitats is occurring

faster than species adaptation (see section 5.4.3). Therefore, salinity is the main factor delimiting the distribution of dinoflagellate species and the main cause of the assemblages' composition. As a result of this, changes in the salinity regime can produce a complete shift in the dinoflagellate community and thus in the biotic community either locally or regionally. This sort of change can also be traced in long-term indicators like resting cysts accumulated in the sediment. This is explored in Chapter 3 of this thesis where salinity was also found to be the main factor affecting dinocyst assemblages in the Black Sea (Fig. 3.5), confirming that dinocysts are a relievable proxy for salinity in the region. Salinity has been proven to be the main parameter explaining shifts in dinoflagellate diversity in the Black and Caspian seas both today as well as in the past (Mudie et al., 2017). For example, Leroy et al. (2019) reported a change in the salinity gradient in the Caspian Sea across the Pleistocene-Holocene boundary. Usually, salinity in the Caspian Sea increases from north to south. Fossil dinocyst assemblages suggest that this has been the case for most of its history, except ~8-8.5 thousand years ago, when the salinity gradient is thought to have reversed because the main source of water of the Caspian Sea was the Amu-Darya River, flowing through Turkmenistan into the Caspian Sea (See Fig. 1 in Leroy et al., 2019), instead of the Volga River as today.

Temperature is well known to be a main parameter affecting the cellular activity of unicellular organisms as well as limiting their distribution (D'Costa et al., 2008; Halac et al., 2013; Sala-Pérez et al., 2016). It is also known to be one of the main constraints for the development of algal blooms (Anderson, 2009; Hallegraeff, 2010, see sections 4.1 and 4.3). Results presented in this thesis confirm that temperature affects dinoflagellate composition and distribution in the Black Sea and it is found the second most important parameter affecting dinoflagellate diversity and biogeography (Fig. 5.6 and 5.7). A similar conclusion is extracted for the analyses carried out with dinocysts, where temperature was, together with salinity, the main factor affecting dinocyst composition and relative abundance, e.g. temperature is the main factor affecting the abundance and distribution of the dominant cyst *L. machaerophorum* (Fig. 3.5).

6.2.3 The relative contribution of environmental and spatial variables in controlling dinoflagellate diversity and distribution

Many previous studies have demonstrated the influence of spatial factors in limiting the biogeography of microorganisms as well as their biodiversity (see section 5.1). The importance of environmental and spatial factors in affecting the biogeography and diversity of dinoflagellates in the Black Sea is tested statistically by applying ANOSIM and Mantle tests to my datasets (see section 5.2.5). Results presented in Chapter 5 found no significant correlation between geographical distance and β-diversity. However, a significant correlation was found with surface salinity as a main factor limiting dinoflagellate biodiversity and distribution. This allows the conclusion that biogeography and biodiversity in this study area are mainly limited by environmental factors (see section 5.4.3). These findings suggest that dinoflagellate diversity and distribution are mostly dependent on niche-based processes (species occurrence and dominance are limited by environmental factors and not due to geographical distance or barriers), in particular salinity and temperature.

6.2.4 The effects of temperature and salinity on the growth of new dinoflagellate cultures

In Chapter 4, this thesis documents the results of testing the first available cultures of *Gymnodinium aureolum* isolated from the Black Sea under different temperature and salinity treatments. This allows for the first time obtaining baseline data on the physiological adaptation of this Black Sea species and its potential to generate blooms under different environmental conditions. Growth experiments showed that this strain of *G. aureolum* developed its maximum growth rate at 20 °C and 15 psu (Fig. 4.5), which suggest that this strain is adapted to Black Sea surface environmental conditions during the bloom season, which supports the idea that this may be a bloom-forming population of *G. aureolum*. Further strains should be analysed to evaluate the growth potential of this species in the Black Sea. In addition, the results suggest that this species should be included in HABs monitoring programs in the region.

6.3 Recommendations for future work

6.3.1 Uneven sampling efforts

This project has contributed to dinoflagellate cyst data and filled some sample gaps in coastal areas of the North and eastern mid-Caspian Sea, which had previously been poorly studied. However, there are still areas across the Pontocaspian region with little or no data available on dinoflagellate cysts or phytoplankton diversity and abundance. These include coastal areas of Turkmenistan, Dagestan, Abkhazia and the northernmost part of the Caspian Sea. Some of these areas, e.g. the North-eastern part of the Caspian Sea, present specific environmental conditions that are only found in marginal areas of the Pontocaspian region, e.g. low salinity regimes ranging from freshwater to ~5 psu. The study of these areas of particular interest is necessary for obtaining a complete picture of the diversity and biogeography of a key group in the ecosystem.

6.3.2 The validity of dinoflagellates as proxies for anthropogenic factors

By comparison with the open ocean, semi-isolated and isolated seas such as the Black Sea and the Caspian Sea have a limited capacity to absorb and disperse pollution (section 1.3.2). In addition, the Black and the Caspian seas are among the most polluted seas in the world, particularly along their coastlines. Several studies have addressed the impact of anthropogenic activities and described their effects at different trophic levels (sections 1.3.1 and 1.3.2). However, very few studies have tested the effects of anthropogenic perturbations on protist communities, such as dinoflagellates in this area and the validity of this group as an indicator of anthropogenic perturbations (see section 3.1). This thesis presents the first region-specific results of statistically testing the effect of shipping activities on dinocyst assemblages (see section 3.4.4.2). The use of dinocysts as indicators of anthropogenic perturbations such as heavy metal pollution, eutrophication and chemical and micro-plastics pollution (see section 1.2.2) has also been suggested by many authors, but again, this has not previously been statistically tested for assemblages in the Pontocaspian region. Further studies are now needed to test dinocyst as proxies for different pollution and anthropogenic perturbations to fully understand the effect of these factors in the region.

6.3.3 Implementing the use of NGS methods in biodiversity studies and monitoring efforts

A few previous studies by Dzhembekova et al. (2017, 2018) together with the results presented in Chapter 5 have demonstrated the advantages that new molecular approaches, such as DNA barcoding and next-generation sequencing techniques can bring to the study of biogeography and biodiversity of microorganisms, including dinoflagellates for this region. These approaches have been successful in the identification of dinoflagellates present in the water column as well as identifying dinocyst in sediment samples (Dzhembekova et al., 2017; 2018; see section 5.1). In addition, these techniques can complement the literature based on traditional taxonomy revealing taxonomic groups that are challenging to identify from their morphology such as parasites. Moreover, the development of a robust DNA barcode database for the Black Sea and the Caspian Sea using the data generating in this project enabled the resolution of inconclusive taxonomical names; clarification of species for taxa previously only identified to genus level; the discovery of new species that have arrived in the basins recently or have been previously undetected; and the revision of current nomenclature (Kaplan-Levy et al., 2016). Besides the benefits of DNA barcoding and high-throughput sequencing (HTS) technologies for biogeography and biodiversity assessments, these techniques also enable fast and accurate monitoring of harmful species of phytoplankton, the majority of which belong to the dinoflagellate group (Ivanova et al., 2019). Monitoring stations for harmful algal species in the Black and Caspian seas are scattered and identification is based on traditional microscopy methods. As a result, seasonal changes in species composition in the water column may go undetected preventing a time-adequate response in case of HAB events. A DNA barcode approach would enable early detection of HAB species in the Black and Caspian seas and monitoring of their abundance and distribution regionally.

6.3.4 Culture experiments with isolated Pontocaspian dinoflagellate species to extend ecological knowledge of important dinoflagellates

Isolating and establishing cultures of phytoplankton species is essential for investigating the ecological adaptation of key species. In the particular case of dinoflagellates, these methods can also be used to study the formation of resting cysts by different dinoflagellate taxa, and to investigate the factors involved not only in the formation of the cyst but also in the excystment process adding information to

the ecological baseline of species of interest. There are dinocyst taxa that dominate or are widely distributed across assemblages of the Caspian Sea of which very little is known of their ecological range, their planktonic phase and their phylogenetic relationships. *Caspidinium rugosum* is an example of a dominant taxon in the Caspian Sea, and *Spiniferites cruciformis* is a widely-distributed taxon. Established cultures from both these cysts would enable the ecological study of these key species in the Caspian Sea and improve the understanding of their potential as indicators of sea surface conditions.

In several previous studies, dinoflagellates have also been shown to have a wide intra-specific variability for different physiological features such as growth, secondary metabolites production and cyst formation (Harvey et al., 2015; Menden-Deuer and Montalbano, 2015; Sala-Pérez et al., 2016). Consequently, to accurately investigate the response of dinoflagellate species to physico-chemical conditions, several isolated strains of the same not previously cultured species must be tested to understand how that species may behave in the ecosystem. This would also permit comparison between strains isolated from different regions and hence allow the investigation of potential ecological adaptation by the Pontocaspian isolates to the environmental conditions present in the Pontocaspian region.

6.4 Final remarks

Dinoflagellates are ubiquitous and one of the main components of the Pontocaspian biota, developing key roles at different trophic levels such as primary producers, decomposers or parasites. In this thesis, I have demonstrated that they are strongly affected by changes in environmental parameters, specifically salinity and temperature. Both of these are predicted to be profoundly impacted by climate change in this area, making dinoflagellates an important and useful group to investigate and monitor. However, on top of this, my work has shown that they also respond to anthropogenic activities and pollution such as shipping activities and eutrophication. For example, rising temperatures and salinity together with increasing eutrophication may result not only in more frequent and intense dinoflagellate blooms but also in the expansion of cosmopolitan taxa replacing the current species assemblages. This may result in a homogenization of the dinoflagellate assemblages dominated by species with a wide ecological range and fast growth rates. This trend can be observed already in some parts of the Black Sea where

the most abundant dinoflagellates are those with a wide ecological range and thriving in eutrophic waters and where algal blooms are recorded frequently.

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APPENDIX A

The content of this appendix belongs to Chapter 3.

Methods to compile explanatory variables

Spatial explicit anthropogenic and environmental variables for the Caspian Sea, known to be relevant for dinocyst assemblages, were compiled. For the Black Sea, the same variables contained in the Bio-ORACLE dataset (Tyberghein et al., 2012) were used. The Caspian Sea variables were resampled to match the Bio-ORACLE dataset, at a resolution of 5 arcmin. Finally, these variables were extracted from the sampled locations.

Table S3.1*:* Summary of variables used in multivariate and correlation analyses.

Variable abrev	Description	Unit
chlmax	Maximum Chlorophyll-*a* content 2011-2015, calculated as the mean of yearly maximum. From https://modis.gsfc.nasa.gov/data/dataprod/chlor_a.php	mg/m^3
chlmean	Mean Chlorophyll-*a* content 2011-2015, calculated as yearly mean. From https://modis.gsfc.nasa.gov/data/dataprod/chlor_a.php	mg/m^3
chlmin	Minimum Chlorophyll-*a* content 2011-2015, calculated as the mean of yearly minimum. From https://modis.gsfc.nasa.gov/data/dataprod/chlor_a.php	mg/m^3
chlra	Range Chlorophyll-*a* (max-min)	mg/m^3
tmax	Maximum Surface Temperature	°C
tmean	Mean Surface Temperature	°C
tmin	Minimum Surface Temperature	°C
tra	Range Surface Temperature (max-min)	°C
smax	Maximum Surface Salinity	unitless
smean	Mean Surface Salinity	unitless
smin	Minimum Surface Salinity	unitless
sra	Range Surface Salinity (max-min)	unitless
dvess	Log-transformed linear distance from the closest vessel route	unitless
dharbor	Log-transformed distance from harbours*harbours size	unitless
vessloc	Number of vessels in sampling cells	unitless

turb	Turbidity, calculated from remote sensing reflectance (Dogliotti et al., 2015). Data from Modis Aqua: https://oceandata.sci.gsfc.nasa.gov/MODIS-Aqua/Mapped/Annual/4km/Rrs_645	FTU (Formazin Turbidity Unit)

Compilation of explanatory variables

1. **chlmax (Maximum Surface Chlorophyll-*a*)**

Layer construction: We averaged the maximum values for each Caspian Sea raster cell yearly mapped Chlorophyll-*a* concentrations from the NASA Ocean Color project (NASA, 2017) for the years 2011 – 2015.

Original resolution: 4 km*4 km (NASA, 2017).

2. **chlmin (Minimum Surface Chlorophyll-*a*)**

Layer construction: We averaged the minimum values for each Caspian Sea raster cell yearly mapped Chlorophyll-*a* concentrations from the NASA Ocean Color project (NASA, 2017) for the years 2011 – 2015.

Original resolution: 4 km*4 km (NASA, 2017).

3. **chlmean (Mean Surface Chlorophyll-*a*)**

Layer construction: We averaged the mean values for each Caspian Sea raster cell yearly mapped Chlorophyll-*a* concentrations from the NASA Ocean Color project (NASA, 2017) for the years 2011 – 2015.

Original resolution: 4 km*4 km (NASA, 2017).

4. **chlra (Range Chlorophyll-*a*)**

Layer construction: We subtracted the Chl_min from Chl_max.

Original resolution: 4 km*4 km (NASA, 2017)

5. **tmax (Maximum Surface temperature), tmean (Mean Surface Temperature), tmin (Minimum Surface Temperature), tra (Range Surface Temperature), smax (Maximum Surface Salinity), smean (Mean Surface Salinity), smin (Minimum Surface Salinity), sra (Range Surface Salinity)**

Layer construction: Our data processing pipeline for these 8 variables consisted of the following steps:

- ❖ Point data measurements were downloaded from the World Ocean Dataset (NOAA, 2018) for the time period from 1914 to 2011 for the Caspian Sea extent including 27315 surface data points.
- ❖ Checked and removed errors in the dataset (data out of the Caspian Sea boundaries, missing decimal punctuation, etc.)
- ❖ Yearly generated metrics over the indicated time period (e.g. average of yearly mean, minimum and maximum values) by Universal Kriging with Automap package in R software (Hiemstra, 2013). Temperature and salinity range (t_range and s_range) were calculated, respectively, as t_max-t_min and s_max-s_min. We used the GEBCO bathymetric raster dataset at 30 arcsec spatial resolution as the initial template (GEBCO, 2014).
- ❖ Clipped raster layers to Caspian Sea bounding box.
- ❖ Filled empty cell values derived by mismatching with the Caspian Sea bounding box with the mean values in a 3*3 focal window, package Raster in R (Hjimans, 2018).
- ❖ Conversion of raster to GeoTiff format.
- ❖ Calculation of evaluation measures to estimate the interpolation performances
- ❖ Finally, the environmental layers were resampled at 5 arcmin to match the Bio-ORACLE dataset

6. dvess (Log-transformed distance from vessel route)

Layer construction: Log-transformation of the linear distance from the closest vessel route in meters.

7. vessloc (Number of vessels in sampling cell)

Layer construction: Discrete classification of the number of vessels navigating in sampling locations, retrieved from mapped oil tankers density for 2015 (AIS Marine Traffic, 2016).

8. dharbor (Log-transformed distance from harbors*harbor size)

Layer construction: Log-transformation of the linear distance of the sum of the two closest harbors in meters multiplied for the harbor size, defined as the number of raster cells laying in vessel routes within a circular buffer of 20km radius centered in the harbor main dock. The reason for including harbor size is that a larger harbor may have a higher impact on sediment distribution and resuspension

what would influence the dinocyst assemblages. The formula assumes that each navigation route has an impact that decays with distance.

9. **turb (Turbidity)**

Layer construction: Values calculated following Equation 1 in the paper of Dogliotti et al. (2015, below).

$$T = \frac{A_T^{\lambda}\rho_w(\lambda)}{(1-\rho_w(\lambda)/C^{\lambda})} \quad [\text{FNU}]$$

ρ_w is the water reflectance, which we download as mapped annual reflectance from Modis at 645 nm band (https://oceandata.sci.gsfc.nasa.gov/MODIS-Aqua/Mapped/Annual/4km/Rrs_645), λ in the equation. A^{λ}_T and C^{λ} are calibration coefficients obtained from Table 2 of Dogliotti et al. paper (2015).

Original resolution: 4 km*4 km (NASA, 2017).

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Table S3.2: Correlation analyses between environmental and shipping variables. R values are presented in the top table and *p*-values in the bottom table. Variables used in these analyses are described in Table S1.

	smin	smax	smean	tra	sra	dvess	tmax	tmin	tmean	vessloc	dharbour	chlmin	turb	chlra	chlmax	chlmean
smin	1															
smax	0.93	1														
smean	0.97	0.99	1													
tra	-0.033	0.32	0.19	1												
sra	0.62	0.86	0.79	0.73	1											
dvess	-0.15	-0.33	-0.27	-0.64	-0.52	1										
tmax	0.38	0.051	0.17	-0.83	-0.41	0.42	1									
tmin	0.22	-0.13	-0.0037	-0.96	-0.59	0.56	0.95	1								
tmean	0.18	-0.17	-0.044	-0.95	-0.62	0.54	0.96	1	1							
vessloc	-0.32	-0.17	-0.23	0.49	0.088	-0.75	-0.44	-0.5	-0.47	1						
dharbour	-0.93	-0.92	-0.03	-0.62	0.26	-0.39	-0.19	-0.17	0.26	0.26	1					
chlmin	-0.59	-0.76	-0.71	-0.48	-0.82	0.21	0.35	0.41	0.47	0.22	0.57	1				
turb	-0.51	-0.54	-0.53	-0.09	-0.47	-0.11	0.091	0.086	0.13	0.43	0.37	0.68	1			
chlra	-0.18	-0.097	-0.13	0.14	0.05	0.022	-0.48	-0.34	-0.34	-0.19	0.24	-0.19	-0.31	1		
chlmax	-0.36	-0.34	-0.35	-0.0019	-0.25	0.016	-0.047	-0.011	0.013	-0.11	0.2	0.3	0.48	0.27	1	
chlmean	-0.19	-0.2	-0.2	-0.015	-0.19	-0.23	0.12	0.068	0.11	0.13	-0.016	0.41	0.59	0.097	0.08	1

	smin	smax	smean	tra	sra	dvess	tmax	tmin	tmean	vessloc	dharbour	chlmin	turb	chlra	chlmax	chlmean
smin	0															
smax	1.6e-16	0														
smean	6.6e-23	2.9e-31	0													
tra	0.85	0.059	0.26	0												
sra	4.8e-05	1.1e-11	1e-08	4.3e07	0											
dvess	0.39	0.046	0.11	2.3e-05	0.0013	0										
tmax	0.023	0.77	0.31	3e-10	0.012	0.012	0									
tmin	0.19	0.44	0.98	2.8e-20	0.00013	0.00042	3.4e-19	0								
tmean	0.29	0.32	0.8	2.3e-18	5.4e-05	0.00062	1.2e-20	2.4e-36	0							
vessloc	0.056	0.33	0.18	0.0022	0.61	1.6e-07	0.0073	0.0018	0.0039	0						
dharbour	6.9e-16	6.7e-13	4.4e-15	4.4e-15	0.86	5.3e-05	0.13	0.019	0.26	0.33	0					
chlmin	0.00017	7.6e-08	1.2e-06	0.0034	1e-09	0.21	0.039	0.012	0.004	0.004	0.00032	0				
turb	0.0015	0.00071	8e-04	0.6	0.004	0.51	0.62	0.44	0.0097	0.0097	4.1e-06	0.68	0			
chlra	0.28	0.57	0.44	0.4	0.77	0.9	0.066	0.04	0.28	0.15	0.27	-0.19	0.064	0		
chlmax	0.033	0.044	0.038	0.99	0.14	0.92	0.95	0.94	0.51	0.24	0.076	0.3	0.0027	0.12	0	
chlmean	0.27	0.24	0.25	0.93	0.28	0.18	0.7	0.52	0.47	0.92	0.012	0.41	0.00015	0.58	6.5e-09	0

Table S3.3: Nonmetric multidimensional scaling (NMDS) analyses by permutation *p*-value based test summary (999 permutations). Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05.

NMDS summary:
Dimensions: 2
Stress: 0.06630709
Stress type 1, weak ties
Two convergent solutions found after 20 tries

	NMDS1	NMDS2	r2	*p*-value
depth	0.51337	0.85817	0.0149	0.785
Chlmax	0.47041	0.88245	0.1223	0.123
Chlmean	0.30175	0.95339	0.0214	0.712
Chlmin	0.96354	-0.26757	0.4347	0.001***
Chlra	-0.03295	0.99946	0.2625	0.011*
Tmin	0.36217	-0.93211	0.5104	0.001***
Smin	-0.74374	-0.66847	0.5295	0.001***
Tmax	0.19985	-0.97983	0.4758	0.001***
Tmean	0.37749	-0.92602	0.5290	0.001***
Tra	-0.54072	0.84120	0.5327	0.001***
Smax	-0.95747	-0.28856	0.6842	0.001***
Smean	-0.89721	-0.44161	0.6330	0.001***
Sra	-0.94334	0.33184	0.7544	0.001***
Dvess	0.89043	-0.45511	0.3634	0.004**
Vessloc	-0.43403	0.90090	0.0478	0.462
Dharbour	0.68456	0.72896	0.5457	0.001***
turb	0.74954	-0.66196	0.1568	0.064
Sand	-0.74849	0.66314	0.3410	0.002**
Silt	0.60371	-0.79720	0.2270	0.017*
Clay	0.92364	0.38325	0.0434	0.539
OM	0.88735	-0.46110	0.0314	0.626

Table S3.4: Dinocyst raw counts.

<https://fluff.bris.ac.uk/fluff/u2/ms16858/7jZkevBMuSiOw_T_OaIhoQzsF/>

APPENDIX B

The content of this appendix belongs to Chapter 4.

Table S4.1: Summary of two-way ANOVA with interaction effect Temperature and Salinity on growth rate. Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

	Df	Sum Sq	Mean Sq	F-value	*p*-value
Temp	1	0.024614	0.024614	43.078	6.53e-06 ***
Sal	3	0.020936	0.006979	12.213	0.000208 ***
Temp:Sal	3	0.010288	0.003429	6.001	0.006113 **
Residuals	16	0.009142	0.000571		

Table S4.2: Tukey multiple comparisons of mean between experimental treatments for growth rate. The left column indicates the salinity treatment comparison.

	diff	lwr	upr	*p*-value
S15-S10	0.075317	0.035832	0.114801	0.000278
S20-S10	0.00725	-0.03223	0.046735	0.951708
S25-S10	0.0345	-0.00498	0.073985	0.098078
S20-S15	-0.06807	-0.10755	-0.02858	0.00078
S25-S15	-0.04082	-0.0803	-0.00133	0.041509
S25-S20	0.02725	-0.01223	0.066735	0.238124

Table S4.3: Summary of two-way ANOVA with interaction effect Temperature and Salinity on cell length. Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

	Df	Sum Sq	Mean Sq	F-value	p-value
Temp	1	298.1	298.14	62.794	2.40e-14 ***
Sal	3	103.4	34.48	7.263	9.43e-05 ***
Temp:Sal	3	271.9	90.64	19.09	1.42e-11 ***
Residuals	392	1861.2	4.75		

Table S4.4: Tukey multiple comparisons of mean between experimental treatments for cell length. The left column indicates the salinity treatment comparison.

	diff	lwr	upr	p-value
S15-S10	-0.69931	-1.49437	0.095749	0.10703
S20-S10	0.624224	-0.17084	1.419284	0.180277
S25-S10	0.433477	-0.36158	1.228537	0.495882
S20-S15	1.323535	0.528475	2.118594	0.000129
S25-S15	1.132787	0.337728	1.927847	0.001533
S25-S20	-0.19075	-0.98581	0.604312	0.925982

Table S4.5: Summary of two-way ANOVA with interaction effect Temperature and Salinity on cell L/W ratio. Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

	Df	Sum Sq	Mean Sq	F-value	*p*-value
Temp	1	0.076	0.07616	5.661	0.0178 *
Sal	3	0.363	0.12094	8.99	9.02e-06 ***
Temp:Sal	3	0.125	0.04157	3.09	0.0271 *
Residuals	392	5.274	0.01345		

Table S4.6: Tukey multiple comparisons of mean between experimental treatments for cell L/W ratio. The left column indicates the salinity treatment comparison.

	diff	lwr	upr	p-value
S15-S10	-0.0173	-0.05962	0.025025	0.717382
S20-S10	-0.00605	-0.04837	0.036272	0.982842
S25-S10	-0.07584	-0.11817	-0.03352	3.03e-05
S20-S15	0.011247	-0.03108	0.053569	0.902553
S25-S15	-0.05855	-0.10087	-0.01622	0.002268
S25-S20	-0.06979	-0.11212	-0.02747	0.000153

Table S4.7: Cell counts of *G. aureolum* for each experimental treatment used to calculate the growth rate.

<https://fluff.bris.ac.uk/fluff/u2/ms16858/7jZkevBMuSiOw_T_OaIhoQzsF/>

Table S4.8: Cell size measurements of *G. aureolum* at each experimental treatment. Length (L) and width (W) are used to calculate the length/width ratio. Measurements are presented in pixels and µm.

<https://fluff.bris.ac.uk/fluff/u2/ms16858/7jZkevBMuSiOw_T_OaIhoQzsF/>

APPENDIX C

The content of this appendix belongs to Chapter 5.

Table S5.1: Environmental variables compiled and extracted for the sample stations used in the analyses included in Chapter 5. Sea surface salinity at the sampling moment (sss.s), sea surface temperature at sampling moment (sst.s), weekly average sea surface temperature of the week of sampling (sst.w), monthly average sea surface temperature of the month of sampling (sst.m), weekly average sea surface chlorophyll-a concentration of the week of sampling (chla.w), monthly average sea surface chlorophyll-a of the month of sampling (chla.m).

Sample	sss.s (psu)	sst.s (°C)	sst.w (°C)	sst.m (°C)	chla.w (mg/m^3)	chla.m (mg/m^3)
Ge_1	0.8	19.9	22.2	21.9	2.8	2.3
Ge_2	1.6	19.1	22.4	21.9	1.5	0.5
Ge_3	0.0	17.6	22.4	21.9	1.5	0.5
Ro_1.10	18.1	18.3	15.9	17.7	2.8	8.7
Ro_1.15	18.1	18.3	15.9	17.7	2.8	8.7
Ro_1.2	18.1	18.3	15.9	17.7	2.8	8.7
Ro_1.20	18.1	18.3	15.9	17.7	2.8	8.7
Ro_10	0.0	15.6	15.2	15.0	2.5	2.6
Ro_2.0	16.8	16.8	17.7	15.6	1.2	1.4
Ro_2.10	16.9	17.0	17.7	15.6	1.2	1.4
Ro_2.15	16.9	17.0	17.7	15.6	1.2	1.4
Ro_2.2	16.8	16.8	17.7	15.6	1.2	1.4
Ro_2.5	16.8	16.8	17.7	15.6	1.2	1.4
Ro_3	5.5	16.2	15.7	15.2	2.4	2.2
Ro_4	5.5	15.4	15.3	15.2	2.4	2.2
Ro_5	5.4	15.8	15.5	15.2	2.4	2.2
Ro_6	4.1	14.2	14.7	15.1	2.5	2.4
Ro_7	4.6	15.3	15.2	15.2	2.5	2.2
Ro_8	4.1	14.4	14.8	15.1	2.5	2.4
Ro_9	3.7	14.6	14.9	15.1	2.5	2.4
Ukr_1	2.1	20.3	18.9	16.8	2.7	2.3
Ukr_2	3.1	17.4	16.5	13.9	1.6	1.1
Ukr_3	3.8	18.5	17.4	14.9	1.6	1.1
Ukr_4	3.2	19.7	18.3	15.9	1.6	1.1
Ukr_5	0.4	20.3	18.7	14.0	1.7	1.2
Ukr_6	0.5	17.5	16.6	14.0	1.7	1.2
Ukr_7	0.8	20.5	18.9	16.0	1.7	1.1
Ukr_8	0.3	20.5	18.9	17.0	1.7	1.1

Table S5.2: ANOSIM results. Zone referred to the different areas of the study and time to the time of the year when was sampled. However, time was not used in the interpretation of the results, it was left out of the analyses, and it was only tested as an exploratory analysis.

Zone		**Time**	
R	*p*-value	R	*p*-value
0.35	<0.001	0.14	0.07

Table S5.3: ANOVA results for the environmental variables used in dbRDA analyses. Sea surface salinity at the sampling moment (sss.s), sea surface temperature at sampling moment (sst.s), weekly average sea surface temperature of the week of sampling (sst.w), monthly average sea surface temperature of the month of sampling (sst.m), weekly average sea surface chlorophyll-a concentration of the week of sampling (chla.w), monthly average sea surface chlorophyll-a of the month of sampling (chla.m).

Variable	**Sum of Sqs**	**F**	***p*-value**
sss.s	2.0468	6.797	0.001***
sst.s	0.1252	0.4158	0.898
sst.w	0.7162	2.3783	0.04*
sst.m	0.4294	1.4259	0.198
chla.w	0.3286	1.0911	0.354
chla.m	0.1445	0.4799	0.842

Table S5.4: Canonical correspondence analysis (CCA) analyses by permutation p-value based test summary (999 permutations). Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05. Sea surface salinity at the sampling moment (sss.s), sea surface temperature at sampling moment (sst.s), weekly average sea surface temperature of the week of sampling (sst.w), monthly average sea surface temperature of the month of sampling (sst.m), weekly average sea surface chlorophyll-a concentration of the week of sampling (chla.w), monthly average sea surface chlorophyll-a of the month of sampling (chla.m).

	Inertia	Rank
Total	4.921	
Unconstrained	4.921	19
Inertia is scaled Chi-square		

Eigenvalues for unconstrained axes:

CA1	CA2	CA3	CA4	CA5	CA6	CA7	CA8
0.8661	0.7306	0.6717	0.5870	0.4671	0.4166	0.3324	0.2360

(Showing 8 of 19 unconstrained eigenvalues)

	CA1	CA2	r2	Pr(>r)
sss.s	0.85232	0.52303	0.5162	0.001 ***
sst.s	-0.72774	0.68585	0.0211	0.737
sst.w	0.66746	-0.74465	0.0234	0.723
sst.m	0.97413	-0.22599	0.1273	0.144
chla.w	-0.62665	-0.77930	0.0115	0.845
chla.m	0.73109	0.68228	0.1380	0.125

Table S5.5: Distance-based redundancy analysis (dbRDA) analyses results.

	Inertia	Proportion	Rank
Total	9.55852	1.00000	
Constrained	3.79066	0.39657	6
Unconstrained	6.32377	0.66158	13
Imaginary	-0.55591	-0.05816	8

Eigenvalues for constrained axes:

CAP1	CAP2	CAP3	CAP4	CAP5	CAP6
2.4935	0.5769	0.3474	0.2965	0.0446	0.0318

Eigenvalues for unconstrained axes:

MDS1	MDS2	MDS3	MDS4	MDS5	MDS6	MDS7	MDS8	MDS9	MDS10	MDS11	MDS12	MDS13
2.1345	1.2822	0.7469	0.6558	0.5593	0.4384	0.2424	0.1534	0.0497	0.0333	0.0164	0.0074	0.0041

Data matrix S.5.1: This excel file compiles the results of analyzing the sequences obtained as outcome of Illumina MiSeq sequencing with QIIME pipeline as detailed in section 5.2.3. Relative abundance is presented for phyla and genera levels in separate tabs. Tabs labelled with "unfiltered" contain the raw data including the relative abundance of unmatched sequences. Abundance plots included in Chapter 5 are built with the data presented in the filtered tabs. Dinoflagellate rows are highlighted in yellow.

<https://fluff.bris.ac.uk/fluff/u2/ms16858/7jZkevBMuSiOw_T_OaIhoQzsF/>

ITS sequences used in the analyses of this Chapter can be downloaded from the following link:

<https://fluff.bris.ac.uk/fluff/u2/ms16858/7jZkevBMuSiOw_T_OaIhoQzsF/>