Universitat de les Illes Balears

IMPLICATIONS OF PHYTOPLANKTON CELL DEATH LOSSES FOR CARBON FLUX IN OCEANIC FOOD-WEBS

TESIS DOCTORAL

Departamento de Biología de la Universidad de las Islas Baleares

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Key words: Phytoplankton, cell death, vector of mortality, releasing processes, dissolved organic carbon, carbon fluxes, bacterial viability

Let us a little permit Nature to take her own way; she better understands her own affairs than we.

Michel de Montaigne (1533-1592)
Essays, Book III - Chap. XIII

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Abstract

Phytoplankton losses by cell death, independent of grazing are occurring in the ocean. Phytoplankton cells have been described to die upon encountering adverse environmental conditions, and cell death and lysis would result in the release of the carbon incorporated in the photosynthesis by the phytoplankton mostly as dissolved organic carbon (P_{DOC}). The availability of dissolved organic carbon (DOC) is expected to be a major constraint for the heterotrophic bacteria and consequently the release by cell mortality of the recently photosynthate carbon may benefit the bacterial community and the channelled transfer of carbon through the microbial loop. All this processes have been however poorly documented yet and the contribution of the phytoplankton cell death to the release of P_{DOC} has not been explored in natural communities. The goal of this PhD Thesis is to provide quantitative information on phytoplankton and bacteria cell death in natural communities and to document the fraction of DOC released by phytoplankton (P_{DOC}) resulting from cell death under contrasting natural conditions. The exploration of the relationships between the phytoplankton cell mortality, DOC released by phytoplankton (P_{DOC}) and microbial survival would contribute to better understand the paths of carbon from photosynthesis to heterotrophic bacteria by cell death processes operating in nature.

Contrasting environments and planktonic communities from different oceanic regions including the Mediterranean Sea, Arctic, Antarctic and Atlantic oceans were studied. Evaluation of the in situ health status of the natural phytoplanktonic communities and bacteria were analyzed by testing their cell membrane permeability a property used to define cell death by cell biologist. On average the 40.5 ± 16.9 % of the total phytoplankton abundance were represented by dead cells in the natural phytoplankton populations analyzed during this study, suggesting the relevance of cell dead processes for phytoplankton dynamics. Bacterioplankton survival averaged 69.8 ± 19.8 % of heterotrophic living bacteria (HLB) along the studied areas, presenting significant positive relationships with nutrients concentration (i.e. phosphate at oligotrophic areas) and with water temperature, as indicated by the increasing percentage in HLB with increasing water temperature.

Abstract

Nutrients concentration and temperature were also relevant factors determining the heath status of phytoplankton. Across the studied areas, with the exception of polar waters where nutrient availability was not a limiting factor, microphytoplankton populations showed higher mortality within oligotrophic waters, and increased survival rates with increasing nutrient concentrations. For instance, diatoms presented higher percentage of living cells at the NE Atlantic waters enriched in nutrients by cyclonic mesoscale eddies and at the waters influenced by the Mauritanian's upwelling and its mortality significantly increased under anticyclonic conditions, deprived of nutrients, and at the oligotrophic waters from the NE Subtropical Atlantic gyre. At the Mediterranean Sea, dinoflagellates and *Synechococcus* spp. survival responded significantly to phosphate availability and their viability increased with increasing phosphate concentration. Moreover, we observed *Prochlorococcus* sp. cell mortality to be significantly influenced by the increase in concentration of dissolved inorganic nitrogen. Additionally, as well as observed for the bacterioplankton, water temperature appeared to have significant effects on the health status of phytoplankton. Picophytoplankton populations' survival responded positively with increasing water temperature, and *Synechococcus* spp. and *Prochlorococcus* sp. presented higher viability associated with warmer waters in the Mediterranean Sea and NE Atlantic Ocean. Particularly within polar waters, nano-microphytoplankton populations presented a distinct relationship to increasing water temperature; nanoflagellate mortality decreased with increasing water temperature, while conversely, diatoms presented higher mortality associated with warm waters.

Phytoplankton mortality explained the 41.4 ± 3.9 % of the percentage of released DOC production (P_{DOC}) relative to total primary production, indicating that cell death is a major process explaining the production of dissolved organic carbon by oceanic phytoplankton. The percentage of P_{DOC} observed here, represented on average the half of the total primary production (54.4 ± 1.5 %) and supported, at the NE Atlantic Ocean bacterial viability.

The results presented in this PhD indicate that phytoplankton cell mortality, by excreting the photosynthesized carbon as dissolved to the medium, is fuelling the dissolved organic carbon pool and significantly affect the carbon fluxes, by favouring the microbial loop and reducing particulate carbon sequestration to the deep ocean.

Resumen

En el océano se dan pérdidas de fitoplancton por muerte celular, independientes de la predación. Se ha descrito cómo las células de fitoplancton mueren al encontrarse en condiciones ambientales adversas; esta muerte celular y lisis puede resultar en la liberación del carbono incorporado en la fotosíntesis por el fitoplancton en su mayoría como carbono orgánico disuelto (P_{DOC}). Una de las mayores limitaciones para las bacterias heterotróficas es la disponibilidad de carbono orgánico disuelto (DOC) por lo que, consecuentemente, la liberación de carbono fotosintético proveniente de la muerte celular beneficiaría a la comunidad bacteriana al ser transportada por la red trófica. Por el momento, estos procesos aún no están totalmente documentados y la contribución de la muerte celular de fitoplancton en la liberación de P_{DOC} no se ha explorado en comunidades naturales. Esta tesis tiene como objetivo proveer información cuantitativa sobre la muerte celular en fitoplancton y bacterias en comunidades naturales y documentar la fracción del DOC que es liberada por el fitoplancton (P_{DOC}) a través de la muerte celular bajo condiciones naturales muy contrastadas. La exploración de las relaciones existentes entre la muerte celular del fitoplancton, el carbono orgánico disuelto liberado por este fitoplancton y la supervivencia bacteriana puede contribuir a una mayor comprensión de los pasos que da el carbono desde la fotosíntesis hasta las bacterias heterotróficas a través de los procesos de muerte celular que operan en la naturaleza.

Se estudiaron comunidades planctónicas y parámetros ambientales en diferentes y contrastadas regiones oceánicas que incluyeron el Mar Mediterráneo y los Océanos Ártico, Antártico y Atlántico. Se evaluó in situ el estado de salud de las comunidades fitoplantónicas naturales y de bacterias a través del examen del estado de permeabilidad de la membrana celular, propiedad que define la muerte celular para los biólogos celulares. Las poblaciones naturales de el estudio demostraron que las células muertas pueden representar en promedio el 40.5 ± 16.9 % de la abundancia total del fitoplancton para todos los sistemas, lo cual sugiere el grado de importancia de los procesos de muerte celular para la dinámica del fitoplancton. La supervivencia bacteriana supuso como media un 69.8 ± 19.8% de las bacterias heterotróficas vivas (HLB) de las zonas estudiadas y presentó relaciones positivas significativas con la concentración de nutrientes (p.e. fosfatos en áreas oligotróficas) y con la temperatura del agua, de modo que el porcentaje de HLB aumenta a medida que lo hace la temperatura del agua.

La temperatura del agua y la concentración de nutrientes también resultaron ser factores relevantes para determinar el estado de salud del fitoplancton. A lo largo de las zonas estudiadas, con la excepción de aguas polares donde la disponibilidad de nutrientes no era un factor limitante, las poblaciones de microfitoplancton presentaron mayores valores de mortalidad en aguas oligotróficas incrementándose los valores de supervivencia en zonas con concentraciones crecientes de nutrientes. Por ejemplo, las diatomeas presentaron un porcentaje de células vivas mayor en aguas del NE Atlántico, enriquecidas en nutrientes por los eddies ciclónicos de mesoescala y en aguas bajo influencia del afloramiento de Mauritania. Su mortalidad aumentó significativamente en condiciones anticiclónicas, desprovistas de nutrientes, y en las aguas oligotróficas del giro Atlántico NE Subtropical. En el mar Mediterráneo, la supervivencia de los dinoflagelados y de *Synechococus* spp. respondió significativamente a la disponibilidad de fosfatos y su viabilidad también aumentó con aumentos en la concentración de fosfatos. También se observó que la muerte celular en *Prochlorococcus* sp. se veía afectada por la concentración de nitrógeno inorgánico disuelto. De manera adicional, la temperatura del agua, al igual que en bacterias, resultó tener efectos significativos sobre el estado de salud del fitoplancton. Las supervivencia de las poblaciones de Picofitoplancton respondió positivamente a aumentos en la temperatura del agua y *Synechococus* spp. y *Prochlorococcus* sp. presentaron mayores viabilidades asociadas a aguas mas cálidas en el mar mediterráneo y en el NE del océano Atlántico. Particularmente, en aguas polares, las poblaciones de nano-microfitoplancton presentaron mayores mortalidades asociadas a aguas mas cálidas.

La mortalidad del fitoplancton constituye un proceso importante en la producción de carbono orgánico disuelto, resultando que el porcentaje de células muertas de fitoplancton explica el $41.4 \pm 3.9\ \%$ del porcentaje de producción liberada (P_{DOC}) en relación a la producción primaria total.

Los resultados presentados en esta tesis indican que la muerte celular del fitoplancton, excretando el carbono fotosintético en forma de carbono disuelto al medio, alimenta la reserva de carbono orgánico disuelto y afecta significativamente a los flujos de carbono, favoreciendo el loop microbiano y reduciendo la sedimentación de carbono particulado hacia el océano profundo.
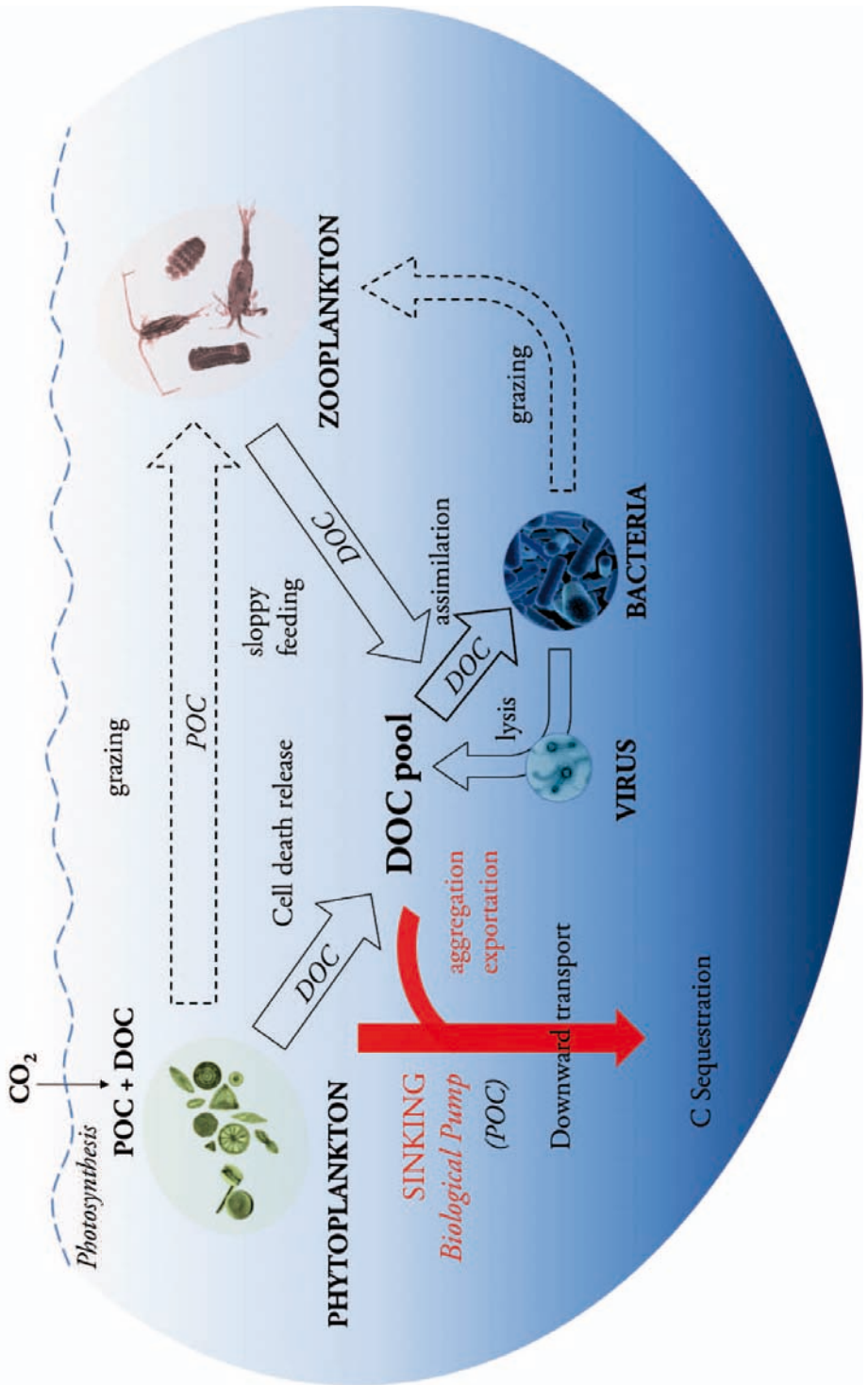
Fig.1. Fate of the photosynthesized carbon by phytoplankton in marine systems. POC: particulate organic carbon – DOC: Dissolved organic carbon. Full black arrows: biological & physiological processes – Full red arrows: physical processes - dotted arrows: trophic relationships

General Introduction

The oceans play a major role in the global carbon cycle and the ultimate fate of anthropogenic CO_2 (Falkowski et al., 2000). Marine phytoplankton are major players in the carbon cycle because of its ability to transform inorganic resources into nutritive organic elements usable by consumers, accounting for about 50% of the global biological uptake of carbon dioxide (Field et al., 1998). CO_2 in solution is converted to organic matter by the photosynthetic activity of phytoplankton and is entering the marine carbon cycle by different processes strongly dependent on the dominant mechanisms of losses operating. In oceanic areas, phytoplankton dominate the primary production and represent the base of the trophic food web, implying that the carbon photosynthesized by phytoplankton is channelled through the pelagic food web via grazing by herbivores (Fig. 1).

Part of the organic carbon could be transported downward from the surface waters to the deep ocean as a result of phytoplankton sinking. This process known as the "biological pump" represents the flux of organic carbon, mostly as particles, that ending at the bottom of the ocean sequestrates atmospheric carbon (Fig. 1). A substantial part of the photosynthesized carbon is however entering the dissolved organic pool by a variety of processes as sloppy feeding, released during grazing, or by phytoplankton dissolved organic carbon release by exudation and cell death. This path of the carbon could be therefore transfer to the food web by the microbial loop (Azam 1998), since heterotrophic bacteria are the major consumers of this DOC pool (Fig. 1). The fate of the carbon photosynthesized by phytoplankton in the system is then dependent on the dominant loss processes and the net balance will be calculated by applying the equation ruling phytoplankton population dynamics:

Net growth = Gross growth + Losses (grazing + sinking + cell death)

Among the losses mechanisms, phytoplankton cell mortality has been receiving less attention until recently but it could be more important than presumed in the past, representing losses up to 50% of the phytoplankton growth (Agustí et al 1998).

In the euphotic layer, the net phytoplankton biomass accumulation and production are a function of the growth rates of species' populations, mostly depending on resources on the one hand, and of their loss rates via sinking, grazing and cell death in the other.

A large effort has been made in the literature to describe the processes influencing phytoplankton growth and productivity. Among the abiotic factors driving oceans primary productivity and biomass, nutrients availability largely influences phytoplankton biomass and production (e.g. Reynolds 1995) as well as modifying community structure, indicated, for instance, by the predominance of small phytoplankton forms (picophytoplankton) under oligotrophic conditions (Platt et al., 1983; Agawin et al., 2000).

The light irradiance, water temperature and hydrological features (such as mixing regime) are also interceding in ruling phytoplankton dynamics and structure (e.g Chan 1978; Litchman 1998; Agustí and Duarte, 2000; Atkinson et al., 2003; Daufresne et al., 2009). The competition between the phytoplanktonic species for the same resources (e.g. nutrients, light) is also influencing the community structure and natural population dynamics (e.g Litchman and Klausemeier, 2001; Litchman et al., 2004) but is difficult to evaluate in situ. The principle of competitive exclusion (Hardin, 1960; Armstrong and McGehee, 1980) suggests that in homogeneous, well-mixed environments, species that compete for the same resource cannot coexist, and one species should finally take advantage over the others.

Thus, while in many natural waters, nutrients and light are the major limiting resources and natural phytoplankton population would be competing, in situ measurements show prolonged coexistence of a large number of phytoplankton species, which was identified by Hutchinson (1961) as the plankton paradox. How a large number of competing phytoplankton species could coexist in marine ecosystems under a seemingly limited variety of resources? Several mechanisms were proposed to explain this plankton paradox; the spatial segregation (Hassell et al., 1994) by which species would differ in their ecological preferences and occupy different niches (Connell 1978); perturbation of the water column would also promote coexistence of species (Tilman 1994). Incomplete vertical mixing through a light gradient affects the growth of the phytoplankton species (Huisman et al., 1999), which might in some cases promote the coexistence, and diversity of phytoplankton (Weissing and Huisman, 1994).

Since Hutchinson (1961) questioned the theoretical foundation of the mechanisms allowing the coexistence of complex planktonic communities, and, despite the aroused effort of the marine biologist and ecologist community to explain the paradox, the analysis of such coexistence in natural planktonic communities results elusive.

For instance, when examining the competitive success of planktonic populations, the classical evaluation focuses in changes in abundance and their distribution, but this approach cannot suffice because factors such as grazing pressure, difficult to embrace in the evaluation, would reduce the abundance of the best competitor (Thingstad et al., 2005).

The inspection of the health status of the natural planktonic species, quantifying the living and dead cells proportion in situ under the environmental conditions, constitutes a relevant approach to inform on the success of the population conforming the natural communities (Agustí 2004; Alonso-Laíta & Agustí 2006; Alonso-Sáez et al. 2006; Gasol et al., 2009a) facing environmental changes and aspires to be broadly apply to document the interactions and ecological preferences of the population. The same factors that are regulating the dynamic of phytoplankton population growth would be vital when limited, representing a stress for the cells inducing cell death. New discoveries and growing empirical evidence on the rates and mechanisms of phytoplankton cell death illustrate that phytoplankton cells die upon encountering adverse environmental conditions, such as limited nutrient concentration (Tilman et al., 1982; Berges and Falkowski, 1998), low and extreme UV and PAR conditions (Berges and Falkowski, 1998; Llabrés and Agustí, 2006), temperature thresholds (Alonso-Laita and Agutí 2006), and viral infection (Suttle et al., 1990; Fuhrman 1999). The same concept will apply to the heterotrophic component of the plankton, with the availability of organic and inorganic nutrients constrain their abundance, production and metabolic activity (del Giorgio et al., 1996; Schumann et al., 2003; Alonso-Sáez et al, 2007; Mével et al., 2008; Gasol et al. 2009b). Water temperature that intercedes as well in bacterial processes such as growth, production, and substrates uptake (White et al., 1991; Kirchman et al., 1997; Pomeroy et al., 2001) would also affect bacterial survival.

The assessments of the percentage of living and dead planktonic cells in the ocean would provide a direct information on the health status of the populations under the growth conditions experienced in situ, and would help in exploring their competitive success and coexistence. The study of such interactions may enable us to acknowledge the underlying mechanisms that govern the structuring of natural plankton communities, such as competition and ecological preferences, identifying the factors affecting health status under changes in environmental conditions (such as nutrient availability and hydrological changes).

Phytoplankton cell death and its role in the release of dissolved organic carbon

Phytoplankton cells may represent a direct source of production of oceanic DOC, which is often overlooked or underestimated. Some studies have estimated that in some systems about 50% of algal production passes to the DOC pool (Karl et al., 1998). The production of DOC by phytoplankton (P_{DOC}) is principally sustained by mechanisms such as sloppy feeding, phytoplankton cell death and lysis, and exudation (Myklestad 2000; Nagata 2000). The sloppy feeding consists in the incomplete feeding by herbivorous that results in cell fragments and in the liberation of organic carbon (Nagata and Kirchman 2000, Nagata 2000). There is also evidence of consistent extracellular exudation by phytoplankton cells in their exponential phase of growth (Myklestad 2000). Cell death and lysis through physical stressors or biological factors such as the attack of viruses would result in the loss of significant amounts of cell material to the external medium and imply the liberation of the organic carbon synthetized during the photosynthesis (Myklestad 2000; Nagata 2000).

Previous studies revealed that cell lysis should be a significant vector for DOC production in oligotrophic waters (Agustí et al., 1998; Agustí et al., 2001; Agustí and Duarte 2012). The extracellular release or production of dissolved organic carbon (P_{DOC}) by phytoplankton would be expected to vary with the health status of the phytoplanktonic populations and the reliable changing environmental conditions. The examination of such contribution of the phytoplankton cell death to the releasing in P_{DOC} has been poorly explored in natural communities.

The dissolved fraction of primary production (P_{DOC}) can represent a significant amount of total primary production (TPP) (Marañón et al., 2004; Morán and Estrada, 2001) though it is often neglected in primary production measurements, typically estimating particulate primary production. The dissolved organic carbon production by the phytoplankton corresponds to the difference between total and particulate primary production (Morán et al., 2001) which measurements are based on the incorporation of radioisotope ^{14}C during the photosynthesis (Steeman Nielsen 1952). Studies describing the dynamic of P_{DOC} indicated higher contribution of the fraction of P_{DOC} with regard to the total primary production, termed the Percentage of Extracellular Release (PER), in oligotrophic waters and a decreased of the percentage of P_{DOC} in more productive waters (Teira et al., 2001; Morán et al., 2002). However, the quantification of DOC production by phytoplankton and its relative proportion to total primary production in oceans remains poorly documented but needed to estimate the contribution of the phytoplankton cell death to the release of P_{DOC} in marine ecosystems.

The fate of released dissolved organic carbon

Heterotrophic bacteria (HB) play a key ecological role in the cycling of carbon and nutrients in aquatic systems (Cole et al., 1988; Ducklow 2000), as they are major consumers of dissolved organic matter (DOM) in the ocean (Sherr and Sherr, 1994; Azam, 1998). HB recycle organic carbon through respiratory processes, and channel significant amounts of dissolved organic carbon (DOC) to higher levels of the pelagic food webs via the microbial loop (Pomeroy 1974; William 1981; Sherr and Sherr, 1988). The availability of DOC is a major constraint for HB dynamics, influencing a range of processes including HB growth efficiency, respiration or cell activity (Kirchman et al., 1991; Kirchman et al., 2004). Since the dissolved organic fraction is almost exclusively accessible to HB (Azam et al., 1983, Ducklow and Carlson, 1992), the amount of labile DOC released as a result of phytoplankton cell death would benefit this particular bacterial community, fuelling its growth and having a notable impact on food web structure and the fate of the recent photosynthate carbon in the ocean (Kirchman et al., 1991).

It exists a common belief that heterotrophic bacteria are strongly dependent on the organic matter synthesized in situ by phytoplankton (Baines & Pace, 1991), consequently the release of the photosynthetate carbon by phytoplankton, that will be the most labile fraction, would represent a major source of organic carbon for bacteria in the ocean, however examination of the consequences of the P_{DOC} released for HB activity and cell health, have received few attention. A high percentage of bacterial cells are either metabolically inactive or dead in natural marine plankton communities (Choi et al., 1996; Smith and del Giorgio, 2003) and the algal-derived carbon that benefits HB processes such as growth (Cole et al., 1988) associated with the potential rapid utilization of this algal-origin organic carbon (Coveney and Wetzel, 1989; Chen and Wangersky, 1996) would probably affect HB viability and should be explored to better understand bacteria processes.

General goal and outline of the thesis

This thesis aimed to in situ document and couple three important but neglected processes: (1) Phytoplankton cells are dying upon encountering adverse environmental conditions; (2) Cell death and lysis would result in the release of the carbon incorporated in the photosynthesis by the phytoplankton as dissolved organic carbon (P_{DOC}); (3) The availability of dissolved organic carbon released by phytoplankton cell mortality may benefit the bacterial community, which will channel this released organic carbon through the microbial food web.
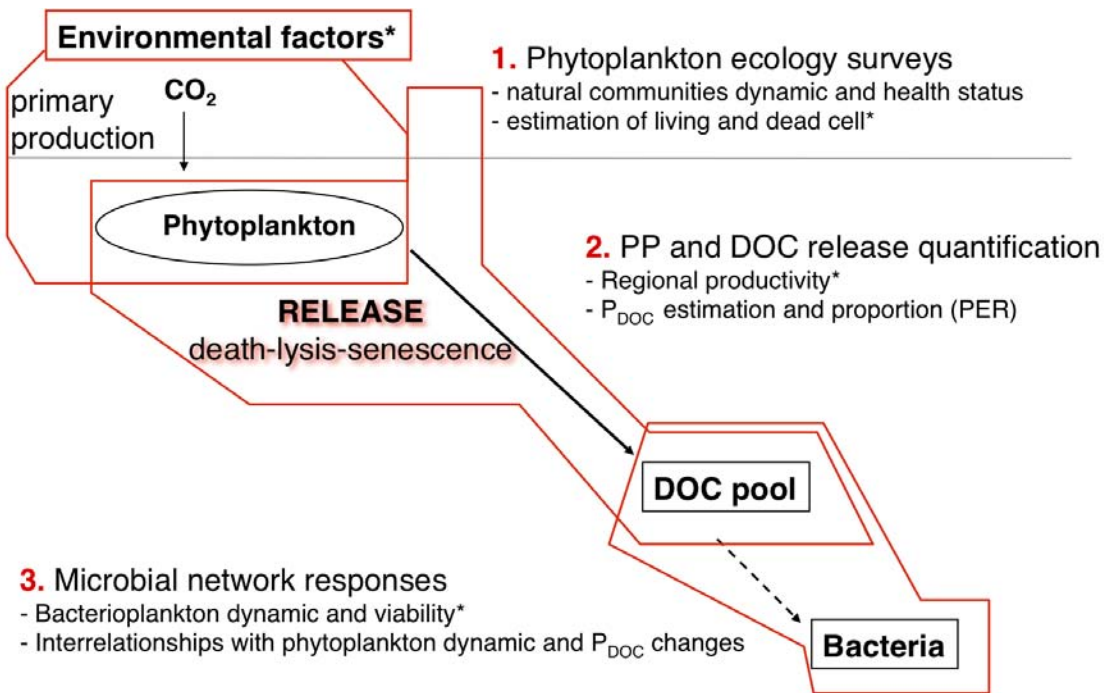
Conceptual scheme: It summarizes the 3 main goals assessed during this thesis and how they couple to respond the general problematic:

Facing recent ENVIRONMENTAL STRESS (natural or anthropogenic), how do PHYTOPLANKTON losses by CELL DEATH in natural communities' affect oceanic CARBON fluxes and MICROBIAL utilization?

The main objective addressed in this work is to provide quantified information on phytoplankton cell death in natural communities and document the fraction of DOC released by phytoplankton (P_{DOC}) in oceans in order to try to relate it to phytoplankton cell death and microbial responses. This goal will be achieved through specific objectives gathered in three major goals:

Goal 1: To assess the natural plankton populations health status, quantified by estimating living and dead cells in natural communities and to examine its relationships with environmental conditions (chemical and physical). The objective is to better understand the ecological constrains of the natural plankton communities facing a wide range of environmental conditions, identifying thus the major factors influencing their dynamics and assessing the competitive success within the coexisting populations.

Goal 2: To quantify and document the production of dissolved organic carbon (P_{DOC}) by phytoplankton communities within different oceanic systems, and analyze whether phytoplankton cell death could explain the excretion of photosynthate carbon as P_{DOC}.

Goal 3: To explore the implications of the dissolved organic carbon produced by the phytoplankton cells (P_{DOC}) on heterotrophic bacterial community, testing the relationship between the recently released labile P_{DOC} and the viability of HB.

These three major goals will be illustrated and addressed within the following chapters:

Chapter 1

Phyto- and bacterioplankton abundance and viability and their relationship with phosphorus across the Mediterranean Sea (2010) Sébastien Lasternas, Susana Agustí and Carlos M. Duarte. *Aquatic Microbial Ecology 60*: 175–191.

This chapter aims to study the changes in plankton community structure and the variability in health status of the major planktonic populations in a synoptic study across the Mediterranean Sea. Here, we analyse the different nutrient regimes and hydrological conditions across the different sub-basins and straits and examine the distribution and health status of both heterotrophic bacteria and planktonic autotrophs (pico, nano and microphytoplankton). We try to relate the survival success of the different populations in relation to environmental conditions, such as, nutrient limitation, water temperature, and judge their competitive capacity with the coexisting species (***Goal 1***).

Chapter 2

Phytoplankton community structure during the record Arctic ice-melting summer 2007 (2010) Sébastien Lasternas and Susana Agustí. *Polar Biology 33*: 1709–1717.

In this chapter, we search for changes in the abruptly altered pelagic arctic ecosystem during the record ice-melting in the Arctic observed in summer 2007, by quantifying and analyzing the differences in phytoplankton abundance and community structure. We examine how water salinity and temperature, as a proxy of waters receiving ice-melt, may explain the differences in the phytoplankton communities and other phytoplankton cell properties examined.

Goal 1 is tackled by testing the state of phytoplankton cell health by examining the proportion of living v. dead cells. Additionally, we quantify the primary production and its fraction released as dissolved organic carbon (***Goal 2***).

Chapter 3

Planktonic community structure and survival success in Antarctic Peninsula waters during Austral summer (2012) Sébastien Lasternas and Susana Agustí. *Submitted to Global Change Biology*

In this study conducted in the Antarctic Peninsula (AP) during the austral summer of 2009, we quantify the percentage of living and dying cells in the heterotrophic and autotrophic plankton communities and explore the variation in the community structure and survival under the environmental variability observed at the AP waters (***Goal 1***). We also quantify the extracellular release by the production of dissolved organic carbon (P_{DOC}) by phytoplankton and its influence on the bacterial community in an effort to evaluate the carbon fluxes at this part of the warming Southern Ocean (***Goals 2&3***).

Chapter 4

Carbon fluxes forced by anticyclonic mesoscale eddies generated by islands at the subtropical NE Atlantic Ocean (2012) Sébastien Lasternas, Marc Piedeleu, Pablo Sangrá, Carlos M. Duarte and Susana Agustí. *Biogeosciences Discussion 9: 10241-10283.*

The distribution and health status of heterotrophic bacteria and phytoplankton communities are investigate within cyclonic and anticyclonic systems of the Canary eddy fields and compared to similar processes in waters outside the eddies system (***Goal 1***). We estimate the phytoplankton lysis rates and their relationship with the quantified production of dissolved organic carbon by phytoplankton (P_{DOC}) at the different sites to analyse how eddy structures amplify the magnitude of cell mortality, influence the microbial community and consequently influence carbon fate (***Goals 2&3***)

Chapter 5

Bacterial survival governed by the release of dissolved organic carbon from senescent oceanic phytoplankton. (2012). Sébastien Lasternas and Susana Agustí. *Submitted to Proceedings of the National Academy of Sciences*

Goal 1 is addressed in this chapter by quantifying the percentage of living and dying cells in phytoplankton and bacteria communities across a range of oceanographic conditions, from ultra oligotrophic to highly productive waters in the NE subtropical Atlantic Ocean.

Goals 2&3 are also addressed by documenting the proportion of DOC released by phytoplankton and its implication on HB viability, and phytoplankton cell mortality.

Methodological highlights

This research focusing in the analysis of natural communities and processes, implied the collection of data from the field during several researches cruises from contrasting oceanic systems. These oceanographic researches provided a great opportunity to confront a variety of communities, and environmental conditions, ranging from cold to warm waters and contrasting nutrient and hydrological regimes (Figure 2).
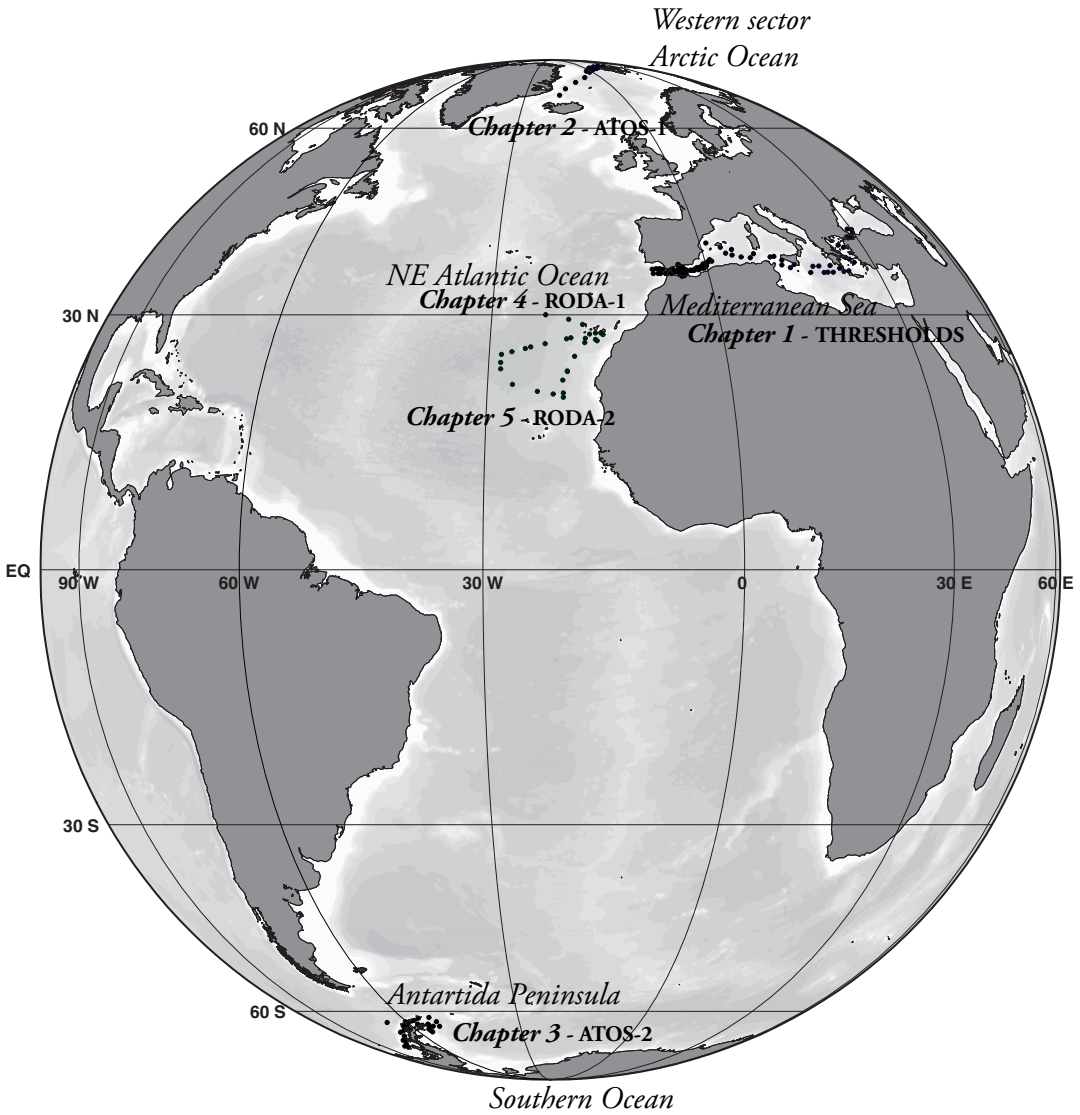

Fig.2. Oceanic areas studied and sampling stations occupied during the various cruises

To achieve the objectives of our research, we applied recent approaches to quantify cell death in natural planktonic communities encountered during the oceanic cruises we participated. These approaches consisted in testing cell membrane permeability, as this property is used to define cell death in Cell Biology (Alberts et al., 1998). The membrane in healthy cells is selectively permeable, regulating what enters and exits the cell, thus facilitating the transport of materials needed for survival (Alberts et al., 1998). Any alteration of the membrane that compromise the permeability would lead to the cell to die with no possible recovery as the process is irreversible (Ellis et al., 1991; Alberts et al., 1998). We utilized two distinct techniques suitable for the study of both autotrophs and heterotrophs.

The cell digestion assay (CDA) developed by Agustí and Sánchez (2002) is a technique used to identify dead or dying phytoplankton cells in natural communities. Originally applied in the field of Cell Biology (Darzynkiewicz et al., 1994), this approach aims in characterizing the dead (necrotic or advanced apoptotic) cells that increasing the membrane permeability have lost their ability to maintain homeostasis (Wyllie et al., 1980; Ellis et al., 1991; Darzynkiewicz et al., 1994). The procedure involves the brief exposure of the natural cells to an enzyme cocktail (Trypsin and DNase I). Compromised plasma membranes (i.e. necrotic or advanced apoptotic cells) can lose their physical barrier properties and permit the free passage of enzymes into the cells' cytoplasm, resulting in the total digestion of the senescent or dead cells. Therefore, this technique allows quantification of living cells remaining in the sample after the CDA test. This technique is as valid in classic or epifluorescence microscopy as in flow cytometry discarding any possible confusion with the autofluorescency of the phytoplankton cells, as opposed to the utilization of staining methods.

The nucleic acid double staining (NADS) method of Gregori et al. (2001) is an accurate technique for discriminating living from dead bacterial cells. This method involves the use of two stains with distinct properties. The SyBR Green (DNA-binding stain) is able to stain, indifferently, viable or dead heterotrophic bacteria. Propidium Iodide (PI) specifically penetrates cells with compromised membrane integrity, hence staining the dead HB. In addition to its simplicity, this double staining method, largely used in natural samples, provides clear fluorescent signals in cytometer that may not be perceived when using a single stain method.

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Chapter 1

Phyto- and Bacterioplankton abundance and viability and their relationship with phosphorus across the Mediterranean Sea

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Aquatic Microbial Ecology 60: 175–191

Chapter 2

Phytoplankton community structure during the record arctic ice-melting of summer 2007

Sébastien Lasternas, Susana Agustí (2010)

Polar Biology 33: 1709–1717

Chapter 3

Planktonic community structure and survival success in warming Antarctic Peninsula waters during Austral summer

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Abstract

During the austral summer 2009, the composition and survival success of autotrophic and heterotrophic planktonic communities of the Antarctic Peninsula (AP) were examined across the waters of the Bellingshausen Sea, the Bransfield Strait and the Weddell Sea. Changes in phytoplankton biomass and productivity (chlorophyll *a* and primary production measurements) at the AP waters as well as the release of dissolved organic carbon by phytoplankton (P_{DOC}) were also examined in this study. The picophytoplankton fraction was little representative, contributing to 3.3 ± 0.9 % (mean $\pm$ SE) of the total autotrophic biomass. Larger phytoplankton (diatoms and dinoflagellates) dominated at the most productive stations at the Weddell Sea (40.6 mg C mg^{-3} h^{-1} maximum total primary production) while the contribution to total phytoplankton biomass of the small autotrophic flagellates (*Cryptomonas* sp.) increased at the others stations. Both heterotrophic and autotrophic picoplankton communities presented similar thermal preferences for warmer temperature, as indicated by increasing survival success of the populations with increasing water temperature. The increasing contribution of Cryptophytes to total phytoplankton biomass was significantly related to the increasing water temperature, identifying the changes in water temperature as a strong factor in structuring the natural phytoplankton population of the AP. Cryptophytes and larger phytoplankton presented distinct ecological preferences inferred from the examination of the proportion of dead cells (%DC) of both Cryptophytes and large phytoplankton (diatoms + dinoflagellates) population. The mixing regime encountered at the Bransfield strait tended to exclude large phytoplankton, as indicated by the highest diatoms %DC (up to 66%DC) and favoured their relative contribution to and Cryptophytes that here presented highest survival. Phytoplankton mortality (particularly Cryptophytes') was identified as a major process explaining the release dissolved organic carbon by phytoplankton (P_{DOC}). The increasing Cryptophytes dominance along with the decline of the larger primary producers (e.g diatoms) attributed to the warming of the Antarctic waters provides indications of major changes in phytoplankton community structure and in carbon flow with the already evidenced warming in the Southern Ocean.

Introduction

The Polar regions are experiencing rapid climatic changes (IPCC 2007; Steig et al., 2009) and the Antarctic Peninsula (AP) has been identified as the area experiencing the higher warming in Antarctica (Steig et al., 2009). Marine ecosystem changes associated to warming effects have been evidenced (e.g. Smetacek and Nicol, 2005; Agustí et al., 2010) in polar regions. For the Antarctic Peninsula, there are reports of consistent changes in the planktonic ecosystem, with a decrease in the occurrence of large phytoplankton blooms (Montes-Hugo et al., 2009) and shifts in the planktonic community structure (Moline et al., 2001) from diatoms to small phytoplankton cells (in this case often Cryptophytes) (Moline and Prezelin, 1996; Moline et al., 2004; Montes-Hugo et al., 2008) and consequently from krill to salps (Atkinson et al., 2004; Smetacek and Nicol, 2005).

Warming in the Antarctic region triggered environmental changes such as rising water temperature (Burrows et al., 2011), decreases in ice extent and duration (Chen et al., 2009), modifying the ice melting extension and affecting the water masses circulation and mixing regime (Vaughan et al., 2003). The depth of the upper-mixed layer (UML) has a large influence on the photosynthetic capacity in Antarctic waters (Helbing et al., 1994) and has been identified as key factor for phytoplankton blooms in the Antarctic Peninsula (Boyd 2002; Vernet et al., 2008; Montes-Hugo et al., 2009) with high phytoplankton biomass and production associated to adequate light availability and shallow UML (Mitchell and Holm-Hansen, 1991; Mura et al., 1995; Vernet et al., 2008). Light irradiance, has been reported to strongly control phytoplankton biomass with induction of large phytoplankton blooms up to 35 µg Chl a L^{-1} under improved light in-situ mesocosms in the Antarctic Peninsula (Agustí and Duarte, 2000).

Warming is also expected to have important direct impacts on picoplankton communities as a significant increase in the contribution of picoplankton to autotrophic biomass and production has been evidenced under higher thermal conditions (Agawin et al., 2000), as well as signs of bacterial processes intensification (growth rates and production) have been described associated to the regional warming at the Antarctic Peninsula (Kirchman et al., 2009; Ducklow et al., 2010). Beside the response at community levels, supplemental studies addressing the straight effect of temperature on plankton population and species focused on allometric relationships, as reduction of population size species has been regarded as one of the main ecological response to global warming in aquatic ecosystem (Daufresne et al., 2009) and shift in community towards small-size species has been foreseen in a warmer ocean (Atkinson et al., 2003; Morán et al., 2010).

Changes in the phytoplanktonic community are difficult to investigate since they could whether be caused by the environmental modifications associated to warming or be attributed to direct metabolic response of single-cell to temperature (Finkel et al., 2010) modifying the physiological status of individual cells. The evaluation of the cell health status in plankton communities has been used recently to examine the status of the natural populations under in situ environmental conditions (Agustí 2004; Gasol et al., 2008). The quantification of the living and dead cells of both heterotrophs and autotrophs population constitute a relevant approach to identify the stressors drifting population dynamics in different oceanic areas (e.g. Grégori et al., 2001; Agustí and Sanchez, 2002) allowing to identify the tolerance and ecological niche of populations in response to changes in nutrients availability or temperature (Alonso-Laita and Agustí, 2006; Lasternas et al., 2010; Lasternas and Agustí, 2010).

In this study, besides the description of the plankton communities' and production distribution, we examined the survival success of the natural planktonic components, mediating the quantification of the percentage of living and dead phytoplankton and heterotrophic bacteria cells under the changes in hydrological properties associated to increasing temperature. We aimed so to evaluate the survival success and ecological preferences of the natural populations in response to the changing environmental conditions at the warming Antarctic Peninsula.

Moreover, phytoplankton mortality is perceived to be one of the important carbon loss processes (Proctor and Fuhrman, 1991; Nagata 2000) affecting carbon fluxes since phytoplankton cells death result in the extracellular release of the dissolved organic carbon (P_{DOC}) recently assimilated during the photosynthesis (Myklestad 1977; Sharp 1977). Therefore, we quantified the extracellular production of dissolved organic carbon (P_{DOC}) by phytoplankton and explore the relationship between phytoplankton cell mortality with (P_{DOC}) in order to address the modification of the carbon fluxes in response to recent changes in water masses and temperature at the Antarctic Peninsula.

Material and Methods

Water sampling and planktonic communities

The study was carried out during the ATOS-Antarctica oceanographic cruise from 26th January to 28th February 2009, aboard the Spanish Oceanography Research vessel *Hespérides.* 28 stations were sampled across three oceanographic regions around the Antarctic Peninsula, the Bellingshausen Sea, the Bransfield Strait and the Weddell Sea (Fig. 1). At each station, vertical profiles of temperature, salinity, and fluorescence were performed using a Seabird 911 CTD. Water samples were collected using 12 L Niskin bottles attached to a Rosette-CTD system, at 5 to 6 depths, from subsurface waters (5m) to mid-depth waters, generally 70 to 100 m, below the deep chlorophyll maximum (DCM) located between 12 and 50 m (28.2 ± 3.2 m, mean ± SE). Additional samples from 1 m depth were taken using 30 L Niskin bottles. Profiles of CTD data were used to calculate the thickness of the upper mixed layer (UML), an index of the stability of surface water column, as the shallowest depth at which σ_t (water density) differs from surface values by more than 0.05 kg m^{-3} (Mitchell and Holm-Hansen, 1991).
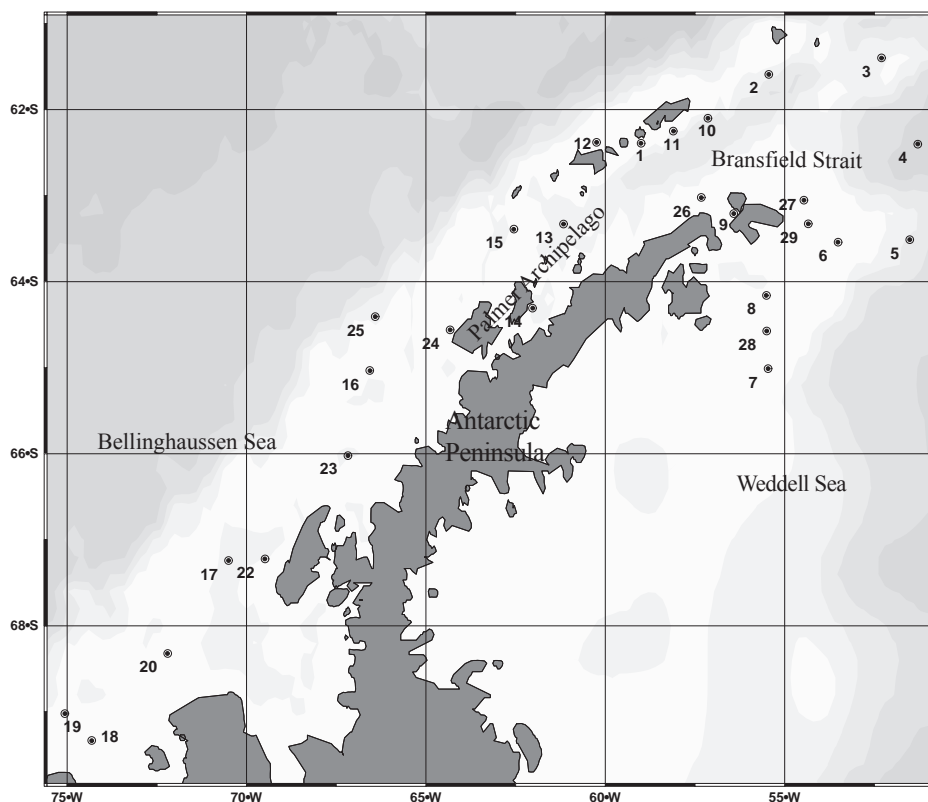

Fig.1. Areas of study and sampling stations occupied during the ATOS 2 cruise in the Antarctic Peninsula sector. Numbers represent the different sampling stations

Underwater light (photosynthetically active radiation, PAR) was measured at each station from the surface to 200 m depth using a PUV-2500 profiling radiometer (Biospherical Instruments) fitted with a PAR (400–700 nm) sensor. The percentage of light received at the different sampling depths was calculated from linear regressions of the natural logarithmic downwelling irradiance against depth, relative to the light received at 1 m depth.

Chlorophyll *a* concentration was determined fluorometrically by filtering 200 ml of water through Whatman GF/F filters, and extracted in 90% acetone for 24h before spectrofluorometric determination using a Shimadzu RF-5301PC spectrofluorometer following Parsons et al. (1984).

Primary production measurements were assessed by the ^{14}C technique (Steeman-Nielsen 1952). Seawater initially sampled at 5 depths including the surface (5 m), two intermediate depths, the DCM and an ultimate depth below the DCM, was delivered into transparent (light) and black masking tape-covered polycarbonate bottles (150 ml), and inoculated with 80 µCi activity of a $NaH^{14}CO_3$ working solution. Inoculated bottles were set up at respective depths along a mooring buoy and thereby incubated in situ for 4 hours. For each sample, two aliquots of 5 ml (replicates) were introduced in scintillation vials (20 ml) for the determination of total labelled organic carbon production (TPP); the sum of ^{14}C incorporated into POC (particulate organic carbon) and released as DOC (dissolved organic carbon). The remaining volume was filtered through 0.22 µm mesh membrane filters (cellulose membrane filters) of 25 mm diameter to determine particulate primary production (PPP > 0.22 µm). To remove inorganic ^{14}C, the liquid samples were acidified with 100 µl of 10% HCl and shacked for 12h, while the filters were fumed with concentrated HCl (37%) for 12h. Then, 10 ml and 5 ml of scintillation cocktail (Packard Ultima Gold XR) were respectively added to TPP and PPP vials and the disintegrations per minute were counted after 24 h with a scintillation counter (EG&G/Wallac).

The dissolved organic carbon production by phytoplankton (P_{DOC}) was calculated as the difference between total and particulate primary production. The percentage of the extracellular production released by phytoplankton (PER) was calculated as the ratio between (P_{DOC} / TPP) x 100.

The quantification and determination of the phytoplankton communities in our samples were performed using two procedures. For quantification of the larger phytoplankton cells (>2µm in diameter) abundance, samples were collected at the surface (1 m) and the deep chlorophyll maximum (DCM). Water samples of 2-3 litres from 1 m and at the depth of DCM were concentrated to 50-70 ml samples by using a Millipore concentrator stirred cell (Agustí and Sánchez, 2002). The time for the concentration procedure was short, less than 30 minutes, and a gentle pressure was applied over the sample to avoid any damage of the cells as recommended in previous studies (Agustí and Sánchez, 2002; Lasternas and Agustí, 2010) with accurate results for phytoplankton, with no effect on the viability or other cell properties (e.g. movement for flagellated cells, integrity of frustules). Ten ml aliquots (duplicates) of the concentrated sample were filtered onto 2 µm pore-size black polycarbonate filters, fixed with gluteraldehyde (1 % final concentration) and stored frozen at – 80°C until counting.

Phytoplankton cells (>2µm) were counted using an epifluorescence microscope (Zeiss© Axioplan Imaging) and classified into 3 majors groups, nanoflagellates, dinoflagellates and diatoms that belong over two phytoplankton size-classes (2-20µm / >20µm). Diatoms species were further grouped into pennate and centric forms while dinoflagellates were separated into armoured and naked forms. The average cell volume for each phytoplankton group identified during the study was computed, using the geometrical approximation of their forms (Sun and Liu, 2003) so biovolume ($\mu m^3 \, l^{-1}$) of the different phytoplankton groups in each sample was calculated as the product of the cell density (cell l^{-1}) by average cell volume ($\mu m^3 \, cell^{-1}$). The abundance and cell-size calculation of smaller phytoplankton cells were assessed using flow cytometry. Size calibration was established with two fluorescent Plain Microspheres (Polysciences, Inc.) of 1.0 and 2.0 µm in diameter and with two marine isolates, *Micromonas* sp. and *Chlorella* sp. (1.4 and 3.2 µm, respective diameter). Diameter was a logarithmical function of forward scatter intensity (FSCH) following the equation: Diameter = – 4.2 (± 0.2) + 3.2 (± 0.1) x Log (FSCH) (R^2 = 0.99, P = 0.001).

Biovolume of the smaller cells (<2µm) were calculated as the product of cell density (cell l^{-1}) by average spherical equivalent cell volume (µm^3 cell l^{-1}). At each station, duplicated 2 ml fresh samples from 6 depths were counted onboard (duplicated counts) using a FACSCalibur flow cytometer (Beckton Dickinson). An aliquot of a calibrated solution of 1 µm diameter fluorescent beads (Polysciences) was added to the samples as an internal standard for the quantification of cell concentration. Red (FL3, bandpass filter>670 nm), green (FL1, bandpass filter 530 nm) and orange (FL2, bandpass filter 585 nm) fluorescence as well as the forward and side scattering signals of the cells and beads were used to detect phytoplanktonic populations (Marie et al., 2005).

Population survival success

The quantification of the proportion of dead cells in the phytoplankton communities was conducted on board in fresh samples using a cell digestion assay (CDA), which tests cell membrane permeability (Agustí and Sánchez, 2002). The CDA requires that the phytoplankton communities are subjected to an enzymatic cocktail (DNAse and Trypsin) that enters the cytoplasm and digests cells with compromised membranes, i.e. removing dead cells from the sample. Cells remaining in the samples after the CDA are the living cells, which can then be quantified using epifluorescence microscopy or cytometry. The CDA was applied to the prepared concentrates of nano- and microphytoplankton cells (2 to > 20µm) to quantify total cell abundance. Specifically, the CDA was applied to duplicated 10 ml aliquots of cell concentrate by adding 2 ml of DNAse I solution (400 µg ml^{-1} in Hanks' Balanced Salt Solution [HBSS]), followed by 15 min incubation at 25°C in a Digital Dry Bath (Labnet©). After this time, 2 ml of Trypsin solution (1% in HBSS) were added, followed by 30 min incubation at 25°C (Llabrés and Agustí, 2008). 25 ºC was recommended by Llabrés and Agustí (2008) as the optimal temperature to run the assay with polar species, as this temperature alleviated the problems derived by the thermal difference with in-situ temperature, and assured the complete digestion of dead cells. At the end of the second incubation, samples were then placed in ice to stop the enzymatic cell digestion process and then were filtered onto polycarbonate 2 µm pore diameter black filters, washed several times with filtered seawater, fixed with glutaraldehyde (1% final concentration) and stored frozen at – 80°C until counting by epifluorescence microscopy.

Fresh samples to quantify the proportion of dead cells for the smallest phytoplankton were sampled from the 6 same depths selected to estimate total abundance at each station. Duplicated 1 ml samples were run with the CDA, by first adding 200 µl of DNAse I solution and then, after 15 min incubation at 25°C, 200 µl of Trypsin solution. Treated samples were incubated for 30 min at 25°C and were finally placed in ice to cease enzymatic activity. Samples were then counted by flow cytometry as described before. The percentage of dead cells (%DC) was calculated as the ratio between the concentrations of dead cells, to the total phytoplankton population observed in the blanks (live and dead cells).

At each station, the abundance and the proportion of living heterotrophic bacteria were quantified from seawater sampled at 6 depths. To do so, we used the Nucleic Acid Double Staining (NADS, Gregori et al., 2001) flow cytometric protocol. This technique consists on the use of two nucleic acid fluorescent dyes, SYBR Green I (SG1; Molecular Probes) and Propidium Iodide (PI; Sigma Chemical Co.). Bacterial membranes are permeable to SG1, whatever the cell viability, resulting in green fluorescence when stained. However, living or viable cells, with intact plasmic membranes, are impermeable to PI. Thus only compromised or damaged cells are stained with PI (Barbesti et al., 2000), showing red fluorescence as described in Falcioni et al., (2008). Subsamples were analyzed immediately after collection. Samples (1ml) were stained with 10 µl of Propidium iodide (PI, 1 mg ml^{-1} stock solution), reaching a final concentration of 10 µg ml^{-1} and incubated for 30 minutes in dark and room temperature. Then, 10 µl of SYBR Green I (10-fold dilution of 10000×commercial solution in dimethyl sulfoxide) were added to subsample and incubated for 10 more minutes. SG1 and PI fluorescence were detected using a FACSCalibur Flow Cytometer (Beckton Dickinson©) in the green (FL1) and the red (FL3) cytometric channels, respectively. Bivariate plots of green versus red fluorescence (FL1 vs. FL3) allowed discriminating live (green fluorescent, impermeable to PI) from dead cells (red fluorescent membrane-compromised cells, stained by PI and SG1). Bacteria concentration was calculated using a 1-µm diameter fluorescent bead (Polysciences Inc.) solution as an internal standard. Total heterotrophic bacterial abundance, in cells ml^{-1}, was calculated as the sum of red and green fluorescent cell abundance, while dead bacterial cell abundance was determined from the red fluorescent cell counts (PI stained).

The percentage of dead heterotrophic bacteria was calculated as the ratio between the concentration of damaged-membrane bacteria stained with PI and total bacterioplankton abundance.

Statistics

The different parameters were averaged by 3 oceanographic regions (Bellingshausen Sea, Bransfield Strait and the Weddell Seas), and the statistical significance of the differences between average values were tested using Student's t-test, with a critical p-value of <0.05. Data on temperature, production, PER, population biovolume and viability were grouped by 0.5ºC temperature bins and by 10% PER bins to search for patterns. The analysis of correlation between variables that departed from normality was performed using the Spearman's rank coefficients (r_s) (Siegel and Castellan 1988). Linear regression analyses were applied to raw data and binned data. Means are presented ± SE throughout.

Results

Waters features

During the study, stations were sampled across the Bellingshausen Sea, the Bransfield Strait and the Weddell Sea (Fig. 1) and encompassed the water masses usually found at the vicinity of the Antarctic Peninsula (Capella et al., 1992; Mura et al., 1995). Cold and saline waters, originated in the Weddell Sea (-0.75ºC/34.5 PSU), converged with less salty waters, from the Bellingshausen Sea (2.75ºC/33 PSU) at the Bransfield strait that corresponds to a transient system ranging values from both seas (Capella et al., 1992). The upper mixed layer (UML) ranged from 5.9 m up to more than 200 m. Mixed water conditions were observed at the Bransfield area while the stratification was stronger established at stations of the Weddell Sea (Stns. #5-6-7-8), in the vicinity of the Palmer Archipelago (Stn. #24) and at southern stations of the west peninsula (Stns. #18-19).
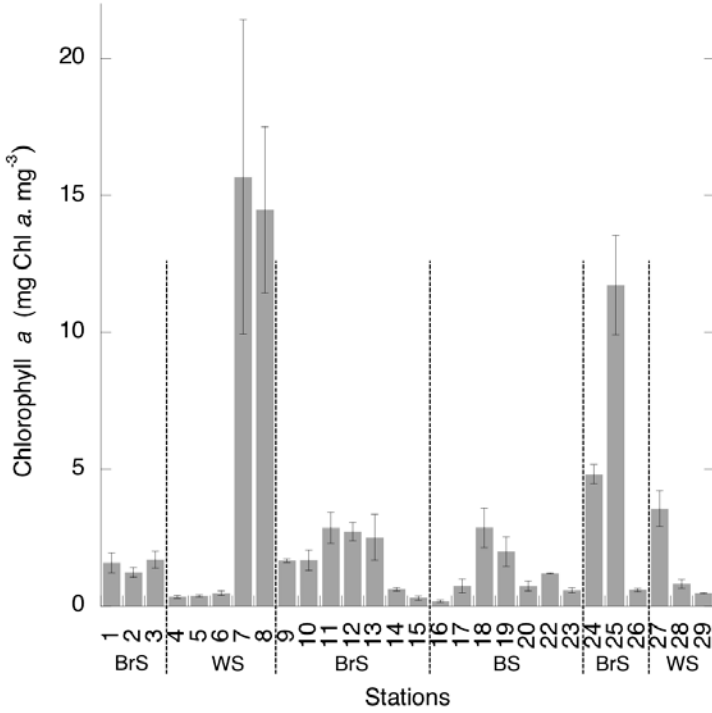
Fig.2. Distribution of the chlorophyll *a* concentration by stations across the study (BrS: Bransfield Strait- WS: Weddell Sea- BS: Bellingshausen Sea)

The most productive waters were found at the Weddell Sea (Stns. #7-8) and at the vicinity of the Palmer Archipelago (stns. #24-25) contrasting to less productive waters from the Bellingshausen region (Fig. 2). Higher chlorophyll *a* concentrations were associated to stratified waters as indicated by the exponential distribution of chlorophyll *a* by UML (Fig. 3).
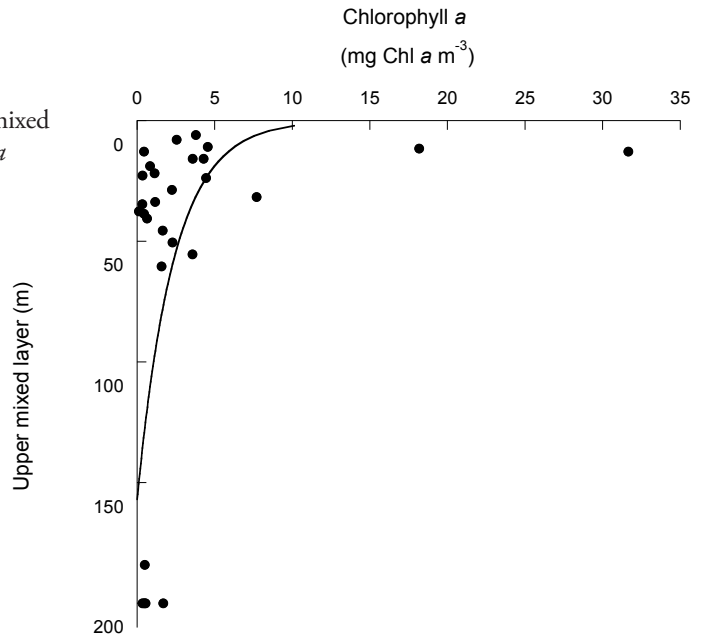
Fig.3. The relationship between the upper mixed layer depth and the maximum chlorophyll *a* concentration at each station

The chlorophyll *a* concentration (Chl *a*) and total primary production (TPP) rates showed a great variation along the study ranging over 600-fold among stations (respectively ranging from 0.05 to 31.66 mg Chl *a* mg^{-3} and from 0.04 to 40.66 mg C mg^{-3} h^{-1}). Chl *a* and TPP were significantly and positively related (Fig 4), displaying highest values at stations located in the Weddell Sea area (Stn#7) and both were positively related to water temperatures (r_s= 0.25, p< 0.005 and r_s= 0.19, p< 0.005 respectively).

The primary production rates were dependent on the available light, being positively related to the percentage of PAR received at the depth of sampling (r_s= 0.36, p < 0.0001) and negatively to depth (r_s= -0.48, p < 0.0001). The percentage of the extracellular carbon released by phytoplankton (PER), averaging 55.4 ± 1.8 % along the cruise, also ranged widely (from 10.3 to 98.9 %) and presented variations independent of the area of sampling (Student's t-test, p < 0.05) but increasing with depth (r_s= 0.49, p < 0.0001), indicated higher fraction of primary production released as dissolved organic carbon at deeper layers.
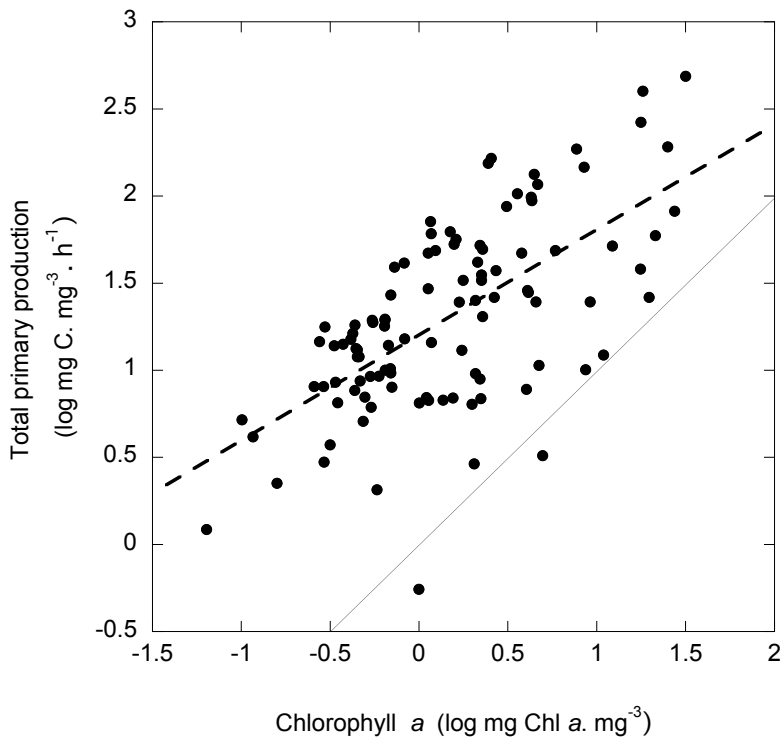

Fig. 4. The relationship between the chlorophyll *a* concentration and the total primary production. The solid line shows the 1:1 relationship and the dotted line shows the fitted regression equation:
Log TPP = 1.2 + 0.61 (± 0.07) log Chl a (R^2 = 0.41, P < 0.0001, N= 105)

Planktonic biomass and communities distribution

Heterotrophic bacteria showed lower biovolume at the Weddell Sea than at the Bellingshausen Sea and Bransfield Strait, which presented similar values (Table 1). Bacterial biovolume was negatively related to depth (Table 2) and higher biovolumes were found associated to warmer waters (Table 2).

		$\mu m^3 \, l^{-1}$	Bellingshausen zone	Brandsfield area	Weddell Sea area
BACTERIOPLANKTON		10^7	2.73 ± 0.24 [A]	2.43 ± 0.12 [A]	1.58 ± 0.08 [B]
	Total	10^8	0.97 ± 0.11 [AB]	0.49 ± 0.14 [B]	2.38 ± 0.79 [A]
	Picophytoplankton	10^6	1.31 ± 0.14 [B]	2.23 ± 0.41 [A]	1.56 ± 0.36 [B]
PHYTOPLANKTON	Nanophytoplankton	10^8	0.57 ± 0.01 [B]	0.31 ± 0.06 [B]	1.16 ± 0.48 [A]
	Microphytoplankton Total	10^8	0.38 ± 0.04 [B]	0.16 ± 0.01 [B]	1.20 ± 0.46 [A]
	Diatoms	10^7	1.92 ± 0.62 [AB]	0.58 ± 0.21 [B]	7.75 ± 0.45 [A]
	Dinoflagellates	10^7	1.91 ± 0.37 [A]	0.96 ± 0.18 [A]	4.27 ± 1.66 [A]

Table 1: Averaged (± SE) biovolume of the planktonic populations among the three oceanographic regions sampled during ATOS-2. Average values for regions connected by different letters are significantly different (p < 0.05)

The phytoplankton cells identified in our samples included the three size-classes of pico-, nano- and microphytoplankton. The total biovolume was significantly related to chlorophyll a distribution along the study (Fig. 5), but the distinct phytoplankton size-classes showed contrasting relationships with Chl *a*. The smallest size-class, represented by a solely picoeukaryotic population (1.3 to 2.1 µm equivalent spherical diameter) was not related to chlorophyll *a* and contribute only to 3.3 (± 0.9) % of the total autotrophic biovolume. Along the study, the biovolume of the picophytoplankton fraction was positively related to water temperature (Table 2) and negatively to UML (Table 2), indicating the association of picophytoplankton biovolume with warmer and mixed waters, consistent with the maximum biovolume observed at waters sampled at the Bransfield strait (Table 1).

Contrarily to picophytoplankton, nano-microphytoplankton groups were strongly related to chlorophyll *a* (Fig. 5) and presented maximum biovolumes at stations sampled at the Weddell Sea, matching with chlorophyll *a* and primary production maxima (Fig. 2 and Table 1). The nanophytoplankton group, mostly represented by the nanoflagellate species *Cryptomonas* sp (11 µm average cell diameter) and the microphytoplankton presented similar dynamics with higher biovolume at stations of the Weddell Sea (Table 1). Diatoms and Dinoflagellates positively related to chlorophyll *a* (R^2 = 0.39, P = 0.002, N= 20) presented an increase in biovolume at stations located at the Weddell Sea area, where diatoms dominated (Table 1).

Variables	rs	p
HB - DEPTH	-0.23	< 0.0005
HB - TEMP	0.62	< 0.0005
PICO - TEMP	0.36	< 0.0001
PICO - UML	-0.39	< 0.0001
DIAT - TEMP	-0.48	< 0.005
HLB - TEMP	0.55	< 0.0001
HLB % - SAL	-0.22	< 0.005
HLB % - DENS	-0.21	< 0.005
PICO %DC - DENS	0.26	< 0.005
PICO %DC - SAL	0.27	< 0.005
NANOMICRO %DC - DEPTH	0.53	< 0.005
CRYPT %DC - PAR	-0.49	< 0.01
PER - NANOMICRO %DC	0.72	< 0.005
PER - DEPTH	0.50	< 0.005

Table 2: Significant correlation coefficients among variables. Temperature (TEMP); Salinity (SAL); Density (DENS); Upper Mixed Layer depth (UML); Photosynthetic active radiation (PAR); Heterotrophic bacteria biovolume (HB); Heterotrophic Living Bacteria biovolume (HLB); Percentage Heterotrophic Living Bacteria (HLB %); Picophytoplankton biovolume (PICO); Percentage of Picophytoplankton dead cells (PICO %DC); Nano-microphytoplankton biovolume (NANOMICRO); Diatoms biovolume (DIAT); Percentage of *Cyptomonas* sp. dead cells (CRYPT %DC) and Percentage of extracellular release (PER)
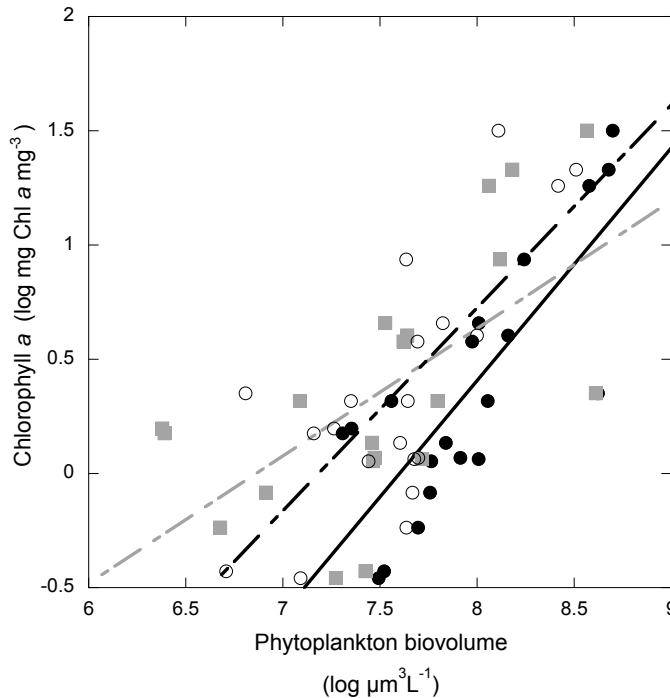

Fig.5. The relationships between Total phytoplankton biovolume (full black dots), biovolume of *Cryptomonas* sp. (empty circles) and the Chl *a* concentration. Full black line shows the fitted regression equation Log Chl *a* = -7.75 + 1.02 (± 0.18) log total biovolume (R^2 = 0.63, P < 0.0001, N= 20), dotted black line shows the fitted regression equation Log Chl *a* = -6.38 + 0.88 (± 0.19) log *Cryptomonas* sp. (R^2 = 0.55, P = 0.0002, N= 20)

Diatoms showed distinct order (centric vs pennate) distribution across the areas of studies. Centric groups prevailed along the cruise and while pennate forms, mainly represented by *Pseudo-Nitzschia Seriata* and *Delicatissima* and *Navicula* sp., presented similar biovolumes at the different areas, we observed a significant increase in the biovolume of centric diatoms at the Weddell stations (Fig. 6a). Centric diatoms presented moreover, a contrasting assemblage along the study, with *Corethron* sp. and *Coscinodiscus* sp. the predominant forms at Bransfield and Bellingshausen zones, while at the Weddell Sea stations, we observed the population being mostly represented by *Thalassiosira* sp.
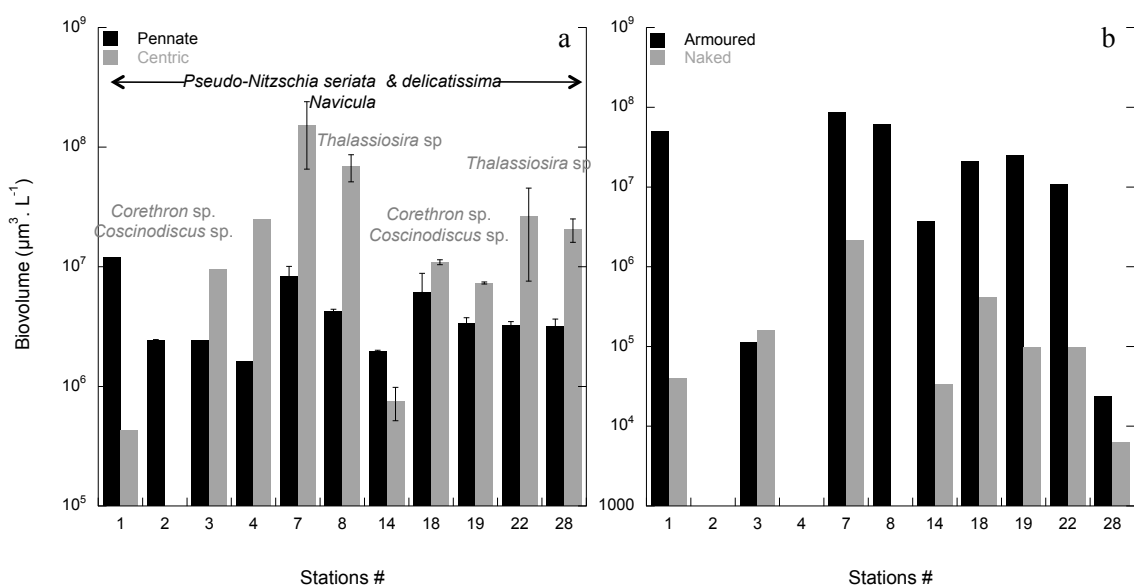

Fig.6. Biovolume distribution of (a) centrics and pennates diatoms groups and (b) naked and armoured dinoflagellates groups by stations across the study

Regarding to dinoflagellates assemblage, the armoured forms, principally *Dinophysis* sp. (16 – 25 μm) and *Prorocentrum* sp (6 - 24 μm), dominated along the study and reached maximum averaged abundance at the Weddell stations (Fig. 6b). The naked forms, belonging as a majority to the order of the Gymnodiniales, showed also a significant increase at Weddell stations.

Microphytoplankton dominated the biomass at the Weddell Sea (66% of total biovolume) although nanoflagelates dominated at the other two zones (56 and 66% of total biovolume at the Bellingshausen and Bransfield area, respectively). We also observed that the percentage of biomass dominated by nanoflagelates (i.e. *Cryptomonas* sp.) was positively related to increased water temperature (Fig 7; R^2 = 0.55, n= 19, p < 0.001) contrasting with the negative relationship between the biovolume of diatoms and the water temperature (Table 2), indicating higher diatoms biovolume associated with colder waters.
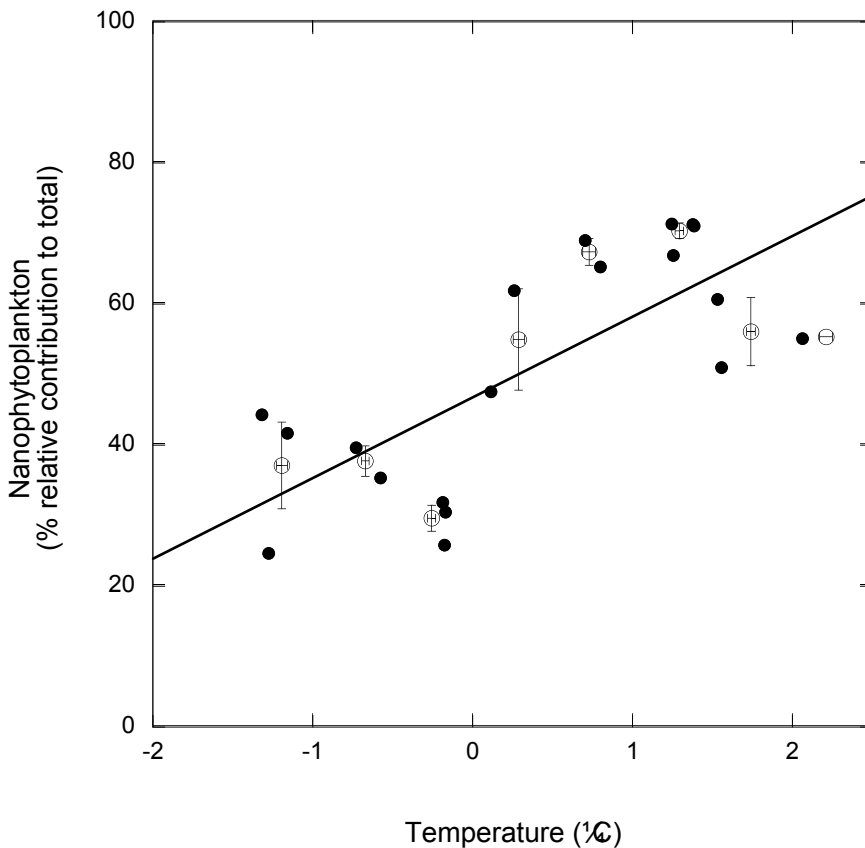

Fig.7. The relationship between temperature and relative *Cryptomonas* cells biovolume contribution to total phytoplankton biovolume. Raw percentage data are represented by empty dots and percentage binned by 0.5°C temperature unit intervals are in full dots. The full line shows the fitted regression equation % *Cryptomonas* biovolume = 46.8 + 11.4 (± 2.5) temperature (R^2 = 0.55, P < 0.001, N= 19)

Plankton cell health and phytoplankton carbon release

We observed changes in the cell status of the planktonic populations across the study. The percentages of heterotrophic living (%HLB) averaged 70.7 ± 1.2 %HLB, ranged from 35 to 95 %HLB, with higher %HLB found associated to less saline and dense waters (Table 2), illustrating higher survival at stations belonging to the warmer Bellingshausen Sea (75.6 ± 2.5 %HLB) than at other regions (Student's t-test, $p < 0.05$). Indeed, we observed a significant relationship between the biovolume of living heterotrophic bacteria and water temperature ($R^2 = 0.69$, $p < 0.005$, n= 10), indicating living bacterial to increase as water temperature increased (Fig. 8).
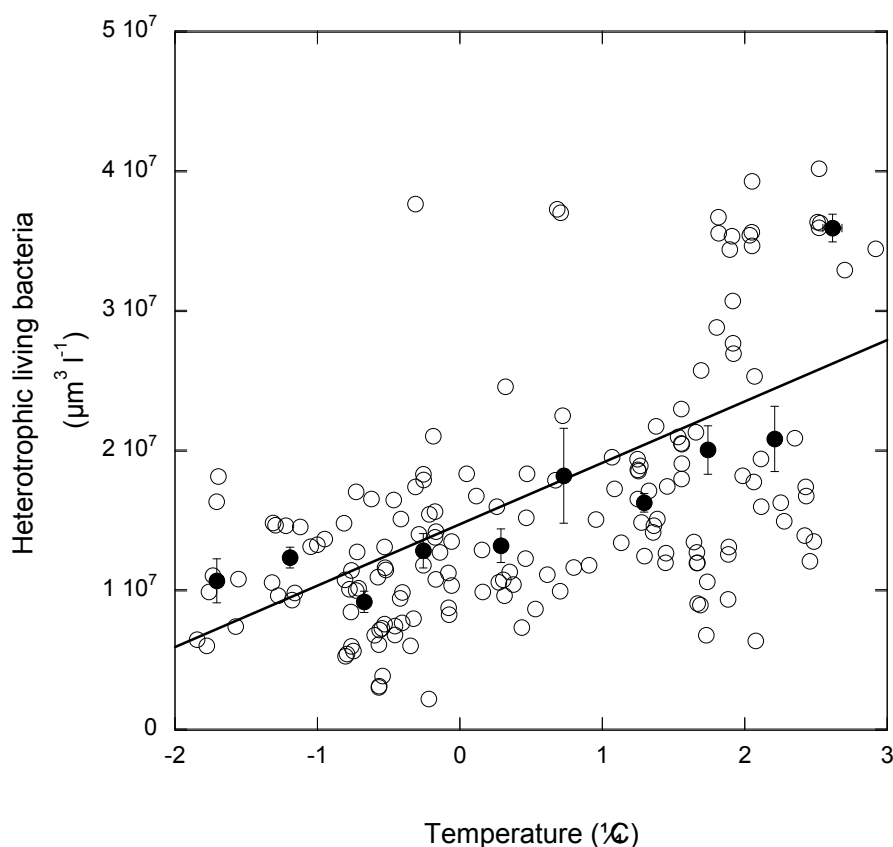

Fig.8. The relationship between the biovolume of living heterotrophic bacteria (HLB) and the temperature. Raw HLB biovolume data are represented by empty dots and HLB biovolume binned by 0.5°C temperature unit intervals are in full dots. The full line shows the fitted regression equation HLB = $4.4\ 10^5$ + 1.3 (± 0.13) 10^5 Temperature ($R^2 = 0.69$, $P < 0.001$, N= 10)

When averaging all the values of cell mortality from the different populations within the communities, total phytoplankton mortality (50.1 ± 4.4 % dead cells, DC) presented similar values across the oceanographic regions (Student's t-test, p < 0.05) but we observed distinct variations among autotrophic size-class groups. Picoautotrophs mortality (from 8.9 to 92.3 %DC) presented higher percentage of dead cells at the Weddell stations (54.9 ± 3.4 % DC) than at the Bransfield Strait (49.7 ± 2.9 % DC) and the lower mortality values were found at the warmer waters of the Bellingshausen Sea (38.2 ± 4.1 % dead cells), indicating the increasing of the picophytoplankton %DC with the increasing in waters salinity and density (Table 2).

The percentage of dead nano-microphytoplankton was positively related with depth and negatively with the percentage of photosynthetically active radiation at the depth of sampling (PAR) especially *Cryptomonas* sp. (Table 2) presenting thus higher mortality of at the lower PAR levels found at deeper layers.

Cryptomonas sp. presented lowest %DC at the Bransfield area, increasing at the Weddell and Bellingshausen stations (Fig. 9a), indicating higher survival at the Bransfield Strait. Conversely, larger cells (diatoms plus dinoflagellates) reached maximum mortality at the Bransfield strait (Fig. 9b), particularly illustrated by diatoms that reached about 70 % of dead cells at this zone while dinoflagellates presented similar mortality between areas.
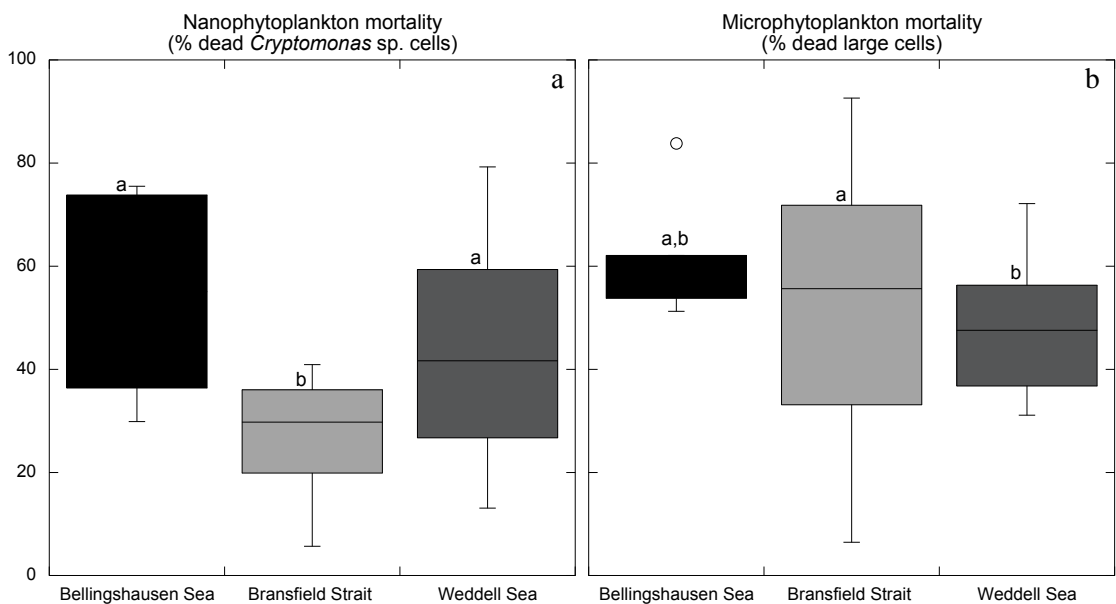

Fig.9. Distribution of the percentage of (a) dead *Cryptomonas* sp. cells and (b) dead large phytoplankton (diatoms and dinoflagellates) cells within regions. Boxes show the lower and upper quartiles, median, minimum and maximum values, and outliers (open circles). Boxes connected by same letter are not significantly different (p < 0.05)

The percentages of dead nano-microphytoplankton cells were significantly and positively related to the percentage of dissolved organic carbon released by phytoplankton (PER, Table 2). Indeed, the percentage of *Cryptomonas* sp. dead cells explained about the 54 % of the production of dissolved organic carbon (P_{DOC}) (Fig. 10), as PER was significantly related to *Cryptomonas* sp. %DC (R^2 = 0.54, p < 0.0005, n= 19), indicating changes in variations the fraction of primary production released as dissolved organic carbon (P_{DOC}) were mainly supported by *Cryptomonas* sp. cell mortality.
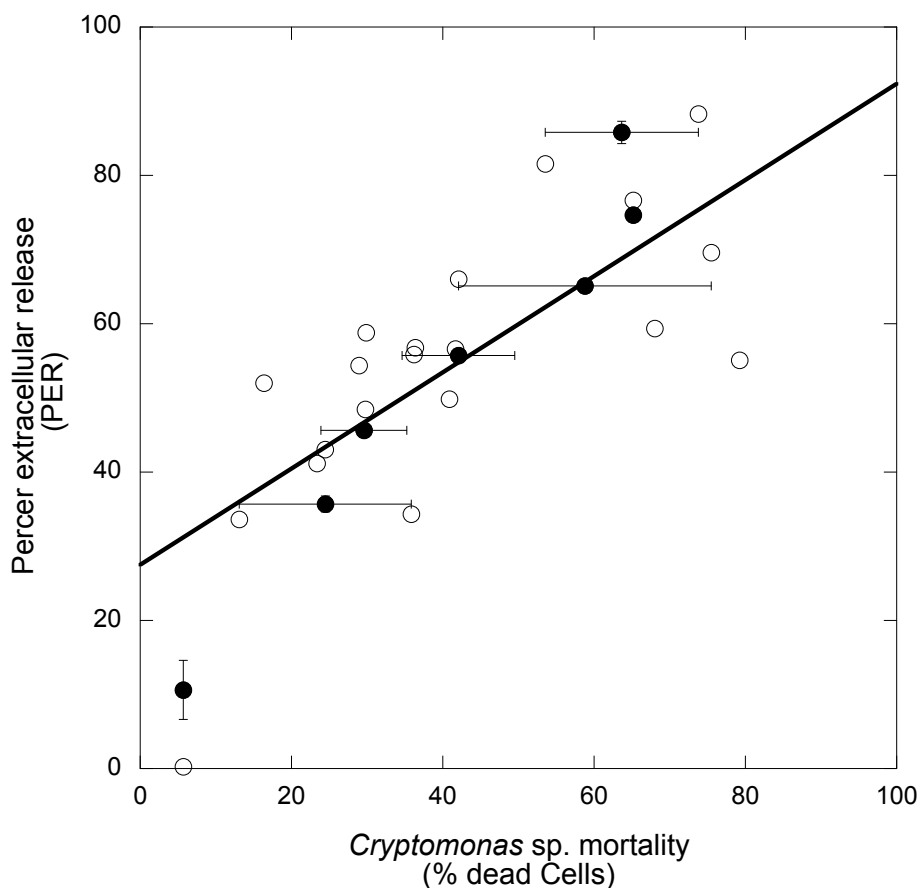

Fig.10. The relationship between the *Cryptomonas* sp. mortality (percentage of dead cells) and the percer extracellular release (PER). Raw percentage data are presented in empty circles and percentages binned by 10% PER unit intervals are in full dots. The full line shows the fitted regression equation PER = 27.48 + 0.64 (± 0.14) *Cryptomonas* sp. %DC (R^2 = 0.54, P < 0.001, N= 20)

Discussion

The results presented here showed contrasting planktonic community distribution and population survival success across the waters around the Antarctic Peninsula (AP) that were largely explained by differences in water temperature associated to the different water masses. The results identified water temperature as an important driver, with the contribution of nanophytoplankton to total phytoplankton biomass increasing with increasing water temperature while larger phytoplankton cells (mostly diatoms) were dominating in cold waters. Moreover, increased water temperature supported higher picoplankton (both autotrophic and heterotrophic) standing stocks, although the contribution of picophytoplankton to total biomass was always low. The evaluation of the auto and heterotrophic picoplankton health status throughout the study revealed direct relationships of the picophytoplankton population survival success with water temperature, salinity and density. Similarly, we also yielded supplemental evidences of the positive effect of the increasing temperature on bacterial survival, as indicated by the higher concentration of heterotrophic living bacteria in warmer waters.

Few studies were performed yet at the Southern Ocean analysing the living heterotrophic bacteria (HB) in natural communities. A previous study by Ortega et al. (2008) presented percentages of living HB consistent with our data in the same area, showing a positive relationship between living HB and Chl *a* concentration. In our study, heterotrophic bacterial abundance was positively related to Chl *a* as expected from the literature (e.g. Bird and Kalff, 1984) but there were no consistent relationships with the abundance of living heterotrophic bacteria. Here, the wider range of Chl *a* concentration compared to Ortega et al. (2008) may explain the different result. In polar waters, it is expected that bacteria growth will be dependent by both DOC availability and temperature (Pomeroy and Wiebe, 2001). HB viability and DOC production by phytoplankton were not correlated, contrary to the positive relationship between P_{DOC} and heterotrophic bacterial survival recently described for the NE Atlantic Ocean (Lasternas et al., 2012). However, we provide here consistent evidence of the bacterial viability control by water temperature, indicated by the significant relationship between the living heterotrophic bacteria and water temperature.

In their recent review, Kirchman et al. (2009) examined the responses of bacteria to temperature, showing a positive relationship between bacterial growth rate and water temperature, but the relationship was not linear, as growth rates increased ten fold more at polar temperatures (from –1.8 to approximately 4 °C), indicating that the activation of bacterial by temperature in polar waters was higher. The bacterial cell survival responded directly to water temperature in our study, strongly in agreement with the results of Kirchman et al (2009). The lack of direct relationship between living HB abundance and DOC produced by phytoplankton found in our results suggest a stronger dependence of the heterotrophic bacteria (HB) community for water temperature than for P_{DOC} availability at the waters sampled. Moreover, higher proportions of P_{DOC} (expressed as PER) were associated to warmer waters, indicating higher dissolved organic carbon released by phytoplankton available for HB at the warm areas. Therefore, despite the lack of a direct relationship between PER and bacterial survival, we could assume a co-effect of water temperature on bacterial community by influencing directly the bacterial cell survival and supporting the availability of substrate such as P_{DOC}. This may likely comfort the expected intensification of bacterial processes with the regional warming at the Antarctic Peninsula (e.g Kirchman et al., 2009; Ducklow et al., 2012).

A similar thermal response is evidenced for the Antarctic picophytoplanktonic cells as indicated by the decreased in cell mortality at warmer waters in our study (r_s = -0.18 n= 158, $P < 0.01$). The Antarctic picophytoplanktonic cells were able to respond fast to improvement of the environmental conditions, as observed by Agawin et al. (2002) that documented a high increase in picophytoplankton production when light availability was optimized experimentally and it is expected its rapid response to the increased temperature as predicted by the metabolic theory (Daufresne et al., 2009; Morán et al., 2010). The contribution of the picophytoplankton fraction to biomass was minor as previously reported for the AP waters (e.g. Garrison and Mathot, 1996; Wright et al., 2009), indicating that despite the positive effect of the increasing water temperature on picophytoplankton cell viability, it subsists factors that limit the picophytoplankton biomass at these polar waters.

Water temperature also affected the phytoplankton community, similarly composed as previous description in the area (Mura et al., 1995; Moline and Prezelin, 1996; Garibotti et al., 2003). The larger forms (diatoms and dinoflagellates) significantly dominated the biomass at the most productive Weddell waters that presented shallower UML.

Previous studies showed as observed here the positive relationship between the reduction of the upper mixed layer and increasing phytoplankton biomass (Mitchell and Holm-Hansen, 1991; Mura et al., 1995; Vernet et al., 2008), being also supported by in situ experiments in mesocosms (Agustí and Duarte, 2000; Agustí et al., 2009) in the Antarctic Peninsula. At the Weddell region where a recent sea-ice melting was observed, diatoms and dinoflagellates presented better survival success. The conditions associated to this marginal ice zone (MIZ) characterized by a recent stratification and receiving recent ice-melt waters from coastal glacier or ice shelves generated favourable stratified conditions promoting biological production and large phytoplankton biomass enhancement (Rivkin et al., 1991; Cook et al., 2005; Montes-Hugo et al., 2009). These conditions may have benefit the cell physiology of the microphytoplankton population as we observed the lowest microphytoplankton mortality at the Weddell waters.

In contrast, higher percentage of microphytoplankton dead cells were observed at the Bransfield mixed waters where the conditions would tended to exclude large forms, principally diatoms as indicated by high mortality rates (up to 66%DC). The weakening health status of large phytoplankton would have favoured the nanoflagellates population as we observed *Cryptomonas* sp. prevailing throughout the open waters of the Bellingshausen Sea and Bransfield straits. The densities observed were lower than ones typical bloom conditions previously observed in the area (Rodriguez et al., 2002; Garibotti et al., 2003) indicating that the environmental conditions encountered were not as optimum for blooming but highest Cryptophytes survival were found at these waters. We found that the light availability was limiting the nanophytoplankton survival during the study and the deepening of the summer mixed layer at the Bransfield strait would have relieve photosynthetically active radiation (PAR) limitation (Vernet et al., 2008) resulting in lower *Cryptomonas* sp. mortality decreasing with higher %PAR in the water column. The distinct survival success of both Cryptophytes and diatoms cells under mixing regimes helped in defining their contrasting ecological niches of both population but no sufficient to explain the increasing contribution of Cryptophytes in the Antarctic waters.

Water temperature constituted a strong factor in structuring the nano-microphytoplankton community as indicated by the significant increasing proportion of *Cryptomonas* sp. as water temperature increased, explaining the 55% of the *Cryptomonas* sp. contribution to phytoplankton biomass.

Shifts from large-dominated community to smaller-forms dominated by nanoflagellates have been documented during summer at the coastal region of the Antarctic Peninsula (Moline et al., 2001; Montes-Hugo et al., 2009) and attributed to climate warming occurring at the Southern Ocean. Along with a significant increase in air mean temperature over the last 50 years, subsequent decline in ice-coverage (Yuan et al., 2004), reduction in the marginal ice zone and early ice retreat occurring at the AP were identified to influence the increasing importance of Cryptophytes, the decreasing in bloom magnitude and the reduction of the large forms as diatoms with associated food web (Moline et al., 2004; Montes-Hugo et al., 2008; Montes-Hugo et al., 2009). In the study of Moline et al. (2004), authors indicated that the higher contribution of Cryptophytes to total phytoplankton biomass was associated to less saline waters and attributed the increasing of Cryptophytes to increasing air temperature. In our study, we present evidence of the direct effect of water temperature on the growing importance of Cryptophytes, identifying thus the water temperature as a strong environmental factor influencing the phytoplankton community structure that favour the "expansion" of small autotrophic flagellates compared to larger phytoplankton forms under warming conditions.

Water temperature also interceded in the production of dissolved organic carbon by phytoplankton (P_{DOC}). When omitting the very productive stations of Weddell Sea (St #7 and 8) we showed that higher percentages of extracellular release (fraction of P_{DOC} relative to total phytoplankton primary production PER) were associated to warmer temperature ($r_s = 0.25$, $p < 0.001$) in our study. This is in agreement with Morán et al. (2006) that experimentally induced an increase in PER of around 50%, when experimentally increased the water temperature of Antarctic plankton to 2ºC. This relationship likely indicates that phytoplankton would produce higher amount of P_{DOC} at warmer waters, where *Cryptomonas* dominated, with subsequent higher PER rates expected under warmer waters conditions. In addition, changes in cryptophytes' death rates significantly supported the observed variability of the fraction of primary production released as dissolved organic carbon (P_{DOC}) across the waters. The potentially large P_{DOC} discharge to the medium (C leakage) would significantly affect the carbon fate by sourcing the system in dissolved organic carbon instead sequestration.

As we identified the mortality of the *Cryptomonas* sp. cells to be the major process explaining the variation of percentage of extracellular release (PER; fraction of P_{DOC} relative to total phytoplankton primary production) at the Antarctic Peninsula waters, the expected predominance of small-forms (nano instead of micro) would thus accelerate carbon fluxes to the dissolved pool. Size-shift to small forms, with the decline of the larger primary producers (e.g diatoms), would alter both the magnitude of carbon fixed and exported into the deep sea favouring the microbial loop (Pomeroy 1974; Azam et al., 1983; Legendre and Le Fèvre 1995; Laws et al. 2000), allowing the foreshadowing of intensified leakage lightening the carbon pump.

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Chapter 4

Carbon fluxes forced by anticyclonic mesoscale eddies generated by islands at the subtropical NE Atlantic Ocean

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Chapter 5

Bacterial survival governed by the release of dissolved organic carbon from senescent oceanic phytoplankton

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Abstract

Bacteria recycle vast amounts of organic carbon, playing key biogeochemical and ecological roles in the ocean. Bacterioplankton dynamics are expected to be dependent on phytoplankton primary production, but the demonstration of a direct relationship between bacterial cell activity and primary production has remained elusive, possibly because of the diversity of processes (e.g. sloppy feeding, cell exudation, viral lysis) involved in the transference of primary production to dissolved organic carbon available to bacteria. Here we show cell survival of heterotrophic bacterioplankton in the subtropical Atlantic Ocean to be determined by phytoplankton extracellular carbon release (PER). PER represents the fraction of primary production released as dissolved organic carbon, and changes in the PER variability was explained by phytoplankton cell death, with the communities experiencing the highest phytoplankton cell mortality showing a larger proportion of extracellular carbon release. Both PER and the percent of dead phytoplankton cells increased from eutrophic to oligotrophic waters, while heterotrophic bacteria communities, including 60 to 95 percent of living cells (%LC), increased from the productive to the most oligotrophic waters. The percentage of living heterotrophic bacterial cells increased with increasing phytoplankton extracellular carbon release, across oligotrophic to productive waters in the NE Atlantic, where lower PER have resulted in a decrease in the flux of phytoplankton DOC per bacterial cell. The results highlight phytoplankton cell death as a process influencing the flow of dissolved photosynthetic carbon in the NE Atlantic Ocean, and demonstrated a close coupling between the fraction of primary production released and heterotrophic bacteria survival.

Introduction

Heterotrophic bacteria (HB) play a key ecological role in the cycling of carbon and nutrients in aquatic systems (Cole et al., 1988; Fuhrman 1992; Ducklow 2000) been the major consumers of dissolved organic matter (DOM) in the ocean (Sherr and Sherr, 1994; Azam 1998). HB recycle organic carbon through respiratory processes and channel significant amounts of dissolved organic carbon (DOC) to higher levels of the pelagic food webs via the microbial loop (Williams 1981; Azam et al., 1983; Sherr and Sherr 1988). The availability of DOC is a major constraint for heterotrophic bacterial dynamics, influencing a range of processes including HB growth efficiency, respiration or cell activity (Kirchman et al., 1991; Carlson and Ducklow, 1996; Herndl et al., 1997; Kirchman 1997; Kirchman et al., 2004). Indeed, a high percentage of bacterial cells are either metabolically inactive or dead in natural marine plankton communities (Choi et al., 1996; Smith and del Giorgio, 2003).

Phytoplankton, in turn, is the main source of DOC to support bacterial dynamics, linking phytoplankton and bacterial dynamics in the ocean. Phytoplankton release DOC as a result of cell lysis or direct exudation (Nagata 2000), and about 50% of daily primary production can be released by phytoplankton as DOC (Karl et al., 1998), potentially providing a source of carbon to HB. Extracellular release or production of dissolved organic carbon (P_{DOC}) by phytoplankton is a process mostly dependent on phytoplankton health (Fogg 1977; Sharp 1977), which is related to nutrient availability (Myklestad 1977; Obernosterer and Herndl, 1995), incident UV and PAR radiation (Berges and Falkowski, 1998; Llabrés and Agustí, 2006), and viral infection (Mühling et al., 2005). Phytoplankton cell death results in cell lysis (Brussaard et al., 1995; Agustí et al., 1998; Agustí and Duarte, 2000) and the subsequent release of the cellular contents and could play, therefore, an important role in driving P_{DOC} and the associated DOC supply to bacteria. Yet, the possible relationship between phytoplankton cell status and P_{DOC}, on the one hand, and the status of heterotrophic bacterial cells, on the other, has not yet been tested.

The status bacterial cells depends on a large number of processes but is ultimately dependent on the functioning of membrane proton pumps and the integrity of the cell membrane that indeed defines the viability of bacteria (Shapiro 2008). The analysis of bacterial cell-membrane integrity allows the discrimination between living and dying cells and has been introduced in recent studies assessing the environmental factors driving bacterial survival (Alonso-Sáez et al., 2007; Gasol et al., 2009; Morán and Calvo-Diaz, 2009; Lasternas et al., 2010).

These new approaches allow the characterisation of bacterial status at the individual-cell level and offer, as identified by Gasol et al. (2008), great potential to further our understanding on the variability of bacterial activity in aquatic systems, beyond the insights derived from previous approaches based on the examination of bulk assemblage properties.

Here we examine the status and survival of heterotrophic bacteria in the subtropical Atlantic and test their hypothesised relationships with the status of photosynthetic plankton cells and the release of dissolved organic carbon. Phytoplankton and bacteria cell health status were investigated by quantifying the percentage of living and dying cells in communities across a range of oceanographic conditions from highly oligotrophic to productive waters in the NE subtropical Atlantic.

Material and Methods

Study site and sampling

This study was conducted in the subtropical NE Atlantic Ocean section during the RODA 2 cruise on board R/V *Hespérides*, from February 2 to February 27 of 2007. A total of 24 stations were sampled, nine stations in the north Atlantic gyre area (Zone 1), eight placed in the vicinity of the Canary Current region (Zone 2) and eight stations in the area influenced by the Mauritania's upwelling (Zone 3; Fig. 1). At each station, vertical profiles of temperature, salinity, and fluorescence down to 200 m depth were performed using a Seabird 911 Plus conductivity–temperature–depth (CTD) system. Seawater samples were collected in 12-Liter Niskin bottles mounted on a General Oceanics rosette sampler from 7 depths from the surface to 200 m. Water samples of 200 ml were filtered through Whatmann GF/F filters to estimate total chlorophyll *a* concentration (Chl *a*), and extracted for 24 h in 90% acetone fluorometric determination (Turner Designs fluorometer) following Parsons et al., (1984).
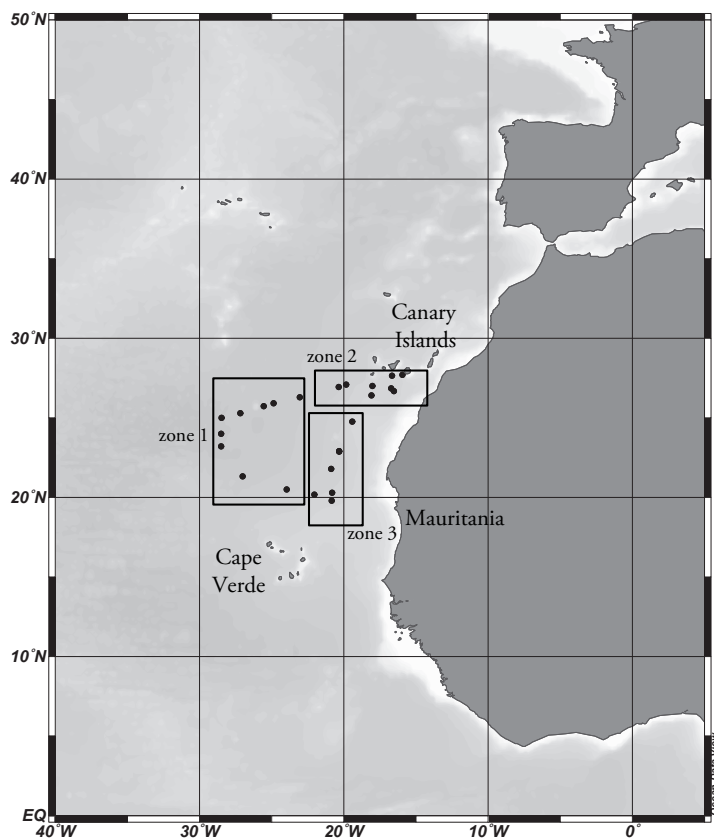

Fig.1. Areas of sampling and stations occupied during the RODA cruise. Zones 1, 2 and 3 correspond to three singular hydrographical and biological conditions: Oligotrophic tropical Atlantic zone (zone 1), Intermediate Gran Canaria Current area (zone 2), Upwelling associated waters (zone 3)

Primary production and percentage of extracellular release (PER)

In situ primary production was measured using ^{14}C additions (Steeman-Nielsen 1952). Seawater sampled at 5 depths including the surface (5 m), two intermediate depths, the deep chlorophyll maximum (DCM) and a depth below the DCM and an ultimate depth below the DCM, was delivered into transparent (light) and obscure (black masking tape-covered) polycarbonate bottles (150 ml), and inoculated with 80 μCi activity of a $NaH^{14}CO_3$ working solution. Inoculated bottles were set up at respective depths along a mooring buoy and incubated *in-situ* for 4 hours. For each sample, two aliquots of 5 ml (replicates) were introduced in scintillation vials (20 ml) for the determination of total labelled organic carbon production (TPP); the sum of ^{14}C incorporated into POC (particulate organic carbon) and released as DOC (dissolved organic carbon). The remaining volume was filtered through 0.22 μm mesh membrane filters (cellulose membrane filters) of 25 mm to determine particulate primary production (PPP > 0.22 μm). To remove inorganic ^{14}C, the liquid samples were acidified with 100 μl of 10% HCl and shaken for 12h, while the filters were fumed with concentrated HCl (37%) for 12h. Then, 10 ml and 5 ml of scintillation cocktail (Packard Ultima Gold XR) were respectively added to the TPP and PPP vials, and the disintegrations per minute were counted after 24 h with a scintillation counter (EG&G/Wallac). The dissolved organic carbon production by phytoplankton (P_{DOC}) was calculated as the difference between total and particulate primary production (Morán et al., 2001) and the percentage of the production released extracellular by phytoplankton (PER = 100 P_{DOC} / TPP) was measured.

Bacterioplankton abundance and viability

At each station, the proportion of living heterotrophic bacteria was quantified from seawater sampled at up to 7 depths. To do so, we used the Nucleic Acid Double Staining (NADS) (Grégori et al., 2001) flow cytometric protocol. This technique consists of the use of two nucleic acid fluorescent dyes, SYBR Green I (SG1; Molecular Probes) and Propidium Iodide (PI; Sigma Chemical Co.). Bacterial membranes are permeable to SG1, whatever the cell viability, resulting in green fluorescence when stained. However, living or viable cells with intact plasmic membranes are impermeable to PI. Thus only compromised or damaged cells are stained with PI (Barbesti et al., 2000), showing red fluorescence as described in Falcioni et al. (2008). Subsamples were analyzed immediately after collection. Samples (1ml) were stained with 10 μl of Propidium iodide (PI, 1 mg ml^{-1} stock solution), reaching a final concentration of 10 μg ml^{-1} and incubated for 30 minutes in that dark at room temperature.

Then, 10 μl of SYBR Green I (10-fold dilution of 10000×commercial solution in dimethyl sulfoxide) was added to subsamples and incubated for 10 more minutes. SG1 and PI fluorescence were detected using a FACSCalibur Flow Cytometer (Beckton Dickinson©) in the green (FL1) and the red (FL3) cytometric channels, respectively. Bivariate plots of green versus red fluorescence (FL1 vs. FL3) allowed for discrimination of live (green fluorescent, impermeable to PI) from dead cells (red fluorescent membrane-compromised cells, stained by PI and SG1). Bacterial concentration was calculated using a 1-μm diameter fluorescent bead solution (Polysciences Inc.) as an internal standard. Total heterotrophic bacterial abundance, in cells ml^{-1}, was calculated as the sum of red and green fluorescent cell abundance, while living bacterial cell abundance was determined from the green fluorescent cell counts. The percentage of viable heterotrophic bacteria was calculated as the ratio between the concentration of undamaged-membrane bacteria stained with PI and total bacterioplankton abundance.

Phytoplankton communities and viability of populations

Samples for the quantification of nano- and microphytoplankton abundance was sampled at the surface (5 m) and the deep chlorophyll maximum (DCM). Samples of 2-3 litres were concentrated into 50-70 ml samples using a Millipore cell concentration chamber. This concentration system has been used in previous studies (Alonso-Laíta and Agustí 2006; Lasternas et al., 2010; Lasternas and Agustí, 2010) with accurate results for microphytoplankton, and no effect on the viability or other cell properties (i.e. movement for flagellated cells, integrity of frustules, etc.). 10 ml aliquots (duplicates) of the concentrated sample were filtered onto 2 µm pore-size black polycarbonate filters, fixed with gluteraldehyde (1% final concentration) and stored frozen at -80ºC until counting. Phytoplankton cells were counted using an epifluorescence microscope (Zeiss© Axioplan Imaging), and were classified into 3 major groups; flagellates, dinoflagellates and diatoms, which were then separated into pennate and centric. Autotrophic picoplankton abundance was assessed using Flow Cytometry. At each station, duplicate 2 ml fresh samples from 7 depths were counted on board (duplicated counts) using a FACSCalibur Flow Cytometer (Beckton Dickinson©). An aliquot of a calibrated solution of 1 μm diameter fluorescent beads (Polysciences Inc.) was added to the samples as an internal standard for the quantification of cell concentration. The red (FL3, bandpass filter >670 nm), green (FL1 bandpass filter 530 nm) and orange (FL2, bandpass filter 585 nm) fluorescence, and forward and side scattering signals of the cells and beads were used to detect picoplankton populations of *Synechococcus*, *Prochlorococcus* and eukaryotes (Marie et al., 2005).

The proportion of living cells in the autotrophic communities examined was quantified by applying a cell membrane permeability test known as the cell digestion assay (CDA) (Agustí and Sánchez, 2002). The CDA consists on the exposure of the phytoplankton communities to an enzymatic cocktail (DNAse and Trypsin), which enters the cytoplasm and digests cells with compromised membranes (dead or dying cells), which are removed from the sample. The cells remaining in the sample after the CDA are living cells, which are then counted by flow cytometry or epifluorescence microscope, as described above. The CDA was applied to the concentrates of nano- and microphytoplankton cells prepared to quantify total cell abundance. The cell digestion assay was applied to duplicate 10 ml aliquots of cell concentrate by adding 2 ml of DNAse I solution (400 µg ml-1 in HBSS (Hanks' Balanced Salts)), followed by a 15 minutes incubation at 35ºC in a Digital Dry Bath. After this time, 2 ml of Trypsine solution (1% in HBSS) were added, followed by a 30 minutes incubation at 35ºC. At the end of this time, samples were placed in ice in order to stop the enzymatic cell digestion process, and were then were filtered onto polycarbonate 2 µm pore diameter black filters, washed several times with filtered seawater, fixed with gluteraldehyde (1% final concentration) and stored frozen at -80ºC until counting by epifluorescence microscopy.

Fresh samples to quantify the proportion of living picophytoplankton cells were sampled from the same 5 depths selected to estimate total picoplankton abundance at each station. Duplicate 1 ml samples were run with the CDA by first adding 200 μl of DNAse I solution, and after a 15 minute incubation at 35 ºC, 200 μl of Trypsine solution. Treated samples were incubated for 30 minutes more at 35 ºC and were finally placed in ice in order to cease enzymatic activity. Samples were then counted by flow cytometry as described above for total picophytoplankton abundance estimates. The percentage of living cells was calculated as the ratio between the concentration of cells after applying the CDA, which represents the living cells abundance, and total population abundance, which includes both living and death cells (Agustí and Sánchez, 2002).

Statistics

Spearman's rank coefficients were used to determine correlation coefficients between variables that departed from normality (Siegel and Castellan, 1988). The statistical significance of the differences between average values was tested using Student's t-test, with a critical p-value of 0.05. Heterotrophic bacteria survival, as the percent of living heterotrophic bacterial cells, were grouped by 20% PER bins to examine a relationships between %LHB and PER. Linear regression analyses were applied to raw and binned PER data.

Results

The waters studied included three distinct oceanographic zones (Fig. 1) including the oligotrophic tropical Atlantic Ocean, which presented significantly warmer and saltier waters and low nutrient concentration (Table 1, Fig. 2); waters influenced by the NW African upwelling system, characterised by cooler and fresher waters and higher dissolved nutrient concentration (Table 1); and the transitional system around the Canary Islands, influenced by the Canary current, exhibiting intermediate temperature, salinity and nutrient concentration (Table 1).

Mean ± SE [min/max]	Oligotrophic	Intermediate	Upwelling
Temperature (°C)	21.46 ± 0.16 [A]	19.15 ± 0.16 [C]	19.94 ± 0.22 [B]
Salinity (PSU)	37.21 ± 0.04 [A]	36.87 ± 0.03 [B]	36.74 ± 0.04 [C]
Dissolved inorganic Nitrogen (μmol N L^{-1})	0.31 ± 0.06 [B]	0.75 ± 0.15 [B]	2.28 ± 0.41 [A]
Ammonium (μmol N L^{-1})	0.10 ± 0.01 [B]	0.11 ± 0.01 [AB]	0.13 ± 0.01 [A]
Phosphate (μmol P L^{-1})	0.21 ± 0.03 [AB]	0.09 ± 0.02 [B]	0.33 ± 0.03 [A]
Chlorophyll (mg Chl a m^{-3})	0.28 ± 0.02 [B]	0.37 ± 0.04 [AB]	0.48 ± 0.05 [A]
Total primary production (mg C m^{-3} h^{-1})	0.70 ± 0.10 [A]	0.96 ± 0.13 [A]	1.14 ± 0.20 [A]
Dissolved organic carbon production by Phytoplankton (mg C m^{-3} h^{-1})	0.58 ± 0.09 [A]	0.64 ± 0.10 [A]	0.41 ± 0.09 [A]
PER	81.9 ± 1.9 [A]	64.4 ± 4.7 [B]	41.3 ± 7.9 [C]
Phytoplankton dead cells (%DC)	51.9 ± 4.2 [A]	39.1 ± 2.7 [B]	44.1 ± 4.4 [B]
Heterotrophic living bacteria (%HLB)	85.7 ± 1.1 [A]	79.9 ± 0.9 [B]	74.8 ± 1.0 [C]
Flux of DOC per bacteria cell (pg C. cells^{-1}. h^{-1})	1.82 ± 0.42 [A]	1.59 ± 0.24 [AB]	0.81 ± 0.21 [B]

Table 1. Average ± SE hydrological properties, nutrients and chlorophyll *a* concentration, primary production rates, percentage of extracellular release (PER), health status of phytoplankton and HB and DOC flux per bacteria cell quantified at the three zones. The average values for the zones connected by same letter are not significantly different ($p < 0.05$)
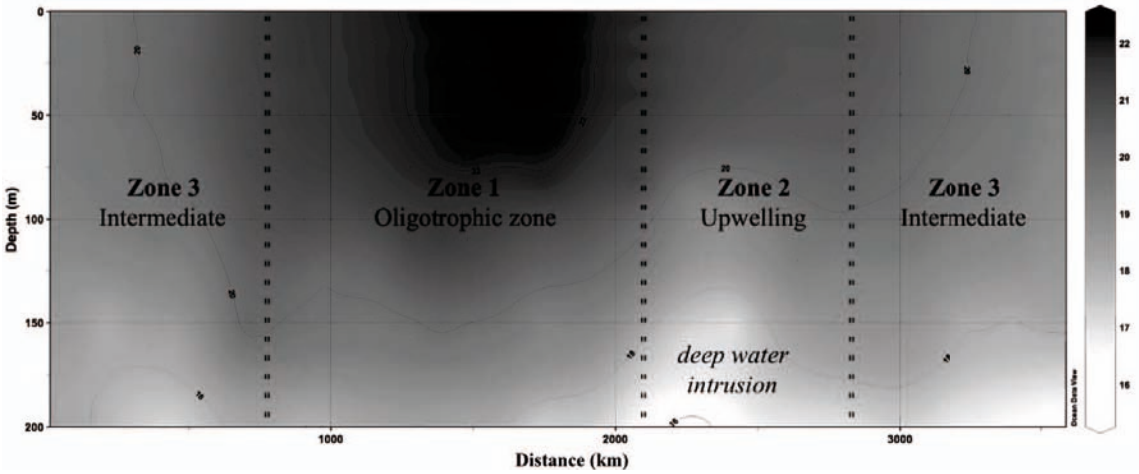
Fig.2. Vertical distribution of Temperature (°C). Sampling depths along the different zones are represented by dots

Total primary production (TPP) declined from the waters influenced by the upwelling, which exhibited the highest values to the most oligotrophic zone, which presented the lowest rates (Table 1). Dissolved primary production (P_{DOC}) was positively related to total primary production (TPP, Fig. 3), and tended to increase as total primary production increased, but with a slope gently lower than 1, indicating that P_{DOC} tended to be proportionally lower in productive waters (Fig. 3, Table 1). Thus, the percentage of extracellular release (PER), which varied greatly across the study (Table 1), was greatest in the most oligotrophic waters sampled and declined towards more productive waters (Table 1).
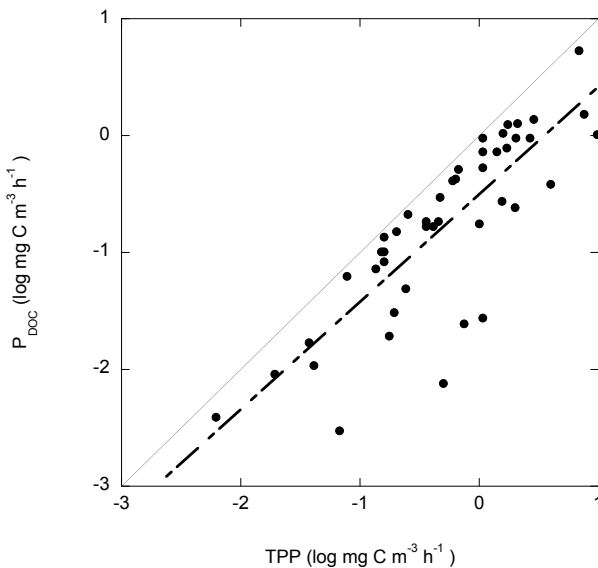

Fig.3. The relationship between the dissolved primary production (P_{DOC}) and total primary production (TPP). The solid line shows the 1:1 relationship and the dotted line shows the fitted regression equation $\log P_{DOC} = -0.501 + 0.92 \ (\pm 0.09) \log TPP$ ($R^2 = 0.68$, P < 0.001, N= 45)

Nano-microphytoplankton communities were present along the study site, and showed higher abundance at the DCM than at the surface waters, with slightly higher abundance within Zone 3, area influenced by the upwelling system (Table 2). Autotrophic flagellates dominated the microphytoplanktonic community throughout the study (Table 2) and presented relatively uniform abundance within the studied zones. Diatoms were poorly abundant within Zone 1 (Table 2) represented almost solely by the pennate genera *Nitzschia* spp., but showed a consistent increase in abundance at the waters influenced by the upwelling (Zone 3, Table 2), with the centric genera *Thalassiosira* sp. and *Chaetoceros* sp. being the most abundant. Dinoflagellates, primarily represented by the naked form *Gymnodinium* spp., displayed low abundance across the cruise (Table 2) and were principally located in surface waters.

Mean (cells/L) ± SE	Oligotrophic	Intermediate	Upwelling
Nano/micro-phytoplankton	$2.22 \pm 0.31 \times 10^3$	$2.83 \pm 0.38 \times 10^3$	$5.89 \pm 1.54 \times 10^3$
Flagellates	$1.09 \pm 0.22 \times 10^3$	$1.53 \pm 0.11 \times 10^3$	$2.78 \pm 0.74 \times 10^3$
Diatoms	$5.18 \pm 0.76 \times 10^2$	$8.41 \pm 1.26 \times 10^2$	$2.23 \pm 0.72 \times 10^3$
Dinoflagellates	$6.09 \pm 0.15 \times 10^2$	$4.63 \pm 1.68 \times 10^2$	$8.77 \pm 3.02 \times 10^2$

Table 2. Mean ± SE of the nano-microphytoplankton abundances in the three zones

Prochlorococcus spp. was the most abundant, during the cruise (Fig. 4), presented significant higher values than both populations of *Synechococcus* spp. and picoeukaryotes at Zones 1 and 2, and decreased at waters associated to the upwelling system (Zone 3). Within this zone, *Synechococcus* spp. abundance surpassed that of *Prochlorococcus* spp. (Fig. 4). Picoeukaryotes's abundance was relatively uniform (about 10^3 cells ml^{-1}) between the 3 zones of study, with maximum values observed at the intermediate zone of the Canary current (Zone 2, Fig. 4). Heterotrophic bacteria presented significantly higher abundance at the oligotrophic zone (Zone 1) and lower ones at zone 2 (Fig. 4).
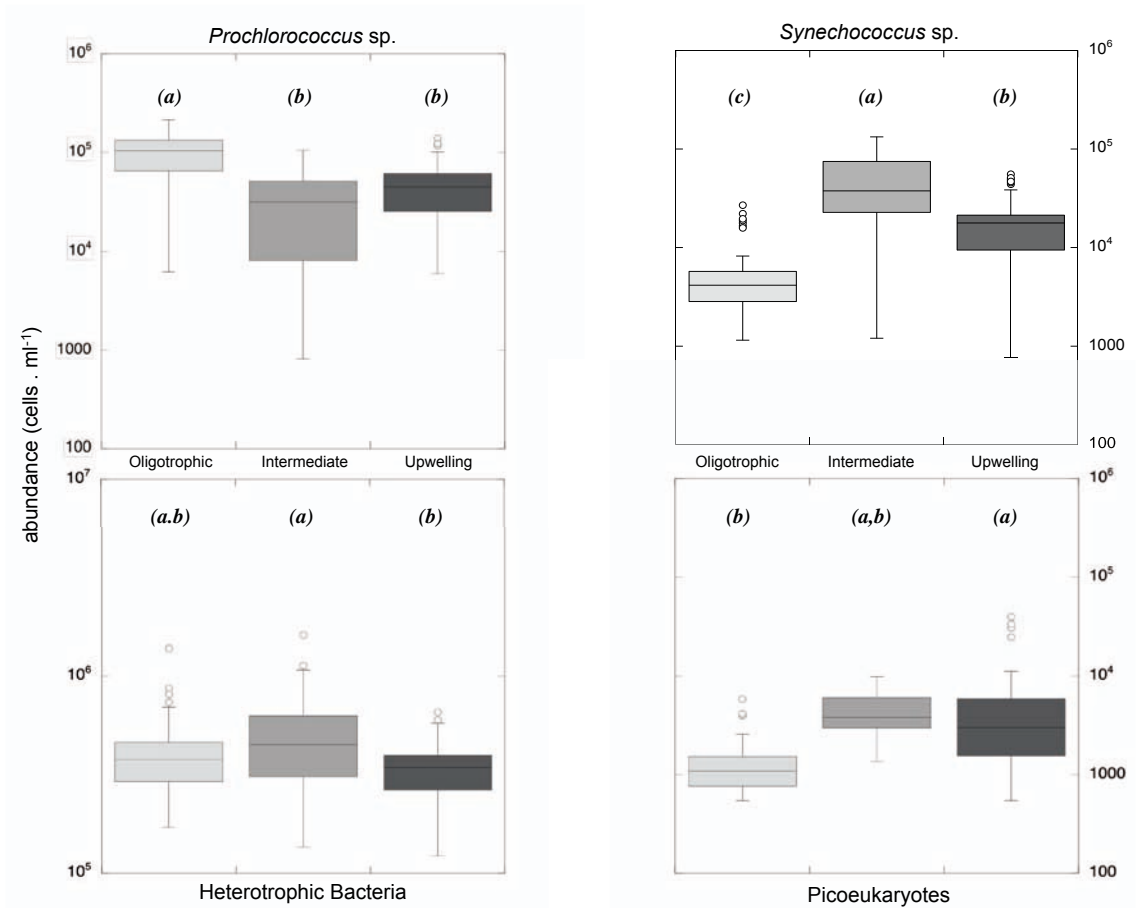

Fig.4. Box plots showing the abundance distribution of the picoplankton populations within systems. The boxes show the lower and upper quartiles, median, minimum and maximum values, and outliers. Numbers correspond to averaged (± SE) abundances within systems. The boxes connected by same letter are not significantly different (p < 0.05)

The proportion of dead phytoplankton cells (%DC) within the different communities showed high variability (Fig. 5). Diatoms dominated the community in the upwelled waters (Table 2) where they showed a low proportion of dead cells, with the highest percentage of dead diatom cells observed in oligotrophic waters (Fig. 5). *Prochlorococcus* spp., the dominant picophytoplanktonic species (Table 2), was less abundant with higher proportion of dead cells at the upwelling zone (Figs. 4 and 5).
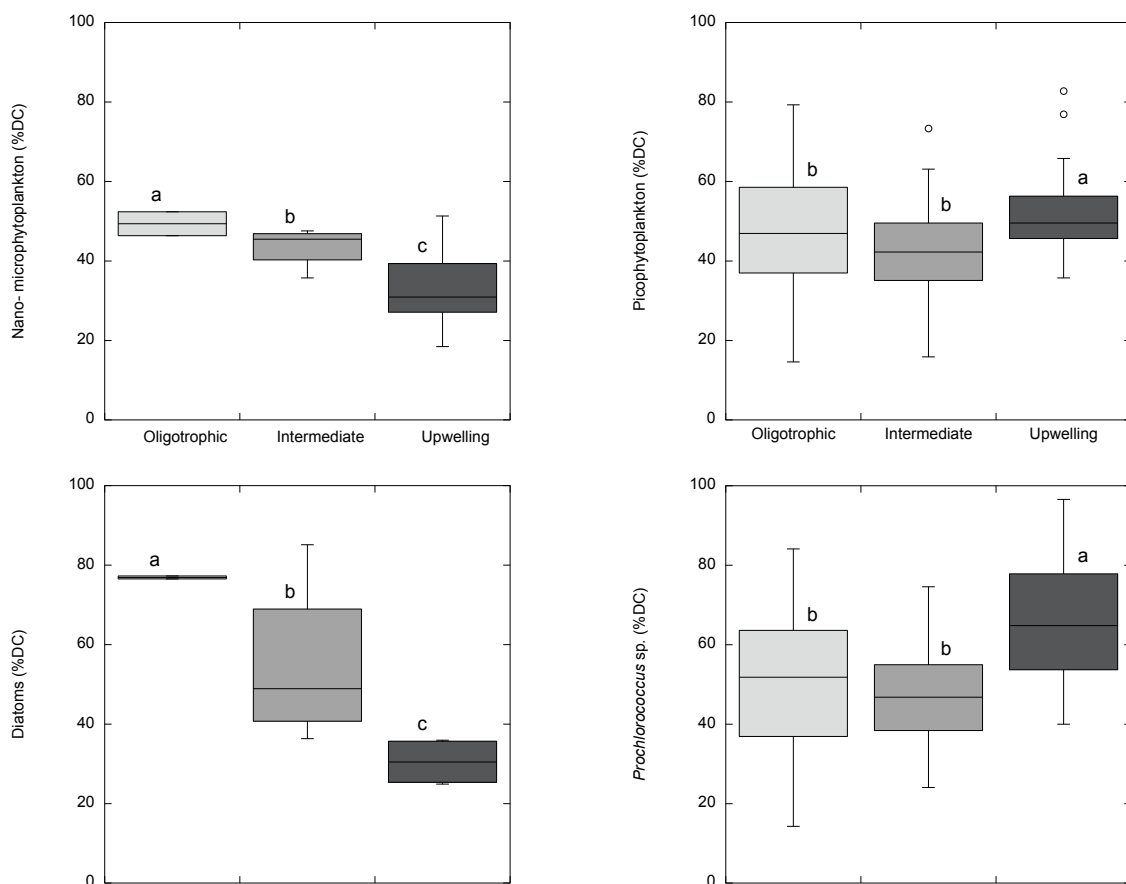

Fig.5. Box plots showing the distribution percentage of dead cells (%DC) of the different phytoplankton groups in the sampled zones. The boxes present the lower and upper quartiles, median, minimum and maximum values, and outliers. The boxes showing the same letter do not have significantly different mean values (t-test, p > 0.05)

The oligotrophic zone presented highest phytoplankton mortality (table 1) associated to highest PER rates. The variability in the percent extracellular carbon release in each station was closely dependent on the status of the photosynthetic community, as reflected in a linear increase in the percent extracellular carbon release with an increase in the percent of dead cells in the photosynthetic community (Fig. 6).
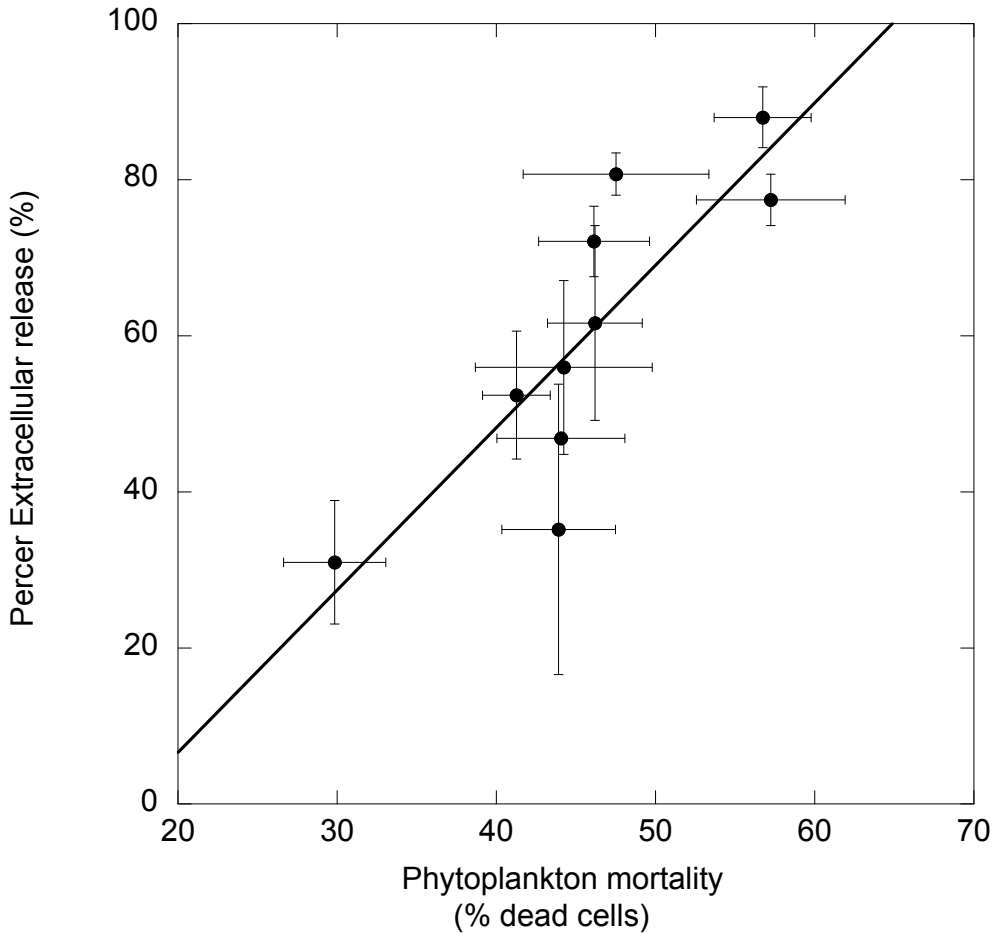

Fig.6. The relationship between the percentage of dead phytoplankton cells (%DC) and the proportion of extracellular carbon release (PER), averaged by stations across the study. The full line represents the fitted regression equation: PER= -35.03 + 2.08 (± 0.49) phytoplankton %DC (R^2 = 0.69, P = 0.0029, N=10)

Heterotrophic bacteria communities included 60 to 95 percent of living cells across communities, with the average %LC of heterotrophic bacteria being higher than that of autotrophic picoplankton (Student's t-test, $P < 0.0001$). While bacterioplankton presented highest abundance in the upwelled waters (Table 2, Fig. 4), the percentage of heterotrophic living bacteria was highest in the oligotrophic waters and declined towards most productive waters (Table 1, Fig. 7).

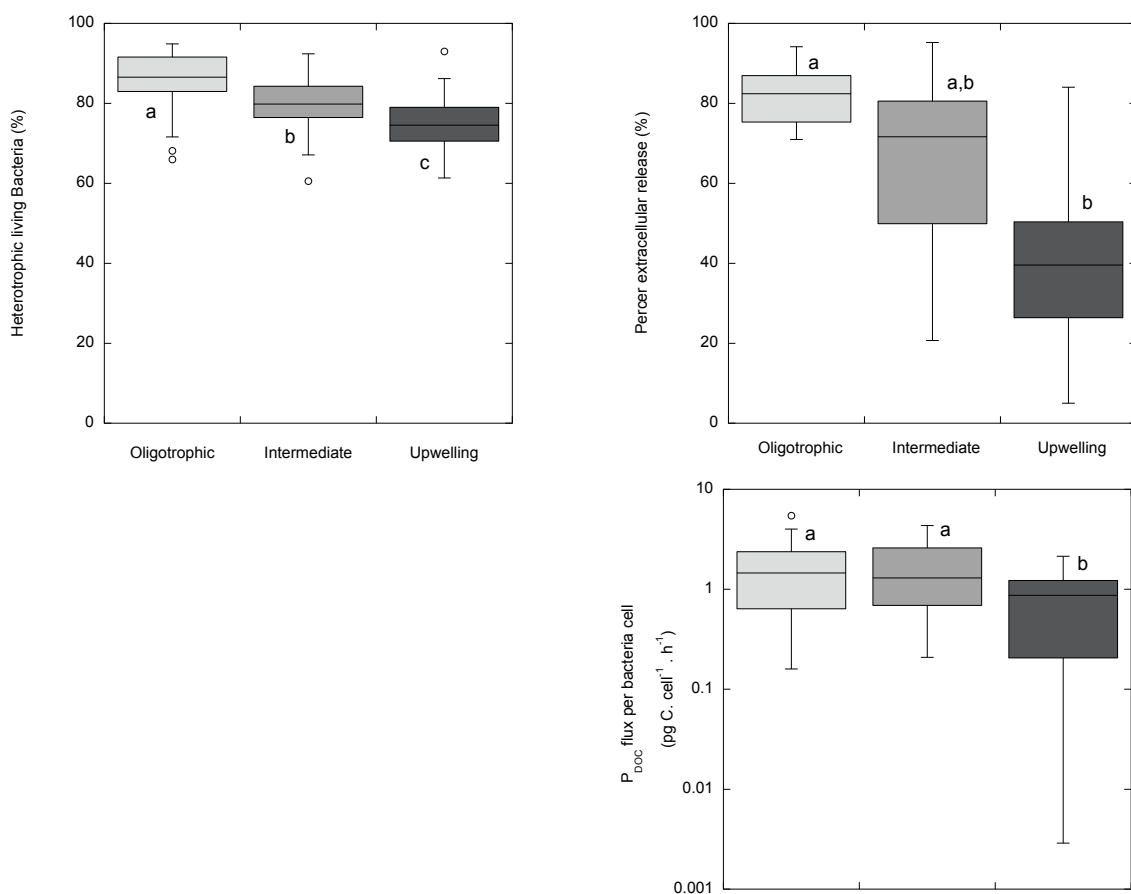Fig.7. Box plots showing the distribution of the percentage of living bacteria cells (%HLB), the distribution of PER and the variation of fluxes of P_{DOC} per bacteria at the three sampled zones. The boxes present the lower and upper quartiles, median, minimum and maximum values, and outliers.
The boxes showing the same letter do not have significantly different mean values (t-test, p > 0.05)

By dividing the production of dissolved organic carbon by phytoplankton and the bacterial abundance, we obtained the flux of P$_{DOC}$ per bacterial cell (pg C. bacterial cell^{-1}) and could appreciate that availability in DOC for heterotrophic bacteria were higher in the oligotrophic waters (Fig. 7) than at the other zone of the study. The percentage of living heterotrophic bacterioplankton cells increased with increasing proportion of extracellular dissolved organic carbon released (R^2 = 0.83, *P* < 0.005, Fig. 8).
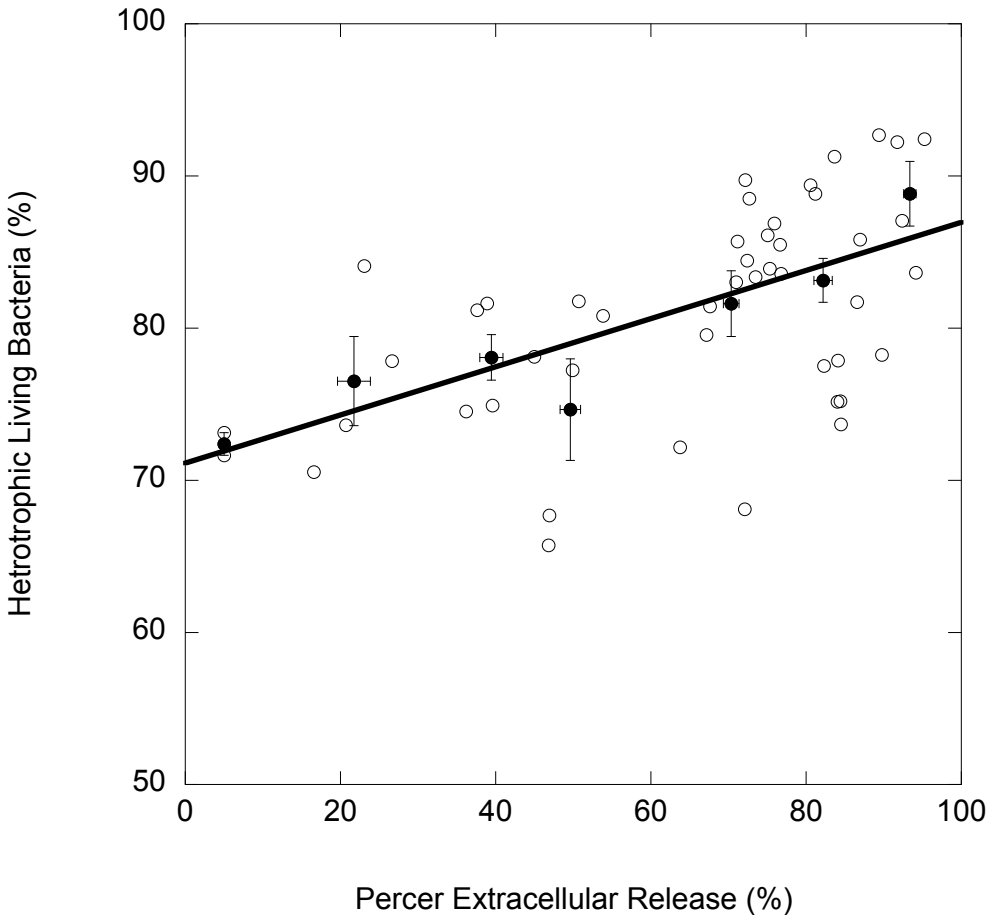
Fig.8. The relationship between the percentage of living heterotrophic bacteria (HLB) and the percent extracellular dissolved organic carbon release. The open and full symbols represent the individual and average (± SE) percentage of living bacteria binned by 20% PER intervals. The doted line represents the fitted regression equation between the percent living heterotrophic bacteria average across 20% PER bins and PER: HLB (%) = 71.6 + 0.16 PER (R^2 = 0.83, P < 0.005, N= 7)

Discussion

The results presented here provide evidence of a close coupling between heterotrophic bacterial survival and the excretion of recently photosynthesized carbon by phytoplankton in the NE subtropical Atlantic. The results presented also suggest a mechanistic pathway linking phytoplankton cell death with high extracellular carbon release and a subsequent increase in the percentage of living heterotrophic bacteria cells. These results confirm the power of approaches based on assessments at the single-cell level (Agustí and Sánchez, 2002; Bidle and Falkowski, 2004; Gasol et al., 2008) to resolve the relationships between the status of phytoplankton cells and that of heterotrophic bacteria, mediated by the extracellular release of organic carbon.

Previous attempts at testing the relationship between P_{DOC} release and bacterial production remained elusive and variable among systems. In open ocean sites, bacterial production and dissolved primary production (DPP) are often tightly linked (Morán et al. 2001; Antarctic off shore waters), while in coastal (Morán et al. (2002a) NE Atlantic coastal system and Morán et al. (2002b) NW Mediterranean) or eutrophic sites (Morán et al., 2002b Antarctic coastal) persists a lack of linkage. Our study provides, to the best of our knowledge, the first demonstration of a direct relationship between recently released labile photosynthate, the preferred carbon source for HB (Norrman et al., 1995), and the survival of heterotrophic bacteria.

A gradient in phytoplankton productivity and community structure from the African upwelling region to the oligotrophic region offshore has been previously reported for the subtropical NE Atlantic (Teira et al., 2003; Pelegrí et al., 2005; Alonso-Laíta and Agustí, 2006), including an increase in phytoplankton mortality rates and the proportion of dead phytoplankton cells along this gradient (Agustí et al., 2001; Alonso-Laíta and Agustí, 2006). The results presented here confirm these findings, with phytoplankton cell viability decreasing from upwelling-influenced waters to oligotrophic waters, particularly for diatoms, which showed a two-fold reduction in the percent of living cells from the upwelling to the oligotrophic waters. However, the patterns displayed by the different populations conforming the phytoplankton community were complex, as phytoplankton show intricate and differentiated niches of cell viability depending on cell size, irradiance, nutrient concentration and temperature (Berges and Flakowski, 1998; Agawin et al., 2000; Agustí 2004; Alonso-Laíta and Agustí, 2006; Agustí and Llabrés, 2007; Lasternas et al., 2010).

The percentage of dead cells tended to increase with decreasing cell size, with more than 40 % dead cells generally found in the picophytoplankton community, consistent with the reported increase in mortality rates with decreasing cell size (Marbà et al., 2007).

Although picophytoplankton communities are typically dominant in oligotrophic waters (Agawin et al., 2000), they showed high variability in cell viability in the most oligotrophic waters sampled here. Surface populations are exposed to high PAR and UV radiation, resulting in high %DC of *Prochlorococcus* spp., which is strongly sensitive to high solar radiation (Llabrés and Agustí, 2006; Agustí and Llabrés, 2007; Llabrés et al., 2010), whereas *Synechococcus* is typically stressed by low light at deep layers but shows higher cell survival in surface waters (Llabrés and Agustí, 2006; Agustí 2004). In addition, the high cell mortality of *Prochlorococcus* sp. in the upwelling waters is consistent with the incapacity of *Prochlorococcus* sp. to use nitrate (Moore et al., 2002) and with the decline in cell viability in waters below 21ºC (Alonso-Laíta and Agustí, 2006).

The patterns of cell survival of the natural phytoplankton populations described here provided compelling evidence that the variation in the proportion of dissolved organic carbon release is driven by phytoplankton cell mortality in the subtropical NE Atlantic Ocean. In agreement with previous studies, communities in unproductive oligotrophic waters tended to release as DOC a higher fraction of their total primary production compared to more productive, nutrient-rich upwelling waters (Teira et al., 2001; Morán et al., 2002a). However, despite the lower PER in productive waters, the magnitude of total organic carbon released by the community was higher, because total primary production was much higher in the upwelling zone. Similarly, whereas the proportion of phytoplankton cells dying in eutrophic waters was low, the total dead biomass in the upwelling region was much larger than that in oligotrophic waters, independently the phytoplankton size fraction. The larger phytoplankton mortality lead to a higher release of primary production as dissolved organic carbon, which, in turn, can support a larger biomass and carbon flux through bacteria in the upwelling zone compared to the oligotrophic waters.

Within the upwelling-influenced area of the NE Atlantic Ocean, bacterial communities have been identified to be carbon-limited (Alonso-Sáez et al., 2007). In our study, we found a significantly higher bacterial abundance in upwelling-influenced waters, consistent with the higher release of DOC from phytoplankton, declining towards the oligotrophic waters offshore. The PER was however, lowest at the upwelling-influenced area. This indicates a lower carbon availability per bacterial cell, and may explain the lower bacterial survival in these waters, in agreement with the carbon limitation of the bacterial community in the upwelling-influenced waters reported by Alonso-Saez et al. (2007). In contrast, high bacterial viability was observed in the oligotrophic waters, where phytoplankton released a much higher proportion of their production as DOC, resulting in a higher flux of P_{DOC} per bacteria cell.

This finding is also in agreement with reports of a strong dependence of bacteria on algal extracellular production in open-ocean environments, while bacterial carbon demand was not related to algal P_{DOC} in coastal and productive systems (Morán et al., 2002a,b).

In oligotrophic areas, allochthonous organic matter from lateral transfer or atmospheric inputs can be an alternative source of carbon to autochthonous production (del Giorgio et al., 1997; Arístegui et al., 2003; Herndl et al., 2008). The lability of atmospheric sources of organic carbon has not yet been established (Dachs et al., 2005), but organic inputs from riverine sources and the majority of the DOC pool in oceanic systems are mostly refractory (Raymond and Bauer, 2001). Accordingly, DOC freshly released by phytoplankton is the source of carbon supporting the most efficient assimilation by bacteria in the oligotrophic ocean (Coveney and Wetzel, 1989; Norrman et al., 1995). Our results support high phytoplankton cell death in the oligotrophic ocean, consistent with previous findings (Agustí 2004; Alonso-Laíta and Agustí, 2006; Lasternas et al., 2010), and demonstrates that high phytoplankton cell death in the open oligotrophic areas of the NE Atlantic results in a large release of DOC relative to primary production, providing a significant flux of labile carbon, that results in high heterotrophic bacteria survival, as demonstrated by the relationship between HB viability and PER presented here.

Acknowledgements

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General Discussion

General Discussion

Throughout this PhD thesis, the assessment of natural planktonic communities cell mortality in addition to the description of their distribution provided relevant information on the functioning and structure of the pelagic community. Evaluation of the survival success of bacteria and phytoplankton populations by the quantification of the percentage of individual living and dead cells constituted an innovative and relevant approach to identify environmental control and species competitive success implementing the description provided by the study of the standing stock of populations. Phytoplankton cell mortality, evaluated among distinct oceanic systems, including the Mediterranean Sea, Arctic, Antarctic and Atlantic oceans, was also identified as a process explaining the release of dissolved organic carbon by phytoplankton (P_{DOC}) indicating that cell mortality may be an important factor influencing the carbon fluxes in oceanic systems. This thesis indentified that the 54.4%, on average, of the primary production is released as DOC, and that substantial proportion of this release is mostly forced by cell death, supporting that phytoplankton cell mortality is a process influencing the carbon fate. This study provides evidence that cell mortality influenced the food web, as the released P_{DOC} significantly explained the survival success of the heterotrophic bacteria in some of the systems studied.

Phytoplankton ecology, survival success and environmental conditions

The first goal of this study aimed to better understand the response of natural phytoplankton populations to environmental conditions, and examine their co-existence in a competitive environment. Regional, seasonal and climatic changes in temperature, nutrients availability or mixing regime constitute stressors for the phytoplankton species growing in the system that may help to explain changes in diversity, since individually affect the health of phytoplankton groups differently. Natural populations die encountering conditions that overcome its limits of tolerance (Putman and Wraten, 1984) and mortality would thus be a proxy of adverse environmental conditions. The responses of phytoplankton to environmental stressors remained difficult to perceive and quantify in situ, but the identification and quantification of the percentage of dead cells, as calculated throughout the chapters, were successfully conducted and resulted useful in reflect the physiological state of the natural phytoplankton populations in situ.

The proportion of dead natural phytoplanktonic cells in the marine systems studied reached a global average of 40.5 ± 16.9 % indicating that in adverse conditions, dying or senescent phytoplankton cells can represent almost half of the total phytoplankton abundance. Depending on the oceanic regions considered we identified distinct key factor controlling phytoplankton cell death, such as nutrients availability at oligotrophic waters or water temperature at Polar Regions. In oligotrophic waters (Chapters 1, 4 and 5), the nutrient availability (such as phosphorus or nitrate), as well as hydrological features such as mixing regimes associated to eddies structures determined the survival success of the larger phytoplankton fraction (nano- microphytoplankton) in oligotrophic conditions. Previous studies assumed mixing conditions that promote nutrient inputs as favourable to large phytoplankton such as diatoms (Margaleff 1978) in oligotrophic waters while dinoflagellates and small flagellates would dominate in areas of increased water column stratification (Béthoux et al., 2002; Tunin-Ley et al., 2009). The evaluation of the health status helped in defining the ecological preferences of the nano- microphytoplankton populations and confirming this assumption. Diatoms presented highest survival success in the turbulent and more nutrient-rich waters of the Sicily Strait and Aegean Sea (Mediterranean Sea, Chapter 1) but also at the upwelled, mixed waters of NE Atlantic cyclonic eddies (Chapter 4) and in the coastal Mauritanian region (Chapter 5). Besides the positive influence of nutrients availability on phytoplankton survival, we were able to identify the stress experienced by diatoms associated to the decline in nutrients. The downward anticyclonic processes tended to deprive the system of nutrients and stratification, resulting in adverse conditions for the health of larger phytoplankton as a result of the progressive oligotrophication, which yielded an increase in larger cells mortality.

At Polar regions (Chapters 2 and 3), contrary to the oligotrophic regions, the waters sampled in both polar hemispheres were not nutrient-limited but are under the influence of climate change and ice melting (ACIA 2004; IPCC 2007; Steig et al., 2009). Climate change may strongly affect oceans primary production (Richardson & Schoeman, 2004; Behrenfeld et al., 2006) and are resulting in an observed phytoplankton biomass decline (Boyce et al., 2010; Gregg et al., 2003) with phytoplankton species responding differently to increase water temperature (Huertas et al., 2011). In our study, we identified water temperature as one of the major key determinants of the cell survival variability and community structure of the polar marine phytoplankton population.

While a majority of the marine phytoplanktonic ecology surveys are inferred from descriptions of the populations' distribution and biomass (as chlorophyll *a*) (e.g Boyce et al., 2010), the examination of the survival success of natural planktonic population provide information on the capacity of population to respond to recent intensified environmental changes and would help in predicting the consequences of such changes for natural communities' distribution and dynamics. The dynamics and distribution of polar phytoplankton communities were strongly influenced by water masses and its associated temperature and the mixing regime (Chapters 2 and 3), with both last properties known to be directly affected by warming in polar regions (ACIA 2004; IPCC 2007; Steig et al., 2009). In the Arctic waters (Chapter 2), the ice-melting event resulted in strong changes in water characteristics (e.g. temperature, salinity) that significantly affected phytoplankton community structure and survival. The increased loads of ice-melt waters negatively affected the biomass fraction of larger phytoplankton producers (diatoms) along with the inhibition of phytoplankton total biomass and production in ice-melting waters (Duarte et al., *data unpublished*). The prymnesiophyte *Phaeocystis pouchetti* dominated the phytoplankton biomass across the study, showing higher percentages of dead cells in cold and lesser saline waters, characterizing the waters dominated by ice-melting. The dominance of *P. pouchetti* was the result of the exclusion of diatoms from ice-melt waters, as opposed to more favourable conditions fulfilling *P. pouchetti* cells preferences. The segregation of phytoplankton communities in the Antarctic Peninsula waters observed in Chapter 3, associated to different environmental conditions, supported existing evidence of shifts in algal community composition from large to small primary producers at the warming Antarctic Peninsula area (Moline et al., 2008; Montes-Hugo et al., 2008). The cell viability of flagellates tended to increase in warmer open waters, resulting in the increased contribution of the cryptophytes *Cryptomonas* sp. to phytoplankton biomass with increasing water temperature. Conversely, larger phytoplankton forms (diatoms and dinoflagellates) showed a different thermal preference, as indicated by the relationship between cell viability and temperature, to the cold inshore waters of the MIZ. The picophytoplankton community even poorly represented at Antarctic Peninsula (Wright et al., 2009), presented also increasing biomass and viability associated with increasing water temperature, confirming the foreseen prevalence of small phytoplankton forms in warming waters (Atkinson et al., 2003; Morán et al., 2010), although its contribution to biomass remained always low, indicative of other factors than temperature constrained their biomass.

In the Mediterraenan Sea, we described for the first time the presence of two different populations of *Synechococcus* spp. (sp#1- sp#2) that presented distinct ecological niches. Viability of *Synechococcus* sp#2 increased in warmer waters and was associated to low phophate concentration (Chapter 1), but *Synechococcus* sp#1 presented higher percentage of living cells (%LC) associated to cold and phosphate-enriched waters suggesting segregated ecological niches of the two ecotypes. *Prochlorococcus* sp. viability in the Mediterranean Sea and the Atlantic Ocean showed a thermal preference for warmer waters (Chapters 1, 4 and 5), in agreement with populations in NE Atlantic waters (Alonso-Laíta and Agustí, 2006) and its geographical distribution, being absent outside the temperate areas (Partensky et al., 1999). Moreover we were able to identify nitrate concentration as an important factor in controlling *Prochlorococcus* sp. survival success across the studies, in agreement with previous studies on its viability (Agusti 2004, Alonso-Laíta and Agustí, 2006) and the documented incapacity of *Prochlorococcus* spp. to use nitrate, which could be toxic for this species (Moore et al., 2002; Scanlan and West, 2002).

Although we observed across the chapters that phytoplankton mortality was consistently affected by water temperature, we didn't found any direct relationship between water temperature and phytoplankton cell mortality when examining all the data set.

		Arctic Ocean	Antarctic Ocean	Atlantic Ocean		Mediterranean Sea
		ATOS-1	ATOS-2	RODA-1	RODA-2	THRESHOLDS
Water temperature	(ºC)	2.13 ± 0.28	0.61 ± 0.11	21.41 ± 0.46	20.20 ± 0.13	16.18 ± 0.31
Chlorophyll *a*	(mg Chl *a* m^{-3})	1.97 ± 0.21	2.85 ± 0.40	0.34 ± 0.05	0.38 ± 0.02	0.67 ± 0.05
Primary Production	(mg C m^{-3} h^{-1})	3.33 ± 0.67	3.57 ± 0.58	0.20 ± 0.04	0.93 ± 0.08	
PER	(P$_{DOC}$/TPP) x 100	55.35 ± 2.48	55.41 ± 1.79	69.47 ± 6.49	64.19 ± 3.61	
Phytoplankton survival	%DC	55.6 ± 6.8	41.2 ± 3.7	49.4 ± 3.2	39.25 ± 3.1	38.1 ± 2.9
Bacteria survival	%HLB		70.7 ± 1.2	62.8 ± 3.4	80.6 ± 0.71	70.4 ± 1.6

Table 1. Averages (± SE) and ranges of the relevant hydrological and biological parameters measured along the different cruises

P_{DOC} *release mediated by phytoplankton cell death*

Different laboratory studies suggested that dead or decaying phytoplankton cells may release significant dissolved organic carbon to the media (Sharp 1977; Myklestad 2000; Nagata 2000). Field studies have provided supporting evidence of high release rates of dissolved organic carbon by phytoplankton in oligotrophic oceans (Karl et al., 1998; Morán et al., 2002a), consistent with reports of high phytoplankton cell lysis and mortality rates therein (Agustí et al., 1998; Agustí et al., 2001). Very recently, Agustí and Duarte (2012) related P_{DOC} with phytoplankton lysis rates. This PhD Thesis explored the relationship between the proportion of living phytoplankton cells and the relative fraction of P_{DOC} released (expressed as PER), quantifying the variation in phytoplankton cell mortality and the production of dissolved organic carbon by phytoplankton (P_{DOC}) across a variety of oceanic waters, from productive to oligotrophic conditions.

We quantified P_{DOC} at the polar waters of the Southern (Antarctic Peninsula, Chapter 3) and Arctic Oceans (Western sector; Chapter 2) and from the temperate-to-tropical waters of the NE Atlantic Ocean (Chapters 4 and 5). The systems studied presented wide gradients in productivity (Table 1) with the polar waters presenting higher primary production and phytoplankton biomass than waters from the NE Atlantic Ocean (Table 1).

The percentage of DOC production with respect to total primary production (expressed as PER) varied across the waters sampled (Table 1) and showed higher values in NE Atlantic (Chapters 4 and 5) than in polar waters (Chapters 2 and 3) (t-student, P< 0.005) showing a significant inverse relationship between PER and total system productivity (r_s = -0.15, P< 0.01, pooled data), in agreement with other studies, documenting a larger percentage of DOC production in oligotrophic waters and lower in productive ones (Teira et al., 2001; Morán et al., 2002b). We observed, however, regional differences, as in Atlantic waters (cruises RODA1-2), the dissolved organic carbon released by phytoplankton (P_{DOC}) tended to decrease as total production increased (slope significantly inferior to 1) while in Antarctic waters (ATOS-2), the P_{DOC} was proportionally increased with productivity (slope significantly superior to 1).

At the arctic, the presence of mucilage produced by *Phaeocystis pouchetii* (Chapter 2) must include dissolved organic ^{14}C forming the mucilaginous structure, resulting in a slope equal to 1, indicating PER and total primary production to vary independently at these arctic waters.
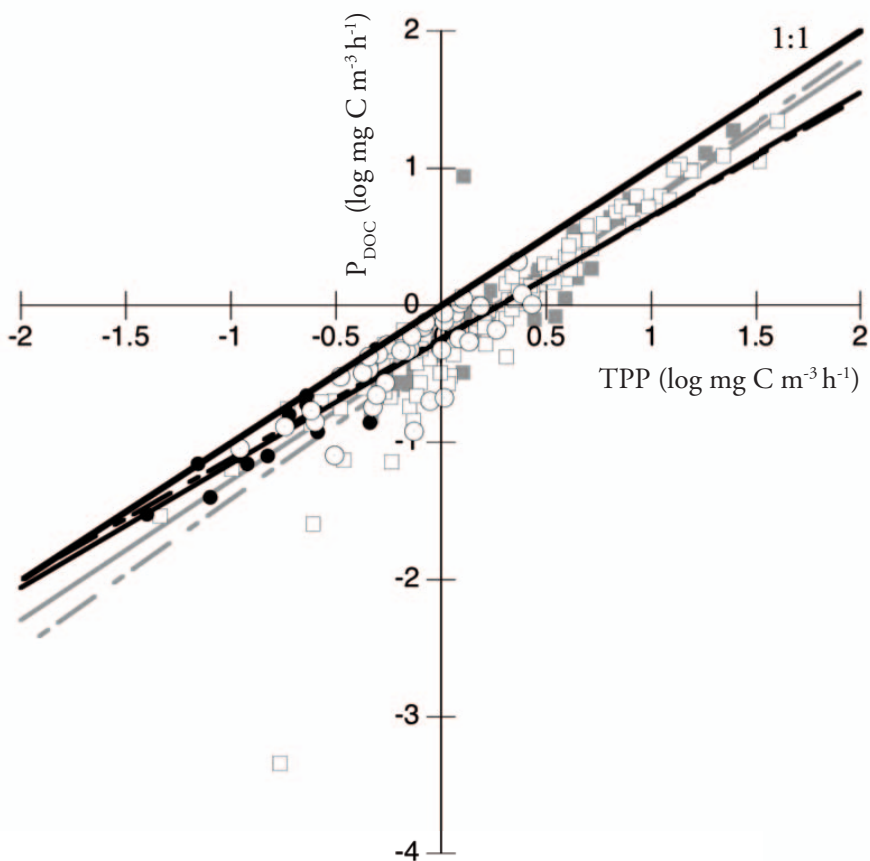
Fig.1. Relationships between the total primary production (TPP, log mg C m^{-3} h^{-1}) and the P$_{DOC}$ release by phytoplankton (log mg C m^{-3} h^{-1}) at the different marine systems. Full and empty grey squares represent data from the Arctic Ocean and Antarctic Peninsula, respectively (ATOS1&2). Full grey line (ATOS1) shows the fitted regression equation Log P$_{DOC}$ = -0.29 + 1.05 log TPP (R^2 = 0.897, P < 0.0001). Dotted grey line (ATOS2) shows the fitted regression equation Log P$_{DOC}$ = -0.32 + 1.09 log TPP (R^2 = 0.821, P < 0.0001). Full and empty black dots represent data from the NE Atlantic cruises RODA1 and RODA 2, respectively (ATOS1&2). Full black line (RODA1) shows the fitted regression equation Log P$_{DOC}$ = -0.25 + 0.90 log TPP (R^2 = 0.782, P < 0.0001). Dotted grey line (ATOS2) shows the fitted regression equation Log P$_{DOC}$ = -0.24 + 0.87 log TPP (R^2 = 0.652, P < 0.0001)

In addition, when integrating all the dada set from the different oceanographic systems, we found that PER was positively correlated with water temperature ($r_s = 0.29$, n=187, p < 0.0001) and depth ($r_s = 0.32$, n=211, p < 0.0001). PER tended to present higher values at deeper layers, indicating a major release at this low light environment, as described by Marañón et al. (2004). PER grouped by temperature bins of 0.5 °C, showed significant positive relationship with the water temperature ($R^2 = 0.565$, P < 0.0001; Fig. 2). This is in agreement with Morán et al. (2006) who experimentally suggested a regional increase in P_{DOC} with temperature rise in Southern Ocean. Our results, independently of the region, showed that the percentage of DOC production by phytoplankton relative to total primary production (PER) increased with increasing temperature (Fig. 2).
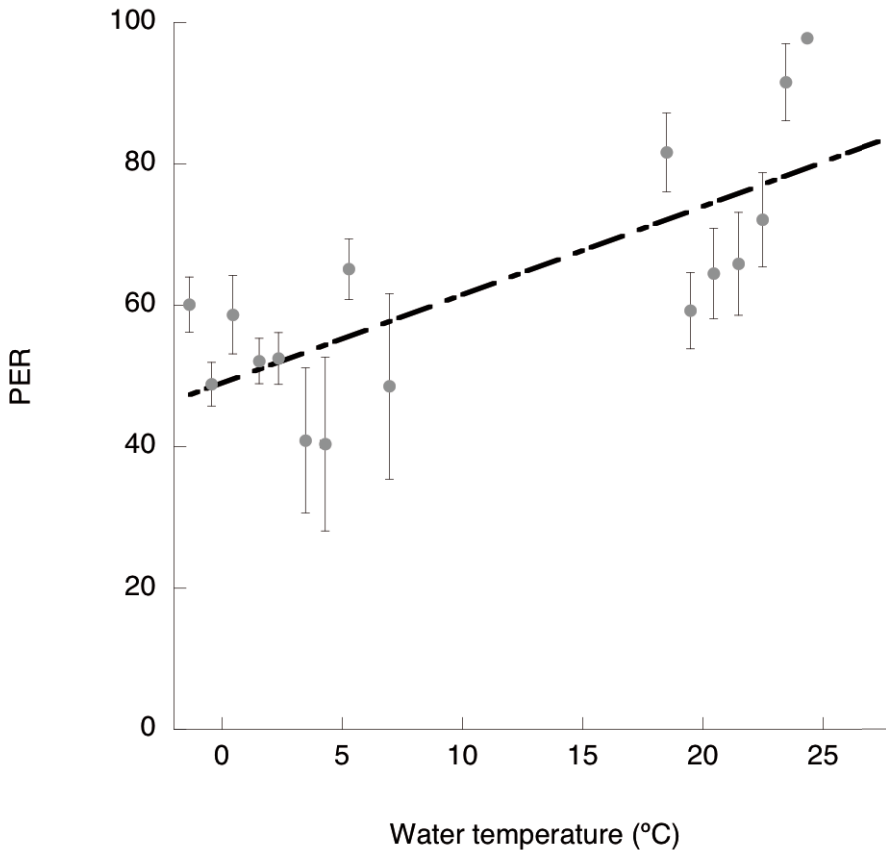

Fig.2. Relationship between the water temperature and the percentage of extracellular carbon release (PER). Percentage data were binned by 0.5ºC unit intervals. Dotted line shows the fitted regression equation PER = 48.9 + 1.24 T (ºC) ($R^2 = 0.565$, P < 0.0001)

The phytoplankton PER variations observed across contrasting regions in the Atlantic Ocean was mostly supported by cell mortality (Chapter 5), and we provided evidence that picophytoplankton mortality, such as that of *Prochlorococcus* sp. and that of the larger phytoplankton community (nano-microphytoplankton) were associated with high P_{DOC} rates depending on water productivity. We then explored whether the percentages of phytoplankton dead cells, independently of the productivity regime of the site of study, explained the changes in PER across communities. We found a significant relationship (Fig. 3) demostrating the mechanistic pathway linking phytoplankton cell death with high extracellular carbon release. The data in figure 3, represent the pooled data of averaged PER and percentages of the nano-microphytoplankton dead cells measured in the stations sampled in Antarctic and NE Atlantic waters (Chapters 3, 4 and 5). We excluded the data from the Arctic Ocean (Chapter 2) because *Phaeocystis Pouchetii* dominated the biomass and the PER release was strongly affected by its capacity to form mucilage, so it was not solely influenced by cell death.
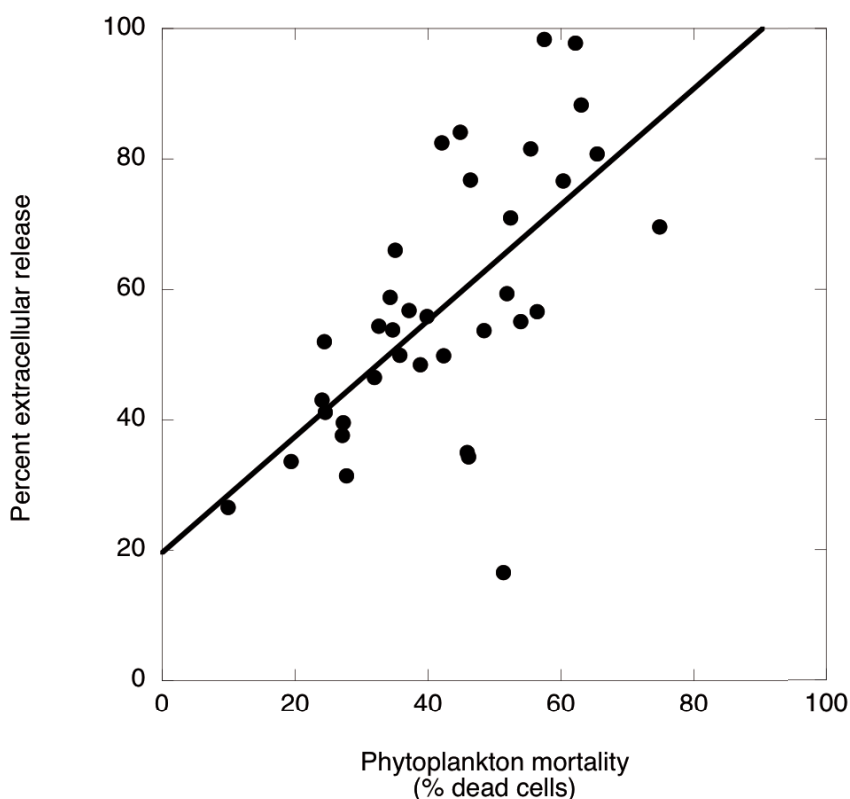

Fig.3. Relationship between phytoplankton mortality (% dead phytoplankton cells) and the percentage of dissolved organic carbon released by phytoplankton. Line shows the fitted regression equation PER = 19.6 + 0.88 %DC (R^2 = 0.414, P < 0.0001)

Studies on DOC released by healthy phytoplankton cells growing in cultures, generally estimated that PER represented a low percentage below 10% of the production in exponentally growing phytoplankton (Sharp 1977, Smith Jr. et al., 1977, Mague et al., 1980, Larsson and Hagström 1982), for which the proportion of dead cells was also very low (Agustí and Sanchez 2002). In this PhD thesis we were able to describe for the first time the relationship between phytoplankton P_{DOC} release and cell mortality for *in-situ* phytoplankton communities. These result identified phytoplankton cell mortality as a process explaining a major proportion of the variation in PER (41.4 ± 3.9 %) in the ocean and confirmed the assumption that dying phytoplankton cells contribute to high DOC release rates (Duursma 1965). The consistent proportion of photosynthate dissolved organic carbon released by dead autotrophic cells imply a leakage in carbon sequestration at the oligotrophic systems and would influence carbon fluxes by contributing to increase the dissolved organic carbon pool, thus favouring the microbial loop (Azam et al., 1983; Legendre and Le Fèvre, 1995).

Control of the bacterial survival success

The P_{DOC} released by phytoplankton across the different systems studied, averaged 54.4 ± 1.5 % of the total primary production (mean ± SE, pooled data), representing a substantial proportion of primary production expected to favour heterotrophic bacteria (HB) growth because the labile photosynthate carbon released by phytoplankton is in fact the preferred carbon source for heterotrophic bacteria (Norrman et al., 1995). Bacterial dynamics and processes (growth efficiency, respiration) have been observed to respond positively to dissolved organic carbon availability in the ocean (e.g Carlson and Ducklow, 1996; Kirchman and Rich, 1997; Herndl et al., 1997). Throughout this study, we confirm the importance of the P_{DOC} substrate to HB, particularly in oligotrophic waters, as observed in NE Atlantic waters (Chapter 5) where bacteria communities have been identified as C-limited (Alonso-Sáez et al., 2007). In this area, we described a strong relationship showing how the proportion of released P_{DOC} from senescent oceanic phytoplankton benefited bacterial survival across the NE Atlantic waters (Chapter 5). When this relationship was analysed for the entire pooled dataset weakened, indicating that other factors than phytoplankton carbon released were important in regulating microbial survival. In fact, heterotrophic bacteria presented similar survival success across the oceanic systems (Table 1), although the factors controlling cell viability was found to vary across systems.

Aside from the availability of P_{DOC} and other organic carbon sources as an important substrate for bacteria, inorganic nutrients could be also act as limiting factor for heterotrophic bacteria growth in marine systems (Kroer 1993; del Giorgio and Cole, 1998). The Mediterranean Sea is characterized by P-limitation increasing to the eastward basin (Krom et al., 1991; Crispi et al., 2001), and we observed there that the survival success of the HB was dependent on phosphate availability (Chapter 1). The last relationship was expected if we consider the influence of phosphate on bacterial production and growth in the oligotrophic waters of the Mediterranean Sea (Thingstad et al., 1998; Van Wambeke et al., 2002) but we emphasized for the first time the importance of such substrate on bacteria cells viability.

We also found a significant effect of water temperature on the regulation of HB survival. As increasing temperatures are expected to positively affect bacterial community dynamics and processes such as production and respiration (White et al., 1991; López-Urrutia et al., 2006; Pomeroy and Wiebe, 2001), an opposite relationship was observed at the Mediterranean waters, although this negative relationship was attributed to the increased availability of phosphorus that presented higher concentrations at cold waters. Across the different chapters of this study, the heterotrophic bacteria examined were exposed to a broad range of temperatures (-1.85 to 28.1 ºC) in the different oceanic systems studied and when examined all the data set together we found a significant relationship between the percentage (averaged by temperature ranges) of living heterotrophic bacterial cells and water temperature (Fig. 4), indicating the increase in bacterial viability with the increase in water temperature ($R^2 = 0.319$, $P < 0.001$) across systems. This result suggests a positive effect of temperature on bacterial cell health status, despite the local adaptations to in situ temperature growth of the populations.
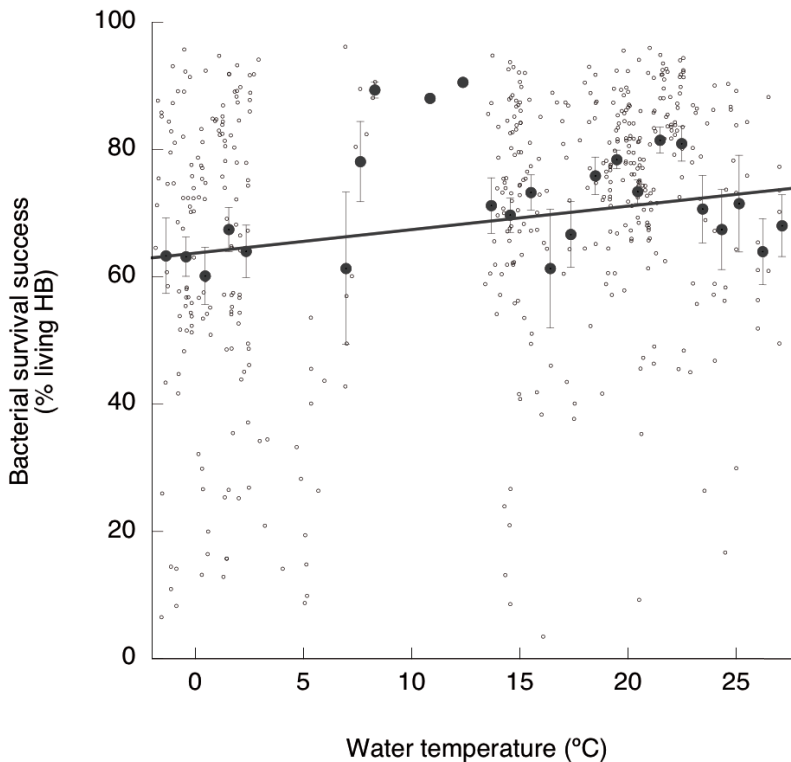
Fig.4. Relationship between water temperature and bacterial health status. Line shows the fitted regression equation. %HLB = 63.75 + 0.37 %DC (R^2 = 0.319, P < 0.0001)

In light of global warming concerns, a recent paradigm has emerged to describe the response of the bacterial community to temperature. Following the metabolic theory (Brown, 2004), increase in water temperature should result in higher bacterial growth and metabolic activity. In their review, Kirchman et al. (2009) verified the consistency of the relationship and found that only at waters bellow 4ºC bacterial growth significantly increased with the increase in water temperature. However, a general pattern relying bacterial community growth and water temperature remained elusive because, as discussed by Kirchman et al. (2009), other limited resources (such as inorganic nutrients or organic carbon) constrained bacterial growth. These factors that co-varied with temperature may themselves have a larger effect on bacterial properties than temperature alone (Lomas et al., 2002). We, however, were able to identify that changes in water temperature significantly explained 31.9 % of the variability in the percentage of heterotrophic living bacteria, indicating that examine in situ bacterial cell death constitutes an alternative and successful approach to identify bacterial response to environmental factors.

In addition to the increasing bacterial production and respiration expected with warming (e.g. Sarmento et al., 2010), the described increasing bacterial viability associated to the increasing water temperature in this study, would suggest the increasing role and importance of the heterotrophic bacteria (HB) and its associated processes in the recent global warming scenario. Moreover, the released of dissolved organic carbon by phytoplankton cell death, significant preferred C-source for heterotrophic bacteria, likely tend to increase with increasing water temperature and would thus reinforce the intensification of the carbon channelling through the microbial loop.

Besides, water warming would disturb the autotrophic population structure and dynamics as it is expected the increasing of small forms abundance and viability in detriment to larger forms (diatoms) and consequently would affect carbon fluxes by weakening the carbon sequestration.

In this PhD Thesis, by analysing the competitive and survival success of different natural planktonic populations under distinct environmental conditions, we illustrated the complexity of the relationships between the different planktonic components and we were able to identify the degree of coupling between the distinct processes that rule planktonic communities and associated carbon fluxes, that we summarize in scheme 1.
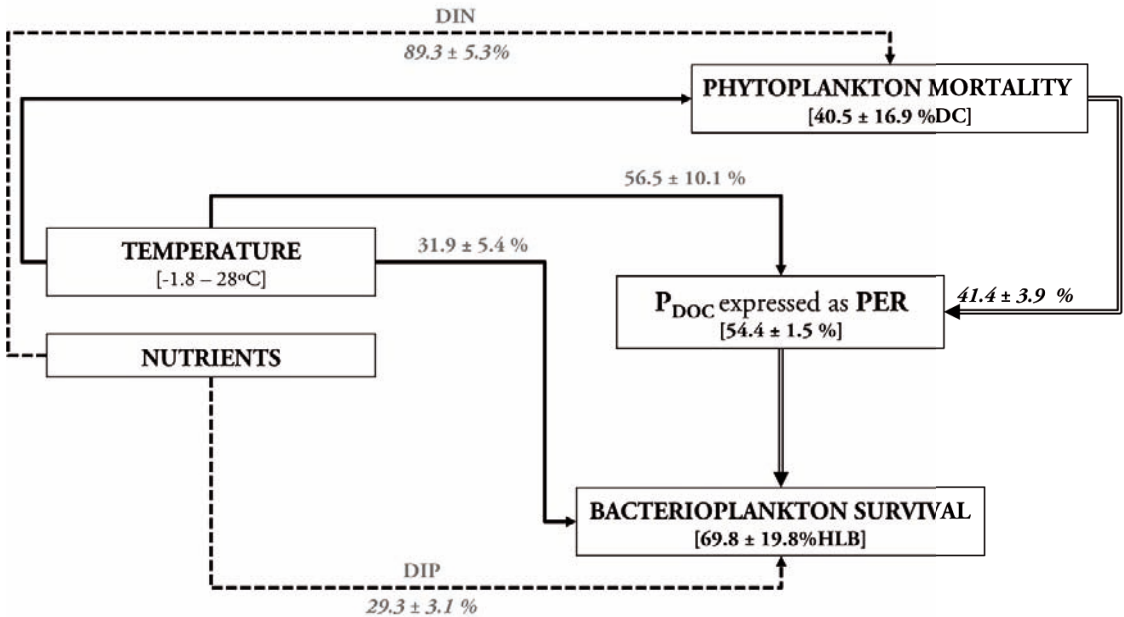
Scheme 1: Summary of the major environmental factors stressing oceanic phytoplankton and bacterial natural populations and influencing the fluxes of carbon.

Numbers in **black bold** represent the averaged percentages (± SE) of Phytoplankton dead cells (%DC), PER and Heterotrophic living bacteria (%HLB) measured along the study. Numbers in ***black italic*** represent the percentage (± SE) of the variability of PER explained by Phytoplankton cell death.

Double line arrows correspond to the phytoplankton to bacteria carbon path. Single and dotted line arrows represent the influence of temperature and nutrients, respectively.

Numbers in **grey bold**, represent the percentages (± SE) of the variability of PER and bacterioplankton survival explained by Temperature. Numbers in *grey italic* represent the percentages of the variability of Phytoplankton cell death (especially here *Prochlorococcus* sp.) and bacterioplankton survival explained by nutrients (DIN: Dissolved inorganic nitrogen; DIP: Dissolved inorganic phosphorus).

As example, at the Mediterranean Sea (MS), DIN significantly affected *Prochlorococcus* sp. mortality as the variation in DIN concentrations explained 89.3 ± 5.3% (at the MS stations) of the variation in percentage of *Prochlorococcus* sp. cell death. Respectively, DIP positively affected bacterioplankton survival, especially at the Mediterranean Sea, where variation in phosphate concentration explained 29.3 ± 3.1% of the variation in percentage of heterotrophic bacteria.

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Conclusions

1- By quantifying the percentage of living and dead cells, we identified in the Mediterranean Sea, phosphate availability as major stressor determining the survival success of heterotrophic bacteria , dinoflagellates and *Synechococcus* sp#2.

2- Two species of *Synechococcus* spp. (sp#1 & sp#2) were identified across the waters of the Mediterranean Sea, presenting distinct vertical distribution and thermal preferences as sp#1 displayed higher percentage of living cells (%LC) at cold waters contrasting with the viability of sp#2 increasing at warmer waters.

3- Temperature positively influenced *Prochlorococcus* sp. survival success in the Mediterranean Sea.

4- Stratification in the Mediterranean Sea favoured small flagellates and dinoflagellates, but negatively influenced diatoms by inducing increased cell mortality.

5- In the Arctic Ocean, during the ice-melting record of the summer of 2007 phytoplankton community was exceptionally 98% dominated by prymnesiophyte *Phaeocystis pouchetti*, although the increased load of ice-melt waters tended to weaken *P. pouchetti* cell viability and exclude diatoms.

6- In the Antarctic Peninsula, changes in the plankton community structure and cell survival were strongly related to the water temperature. Higher temperature increased the survival success of the auto and heterotrophic picoplankton, and the contribution of the Cryptophytes *Cryptomonas* sp. to total phytoplankton biomass.

7- In the Antarctic Peninsula, the survival success of the large phytoplankton (diatoms and dinoflagellates) was associated to cold and stratified water conditions, supporting higher production.

8- Warming in the Antarctic Peninsula favoured a community-shift to small forms, resulting in the dominance of *Cryptomonas* sp. and supporting higher dissolved organic carbon production (P_{DOC}).

9- At the vicinity of the Canary Archipelago in the NE Atlantic Ocean, the upwelling stream at the cyclonic eddies, enhanced nutrients inputs generating favourable conditions for the phytoplankton community as indicating by lower diatoms cell mortality. Conversely the deprivation in nutrients and intensified water mixing, triggered by the active downwelling at the anticyclonic eddies, determined stressing conditions for phytoplankton communities, inducing higher cell mortality than at cyclonic and FF conditions.

10- At the anticyclonic eddies, the increase in phytoplankton cell mortality and lysis resulted in consistent release of dissolved organic carbon (P_{DOC}) to the medium. Heterotrophic bacteria benefited from the larger production of P_{DOC} at AE although their survival success appears to rely more on inorganic nutrients (phosphorus, ammonium) than organic carbon, suggesting inorganic nutrients limitation.

11- At the subtropical Atlantic, along a wide gradient in nutrients concentration, from ultraoligotrophic subtropical Atlantic waters to nutrient-rich NW African upwelled waters, we were able to identify strong relationships between phytoplankton mortality, dissolved organic carbon production by phytoplankton and carbon flux to heterotrophic bacteria.

12- The phytoplankton survival success of the nano-microphytoplankton and picophytoplankton presented contrasting variations from upwelled-productive conditions to oligotrophic waters, as indicated by higher diatoms mortality at the oligotophic waters, where higher production of P_{DOC} relative to total primary production (PER) was found. In contrast, *Prochlorochoccus* sp. presented higher mortality at the nutrient-rich waters of the upwelling area.

13- Phytoplankton mortality significantly explained the variation in P_{DOC} (expressed as PER) at the subtropical Atlantic Ocean.

14- The cell survival of HB in the subtropical Atlantic Ocean appears determined by phytoplankton extracellular carbon release (PER), presenting higher survival at the oligotrophic waters where higher available P_{DOC} was encountered.

15- The proportion of dead natural phytoplanktonic cells in the marine systems studied reached a global average of 40.5 ± 16.9 %, with nutrients availability (such as phosphorus or nitrate) and water temperature identified as key determinants of their survival success.

16- The relative proportion of the total primary production released as dissolved organic carbon by phytoplankton averaged 54.4 ± 1.5 %. The cell mortality occurring in phytoplankton community under natural or stressing environmental conditions explained 41.4 ± 3.9 % of the PER variations consistently representing one of the process implicated in the production of dissolved organic carbon.

17- The large P_{DOC} released by phytoplankton could benefit bacterial survival but do not suffice to explain its general variability. This relationship was stressed at the Atlantic waters, where carbon is limiting the bacterial community.

18- Water temperature has been identified as a strong key determinant for the heterotrophic bacterial survival, changes in water temperature across the contrasting oceanic areas explained 31.9 ± 5.4 % of the variation in percentage of living heterotrophic bacteria.

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