BACTERIAL GROWTH CONTROL IN MARINE ENVIRONMENTS

FROM EXPERIMENTAL APPROACHES TO COMPARATIVE ANALYSES

Luis Antonio Cuevas

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To Judith

to whom with the memories of her smile has removed my fears

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Abstract

To understand (1) the role of heterotrophic bacteria in the biogeochemical cycles, (2) the fate of the organic carbon and mineral nutrients, and (3) the flow of energy to higher trophic levels, we need to understand how bacterial growth is controlled in marine environments. The main hypotheses about the control of bacterial growth are based on the studies about bottom-up control (limiting resources) and top-down control (predators and viruses), that is, how bacteria interact with their 'neighbors' in the microbial food web. Using micro- and mesocosm experiments, comparative analyses, plus an idealized model to test different predictions, this thesis evaluates the role of heterotrophic bacteria in the utilization of organic matter in different marine environments. The effects of mineral nutrients, organic carbon and predator control over bacterial growth are also investigated. Bacterial growth rates can be controlled by carbon or/and mineral nutrients depending on the biological oceanographic conditions (**Papers I and II**). Both types of bacterial growth limitation (carbon and mineral nutrient) can co-exist simultaneously depending on the structure of the microbial food web, mineral nutrient and labile organic carbon concentration. Dominance of nano-phytoplankton, which can be grazed by rapidly responding micro-zooplankton (e.g. idealized model in **Paper IV**), will shifts more rapidly to a carbon limited bacterial growth, thus, heterotrophic bacteria may use all labile organic carbon preventing its accumulation in the euphotic zone (**Paper I**). Contrary, dominance of large diatoms, which can be grazed by slowly responding copepods, can keep labile organic carbon produced by copepod (e.g. sloppy feeding) and bacterial growth can be limited by mineral nutrients (i.e. competition of nutrients with diatoms) (**Paper II**) shifting the system slowly to carbon limited bacterial growth control. Different rates of organic carbon utilization by bacteria can be estimated in manipulated mesocosm experiments by adding mineral nutrients (i.e. silicate, ammonium and nitrate) and by the effect of supply

ratios of glucose-C to mineral nutrients ratio. Total organic carbon accumulation rates are influenced by the dominant phytoplankton communities and by the availability of glucose for bacteria and phosphate for bacteria and phytoplankton. Low availability of mineral nutrients can reduce the bacterial capacity to consume degradable organic carbon (**Paper III**). Investigating the effect of carbon and mineral nutrients on the fate of organic carbon, it seems possible to explain the responses of the system using a 'simplest possible' idealized food web model (**Paper IV**). Thus, different system attributes (e.g. flexible stoichiometry and predatory processes), can affect the dominant nutrient pathway and speed the nutrients transfer in different cases (**Paper IV**). In a global perspective, the variation in the slope (k) and the intercept (β) of the relationship between bacterial productivity and biomass can change observing contrasting trophic ocean basins. Variation in these two parameters (k and β) implies an effect on bacterial growth rates across a gradient of productivity (**Paper V**). Using micro- and mesocosm experiments as well as comparative analyses, can be possible to improve our understanding on the role of heterotrophic bacteria by adding *ad hoc* assumptions to the basic model structure and by looking the global regulation on bacterial growth. Is also necessary to take in account that several aspects complicate the idealized view of the microbial food web, being an enormous task to keep a minimum of interactions to produce a simple conceptual model with prediction power.

List of publications included in this thesis

The thesis contains the following articles. They will be referred using their roman numeral in the text.

PAPER I — **Cuevas LA**, Egge JK, Thingstad TF, Töpper B. Organic carbon and mineral nutrients limitation of oxygen consumption, bacterial growth and efficiency in the Norwegian Sea. Submitted to *Polar Biology*

PAPER II — Vargas C, **Cuevas LA**, González HE, Daneri G. (2007) Bacterial growth response to copepod grazing in aquatic ecosystems. *Journal of the Marine Biological Association of the United Kingdom* 87, 667-674

PAPER III — Cuevas LA, Tanaka T, Thingstad TF, Børsheim KY, Egge J, Skjoldal EF, Thyrhaug R, Töpper B. Effect of supply ratios of glucose-C to mineral nutrients on availability of glucose for bacteria and of phosphate for phytoplankton and bacteria for two arctic mesocosm experiments. Manuscript.

PAPER IV — Thingstad TF, **Cuevas LA**. (2010) Nutrient pathways through the microbial food web: Principles and predictability discussed, based on five different experiments. *Aquatic Microbial Ecology* (in press)

PAPER V — Cuevas LA, Thingstad TF. Global patterns of bacterioplankton dynamics: Relationship between bacterial biomass and production in open ocean regions. Manuscript.

List of other publications

During the period of my PhD work I have also contributed to the following publications in marine microbial ecology.

Published and submitted articles

1 - Molina V, Morales CE, Farias L, Cornejo M, Graco M, Eissler Y, **Cuevas LA**. Contribution of planktonic components to nitrogen regeneration in the coastal area off central-southern Chile during non-upwelling conditions. *Progress in Oceanography* (submitted)

2 - Eissler Y, Letelier J, **Cuevas LA**, Morales CE, Escribano R. (2010) The microbial community in the coastal upwelling system off Concepcion, Chile, 36^oS, 2002-2003 period. *Rev Biol Mar Oceanog* 45(1), 1-18

3 - González HE, Calderón MJ, Castro L, Clement A, **Cuevas LA**, Daneri G, Iriarte JL, Lizárraga L, Martínez R, Menschel E, Silva N, Carrasco C, Valenzuela C, Vargas CA, Molinet C. (2010) Primary production and plankton dynamics in the Reloncaví Fjord and the Interior Sea of Chiloé, Northern Patagonia, Chile. *Marine Ecology Progress Series* 402, 13-30

4 - Pantoja S, Rossel P, Castro R, **Cuevas LA**, Daneri G, Córdova C. (2009) High microbial degradation rates of small peptides and amino acids in oxygen depleted waters. *Deep-Sea Research II* 56(16), 1019-1026

5 - Montero P, Daneri G, **Cuevas LA**, González HE, Jacob B, Lizárraga L, Menschel E. (2007) Productivity cycles in the coastal upwelling area of Concepcion: the importance of diatoms and bacteria in the flux of organic carbon. *Progress in Oceanography* 75, 518-530

6 - Vargas C, Martínez R, **Cuevas LA**, Pavez M, Cartes C, González HE, Escribano R, Daneri G. (2007) The relative importance of microbial and classical food webs in a highly productive coastal upwelling area. *Limnology and Oceanography* 52(4), 1495-1510

Book Chapters

1 - Alder VA, **Cuevas LA**, Franzosi C. (2009) Picoplancton. In: Morales CE, Alder VA (eds) Manual de métodos para el estudio de sistemas planctónicos marinos. Eudeba, Buenos Aires, p. 27-64 (In Spanish) ISBN: 978-950-23-1663-5

2 - **Cuevas LA**, Alder VA, Santoferrara LF. (2009) Nanoplancton. In: Morales CE, Alder VA (eds) Manual de métodos para el estudio de sistemas planctónicos marinos. Eudeba, Buenos Aires, p.65-94 (In Spanish) ISBN: 978-950-23-1663-5

Main Definitions

Anabolism: reaction that utilize energy e.g. in the form of adenosine triphosphate (ATP), to synthesize molecules. Anabolism metabolism results in increase of biomass and storage products.

Bacterial growth efficiency (BGE) or yield: ratio of biomass produced to substrate assimilated or the quantity of biomass synthesized per unit of substrate assimilated.

Bacterial growth rate (BGR): is the increase in cell number per time units, can also involve synthesis of bacterial proteins (e.g. leucine incorporation) and cell division (e.g. thymidine incorporation) between others, per time units.

Bottom-up control: resource limitation influences the consumer and the consumer's predator (control over growth or population size), and so on, up to the food chain. Nutrients and energy sources can be included as main resource factors.

Catabolism: reaction that release energy e.g. in the form of ATP, to break down large molecules (e.g. polysaccharides, lipids, nucleic acids and proteins) into small units (e.g. monosaccharides, fatty acids, nucleotides and amino acids)

Heterotrophic bacteria (also mentioned as bacteria in this thesis): applies to *Archaea* as well as those prokaryotes in the *Bacteria* domain.

Limitation factor: factor that controls a process, organism growth, species population, size or distribution. In marine environments, the availability of food and predation pressure are examples of factors that could be limiting for an organism.

Microbes: all organisms smaller than about 100 μm, which can be seen only with a microscope. These organisms include autotrophs, heterotrophs, and mixotrophs, and refer to both prokaryotes and eukaryotes (e.g. bacteria, archaea and protists or single-celled eukaryotes)

Microcosm experiment (or bottle experiments): a self-contained miniature and representative world that have analogies to a large system, which allows the study of closed systems in a

small sample volume (usually below 5 liters). Natural systems, nutrients and/or predator/viruses manipulation are usually tested.

Mesocosm experiment (or tank experiments): experimental system that simulates real-life conditions as closely as possible, and allows the manipulation of environmental factors (e.g. nutrients, light conditions, etc) in a large sample volume (e.g. above to 100 liters).

Osmotroph: microbes that feed on dissolved nutrients

Phagotrophs: microbes that eat particles

Specific affinity (α): Volume of water cleared for substrate per unit of biomass and per unit of time.

Steady state: Growth occurs at a constant rate and all culture parameters remain constant (e.g. culture volume, pH, cell density, concentration of dissolved oxygen, nutrients and products). In addition, the experimenter can control experimental conditions.

Top-down control: cascading effects of predators controlling their prey (control over biomass), which again may control their prey, and so on, down the food chain. Factors included are predation or viral lysis.

Abbreviations and Units

k: slope of the relationship between bacterial production versus biomass

BA: Bacterial Abundance (cells mL^{-1})

BB: Bacterial Biomass (μg C L^{-1})

BGE: Bacterial Growth Efficiency (no units)

BGR: Bacterial Growth Rate (d^{-1}) also expresed as μ (i.e. specific growth rate)

BP: Bacterial Production (μg C L^{-1} h^{-1})

BR: Bacterial Respiration (μmol O$_2$ L^{-1} d^{-1})

Chla: Chlorophyll-a (μg chla L^{-1})

DGGE: Denaturing Gradient Gel Electrophoresis

DIN: Dissolved Inorganic Nutrients (μM)

DOC: Dissolved Organic Carbon (μM C)

HNA: High nuclei acid content bacteria

β: intercept of the relationship between bacterial production versus biomass

LNA: Low nuclei acid contect bacteria

PCR: Polymerase Chain Reaction

PFT: Plankton Funtional Type

TOC: Total Organic Carbon (μM C)

1. Introduction

1.1 Importance of microbes in marine environments.

The world oceans cover around 70% of the Earth surface and only a minor percentage has been studied, leaving a large unexplored area. It is responsible for about 50% of the global primary productivity and seems to absorb 25% of the present carbon dioxide emissions from fossil fuel combustion (Canadell et al. 2007). In addition, the ocean seems to be a less complex system in term of physical barriers compared to terrestrial systems.

Primary production in the ocean is dominated by unicellular phytoplankton, leading to an ecosystem where not only organic matter degradation is driven by microbes and their interactions, as in terrestrial ecosystems. In terms of marine microbes, early studies (e.g. Krogh 1934) predicted the presence of a 'microbial loop' in marine environments with a late recognition of the key role of microbes in structuring marine food webs and biogeochemical cycles (Pomeroy 1974, Azam et al. 1983). Thus, from the early experiences, studying the whole microbial community, or sometimes using the so-called 'black-box' approach, rather than individual microorganisms has helped to answer fundamental global questions such as, how microbes regulate ocean biogeochemical cycles.

Marine microbes are responsible for 99% of the cycling of the world's gases and nutrients (Gilbert, 2010). In addition, microbes or small picoplanktonic cells (<2 to 3 µm in diameter) can play a very important role in the planktonic community, especially in oligo- and mesotrophic regions of the ocean where they make a large contribution to production, biomass and energy transfer (Stockner 1988). Mentioning only two important biogeochemical processes of global significance, heterotrophic bacteria can hold most of the planktonic

biomass (del Giorgio & Gasol 1995, Gasol et al. 1997), and in terms of activity, they are often considered to be responsible for the net heterotrophy of many aquatic systems (del Giorgio et al. 1997, Cole et al. 2000). Interests of knowing the mechanisms of ecological regulation of bacteria (e.i. mechanisms that regulate bacterial abundance and activity) are clearly a major goal in microbial ecology studies. Thus, is important to improve our understanding to predict these variables and to understand what factors (also in temporal and spatial scales) have major impacts limiting bacterial abundance and activity.

1.2 Marine microbial food webs.

Over the past 30 years our vision of the pelagic food web structure has changed considerably. We now view the classical food web as a relative minor component or in some cases restricted in time and space (e.g. hyper-eutrophic systems, spring blooms). The microbial food web consistently present in all marine habitats is based on pico- and nanoplankton sized autotrophs and heterotrophs (also called osmotrophs), which are efficiently grazed by flagellates, ciliates and copepods (also called phagotrophs) (Fig. 1). The addition of the microbial components into the classical food web structure leads to an increase in the number of interactions between these components. Thus, a fundamental question seems to be how much of the system's complexity need to be explicitly represented in a model when the model's goal is predictive power? for example, trying to predict the magnitude of the different pathways of nutrients flow through the microbial food web. Some examples 'out of the general rule' that focus on 'biological details' include mixotrophic protists (Riemann et al. 1995), parasites or small organisms that feed on larger organisms (Rice et al. 1998) and organisms that feed on particles several orders of magnitude smaller than themselves (e.g. appendicularians, King 1982). Contrary, similarities can be more interesting than differences being possible to build understanding from repeating structures and general patterns. Thus, it

is necessary to define minimum requirements in the conceptual model without losing explanatory and predictive power.

A modified version of Fenchel's (1988) conceptual model and a simple build-up in complexity from the classical food web model (mineral nutrients-phytoplankton-zooplankton) is the three pathways model shown in Fig. 1 with a (1) bacterial, a (2) autotrophic flagellates, and a (3) diatoms entry point for the mineral nutrients. Each of these osmotrophs plankton functional types (PFT) has its separate phagotrophs predator, forming three parallels 'vertical' food chain. The three PFT showed as phagotrophs are also connected through the 'horizontal' carnivorous food chain from heterotrophic flagellates via ciliates to copepods. This conceptual model tries to keep microbial food web models as simple as possible for later use in physical/biological systems and biogeochemical predictions (e.g. Legendre & Rassoulzadegan 1995).
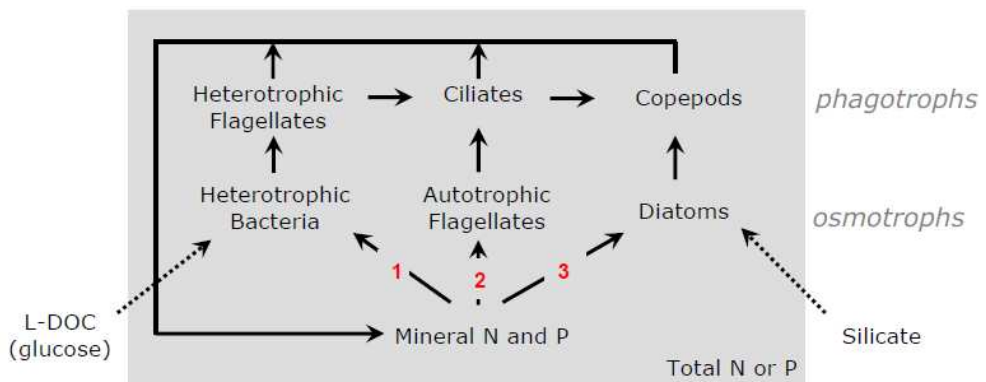

Figure 1. Idealized conceptual model of the microbial part of the food web emphasizing three main pathways for mineral nutrient (red color numbers). Grey area represents steady state conditions in the system.

1.3 Resources and losses processes for heterotrophic marine bacteria.

In the surface ocean the primary source of energy to plankton food webs is sunlight and part of the photosynthetically fixed organic matter and energy can be transferred to the deep sea. The high-energy surface ocean tends to be nutrient poor mainly by the osmotrophs uptake of mineral nutrients, while the low-energy subsurface region of the ocean remains relatively enriched in mineral nutrients manly by the organic mater oxidation in deep waters. Thus, in the upper ocean organic matter production is often limited by the availability of nutrients. The oxidation of dissolved organic matter (DOM) is the main process that heterotrophic bacteria use to obtain energy. DOM is a reduced carbon source that also contains essential mineral nutrients that can support bacterial growth including nitrogen, phosphorous, sulfur and iron. The vast majority of the DOM is biochemically resistant, then it is assumed that reflects its poor nutritional and energetic content (Williams 2000). Bacterial abundances are usually greatest in the upper ocean and in coastal areas, where organic matter fluxes are higher. Generally, in the pelagic ecosystem the DOM production is constrained by photosynthesis, and a variable fraction of the photosynthetically fixed energy (10 to 20%) channels directly to the DOM pool via phytoplanktonic exudation or excretion (Nagata 2000). Other sources of organic matter, like predatory processes such as sloppy feeding and viral lysis can constitute further sources of dissolved organic matter (Nagata 2000, Peduzzi & Herndl 1992).

Mineral nutrients in the euphotic zone of open ocean areas usually range from subnanomolar to micromolar concentrations, however processes such as wind-driven mixing and upwelling results in elevated concentration of mineral nutrients changing the conditions for the osmotrophs communities. Nitrogen (e.g. NO_3^-, NH_4^+) is an essential nutrient for growth and an important source of energy for bacteria, but also show low concentrations in the euphotic zone increasing with depth. In tropical and subtropical regions, NO_3^- is usually found limiting

plankton growth, being a major limitation source for phytoplankton instead of bacteria. Differently, bacteria appear to have efficient NH_4^+ uptake systems, being an efficient competitor for NH_4^+ at the low concentrations found in most oceanic regions. Phosphorous is also an essential nutrient that is compromised in cellular energy storage, membrane structure and genetic exchange. Orthophosphate (PO_4^{3-}) range from nanomolar to micromolar concentrations in the upper ocean and also decrease with depth like other mineral nutrients. Both Mediterranean and Sargasso Sea have been recognized as areas of low PO_4^{-3} concentration suggesting that PO_4^{3-} availability can limit bacterial growth (Cotner et al. 1997, Thingstad et al. 2005a)

Bacterial loss processes are mainly driven by grazers and virus lysis. The remarkably constant number of bacteria in pelagic environments ($\sim 1 \times 10^6$ cells mL^{-1}) and high bacterial growth rates imply that bacterial mortality rates have to be in a similar range as bacterial production. A good correspondence between bacterial growth and grazing loss have been observed that for a wide range of aquatic systems (Sanders et al. 1992) with values of growth and grazing aggregated around the 1:1 line. A large variety of bacterial grazers are possible to find in marine environments, but certainly heterotrophic flagellates (mostly in the size range 2-5 µm) exert a major control on bacterial numbers (Fenchel 1982, Sieburth 1984). Bacterivory has been then an essential estimation for an understanding of the cycling of organic matter in marine environments. Marine viruses constitute the ocean's second largest biomass exceeded only by the total bacterial biomass (Hambly and Suttle 2005). Viral infection has a significant influence on bacterial community dynamics, diversity and nutrient cycling. It has been estimated that viruses are responsible for about 10-50% of the total bacterial mortality in surface waters (see review by Furhman 1999). Bacterial death mediated by viruses produced

more DOM for bacterial consumption, and increase the cycling rates of carbon and mineral nutrients in marine environments (Furhman 1999).

1.4 Competition between osmotrophs.

The idealized food web in Figure 1 emphasizes the osmotrophs versus phagotrophs division, rather than the more traditional autotrophs vs heterotrophs division of the microbial food web. This places the bacteria as potential competitors to the phytoplankton, a view quite different from the classical concept of bacteria as remineralizers. Bacteria obtain most of their nutritional and energetic requirements through active transport of inorganic and organic substrates and they are also uniquely involved in both taking up and releasing mineral nutrients. Contrary, phytoplankton are sink for mineral nutrients whereas heterotrophic protists (heterotrophic flagellates and ciliates in Fig. 1) prey on bacteria and small phytoplankton. In addition, while phytoplankton acquires carbon (C) from inorganic compounds when obtaining energy from light, heterotrophic bacteria acquire C from organic material, especially in its dissolved form. However, the biochemically characterized fraction of DOM (e.g. monosaccharide, amino acids) can account for less than 15% of the total pool in marine waters (Benner et al. 1992) and little is known about the utilization of different components of the DOM pool.

Heterotrophic bacteria appear to account for a large fraction of orthophosphate (PO_4^{3-}) uptake both in the oceans and in freshwaters (Kirchman 1994). The median percentage of PO_4^{3-} uptake attributable to bacteria is 60% when both freshwater and marine systems are considered, but the percentage varies greatly among diverse ecosystems. In terms of N, there are two important sources for bacteria and phytoplankton growth: ammonium (NH_4^+) and nitrate (NO_3^-), respectively. Ammonium uptake by bacteria has been examined in more

details compared to other N sources (e.g. nitrate and urea). Similar to PO_4^{3-}, ammonium uptake by bacteria varies greatly from 5−78% of total uptake (Fuhrman et al 1988, Kirchman 1994). The overall median is about 40% which is two-fold higher than maximum expected percentage, assuming a BP:PP ratio of 0.2 and similar C:N ratios for bacteria and phytoplankton (Kirchman 2000).

Competition between microbes for the same substrate has been linked to cell size (Thingstad et al. 2005b). Because of low surface area to volume ratios, heterotrophic protists are at disadvantage in competing with smaller microbes such as heterotrophic bacteria and many small phytoplankton species (e.g. cyanobacteria) for dissolved compounds. In addition, utilization of NO_3^- as a nutrient source is energetically demanding, requiring intracellular reduction to NH_4^+ prior to assimilation (Vallino et al. 1996). Thus, NH_4^+ usually accounts for relatively high N uptake compared to NO_3^- uptake is small size cells like bacteria, pico- and nano-phytoplankton (Lipschultz 1995). In the Barents Sea, for example, 53% of the NH_4^+ uptake has been attributed to organisms <0.8 µm (Kristiansen et al. 1994).

In pelagic carbon and nitrogen models, the role of heterotrophic bacteria in the consumption of dissolved inorganic nutrients has been seldom considered (Fasham et al. 1990). In order to make reliable conceptual models it is therefore needed to answer how uptake of P and N from the DOM-pool is distributed between bacteria and phytoplankton (Fig. 1)

1.5 Bacterial growth rates and efficiency.

The study of bacterial growth is directly linked to the ability of estimate bacterial abundance (and converted to biomass) as well as production rates. The development of new methods for

assessing both variables is critical to improve our understanding of bacterial growth control in marine environments.

To define bacterial growth rate it is necessary to define bacterial production (or secondary production) as the synthesis of bacterial biomass, primarily from organic sources (e.g. dissolved organic carbon) and mineral nutrients, transferring the organic matter from one pool to another as net effect. Thus, bacterial production (BP) can be expressed as the rate of synthesis of cells or bacterial biomass (BB):

$$BP = \mu BB \qquad (1)$$

where μ is the specific growth rate of the bacterial population expressed in units of inverse time (e.g. d^{-1} or h^{-1})

$$\mu = \frac{1}{BB}\frac{dBB}{dt} \qquad (2)$$

In most of the studies reported, BP is not derived through direct measurements of μ and BB using equation 1. Thus, the determination of *in situ* values of μ still remains ambiguous. The straightforward method to estimate bacterial growth rate has been to give values to the right-hand terms in equation (2) but this approach shows problems with the net changes in BB. In practice, it is more common to measure bacterial production and biomass, and calculate bacterial growth rate (μ) from equation (1) as the ratio between bacterial production and biomass. Thus, measuring bacterial production and biomass remains our best approach to obtain large data sets and accurate measurements of bacterial growth rates that can be used to parameterize food web models (Ducklow 2000).

In dilute environments as marine waters, bacterial communities may have an enormous catabolic versatility and flexibility, which is probably the key to survival and growth in oligotrophic environments. The adaptation of bacteria to dilute and variable conditions is of great advantage in the utilization of the available energy, so that in any given set of environmental conditions, survival and growth can be maximized (Morita 1997). The distribution of carbon into anabolic and catabolic processes results in bacterial growth efficiency (BGE). By definition BGE (or yield) is the quantity of biomass synthesized (or BP) per unit of substrate assimilated (A) where A is the sum of BP and bacterial respiration (BR). Thus BGE can be expressed as follow:

$$BGE = \frac{BP}{A} = \frac{BP}{(BP + BR)} \qquad (3)$$

BGE is a fundamental attribute for microbial metabolism, which largely determines the ecological and biogeochemical role of bacteria in microbial food webs and in aquatic ecosystems (del Giorgio & Cole 1998). The measurements of bacterioplankton growth efficiency are still a challenge for marine microbial ecologist because direct measurements of substrate consumption can seldom be made at realistic time scales, and metabolic rates are often extremely slow (Søndergaard & Theil-Nielsen 1997). Early studies follow the uptake, incorporation and respiration of simple radiolabeled compounds, allowing measure rates of uptake and respiration in short incubations with high sensitivity (Crawford et al. 1974). But during this short incubation times, the intracellular carbon pool often do not attain equilibrium, with the result that BR is underestimated and BGE overestimated. In addition, the single-model compounds may not be representative of the range of substrates utilized by heterotrophic marine bacteria. This approach has largely been replaced measuring BGE using the *in situ* pool of organic matter. For example, BGE can be calculated from the rate of decline in substrate (DOC) and the rate of increase of bacterial biomass (POC) using days or weeks as incubation time. Another approach using the *in situ* pool of organic matter is the

simultaneous measurements of BR and BP in relatively short (<36 hours) incubations. Several difficulties can be observed using this approach; mainly considering long extra incubations to obtain measurable changes in O_2 consumption or more rarely, as CO_2 production. In addition, the physical separation of bacteria from other planktonic components (range of filtration from 0.6 to 2 µm) may disrupt the structure of the bacterial assemblage and the supply of substrates. In that sense, there is still uncertainness in the estimation of BGE that needs to be considered for the interpretation of microbial metabolism and their biogeochemical role in aquatic ecosystems

1.6 Global relationships in marine microbial ecology.

The discussion of BGR control in microbial food webs is often centered on the concepts of 'bottom-up' and 'top-down' control (e.g. Billen et al. 1990, Fig. 2). The criterion that growth must balance loss for any component in steady state means that bottom-up and top-down forces in the sense must be of exact equal importance, at least when considered over sufficiently long time scale.

Suspecting the key role of heterotrophic bacteria in marine food webs (Pomeroy 1974, Azam et al. 1983) there has been a massive campaign to develop new methodologies and techniques that lead to an increase in empirical databases in marine environments. At the same time, questions addressing the relative importance of bottom-up and top-down processes have been approached by the use of conceptual and mechanistic models (Fasham et al. 1990, Anderson & Ducklow 2001) and comparative ecosystem analysis (Gasol & Duarte 2000). Most of comparative ecosystem analyses are used to define relationships between two ecosystems properties or processes through assessment of a statistical relationship. The resulting

relationship can be used to define a predictive range of variability between two ecosystem properties (Gasol & Duarte 2000) within the euphotic zone or in general in the surface ocean.

Nowadays, it is possible to find a number of syntheses studies that identify the comparative analysis of marine bacteria. One of the most robust trends identified from comparative analyses across diverse aquatic ecosystems is that BA generally shows a positive relationship to chla (Bird & Kalff 1984, Cole et al 1988, Ducklow & Carlson 1992, Gasol & Duarte 2000). The power slope of the relationship between BA versus chla has been used to identify two general trends: (1) the upper limit of the bacterioplankton biomass is controlled by the availability of phytoplankton derived sources (Li et al. 2004), and (2) the bacterial biomass becomes more important (higher percentage compared to phytoplankton) in ecosystems where autotrophic biomass is low (Gasol et al. 1997).
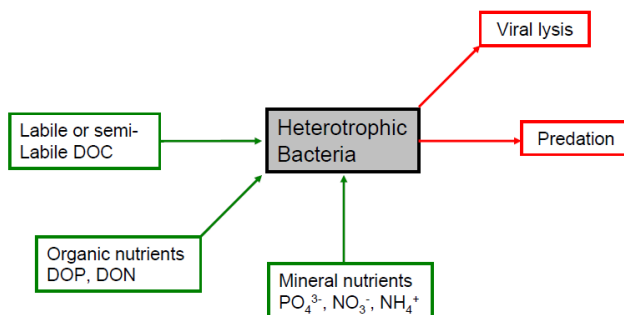

Figure 2. Interaction between heterotrophic bacteria and their 'neighbors' in the microbial food web (modified from Thingstad, 2000). Mineral and organic nutrients and DOC as examples of bottom-up factors (green compartments). Predators and viruses as examples of top-down factors (red compartments).

Comparative studies have also been used to assess the relationship between rates of BP and PP (both volumetric and areal relationships). It has been assumed that this relationship reflects the degree of coupling of bacterial growth and PP. Over large time and space scales, the variation of PP appear mirrored in BP, thus BP is assume to be equivalent to 10-30% of PP (Cole et al 1988, Ducklow 1999).

In comparative studies of data from a large number of systems, bottom-up resource availability for bacterioplankton is commonly identified as the main factor controlling bacterial numbers (e.g. Gasol & Duarte 2000). It is well known, however, that protozoan's have the potential for reducing bacterial abundance and even to occasionally create Lotka-Volterra-like oscillations between bacteria and predator abundance (Fenchel 1982, Azam et al. 1983, Tanaka et al. 1997). In addition, several analysis of mechanistic models predict that BP is controlled by the rate of nutrient supply (i.e. bottom-up control) while final abundance and BGR can be determinate by predation pressure, by substrate supply or by both control modes (Pace & Cole 1994, Thingstad & Lignell 1997). Thus, more nutrient availability would mean more biomass and production, and more predators could mean less biomass or activity. So far, there are no clear conclusions about which mode of control (bottom-up or top-down) is more important in determining bacterial abundance, production and growth.

By the comparison of bacterial growth and loss rates it seems possible to answer the question of weather bottom-up or top-down controls predominate in different oceanic regions. If there is a tendency for values of growth to be 'uncoupled' from those of losses, would be possible to discover either the dominance of bottom-up processes (loss would be below of those of growth) or the dominance of top-down processes (if losses tend to be higher than growth).

A general conclusion from previous studies is that limiting resources presumably regulate heterotrophic bacteria in most of the eutrophic environments, and organic carbon can be the main limiting factor. There are relatively less studies and maybe more contradictive results on: the effect of mineral nutrients, the competition of osmotrophs for both sources of energy and the effect of predator over heterotrophic bacteria. In that sense, this thesis will fill part of the gap by looking the effects of mineral nutrients and carbon sources on bacterial growth in different experiments as well as the effect loss processes by using comparative analyses.

2. OBJETIVES

The general objetive of this thesis was to improve the understanding on the control of bacterial growth rates in marine ecosystems using 3 diffenrent approaches: microcosm experiments, mesocosm experiments and comparitive analysis. In particular this thesis also focuses on the following 3 especific objetives:

Objetive 1. How marine heterotrophic bacteria are limited by mineral nutrients and carbon sources in coastal and oceanic areas (**Papers I, II and III**)

Objetive 2. How the competition between phytoplankton and heterotrophic bacteria for mineral nutrients regulate bacterial growth in manipulated experiments and predicted from the idealized plankton food web (**Paper III and IV**)

Objetive 3. Which are the main mechanisms (sources or predators) regulating bacterial growth at global scale from oligotrophic to eutrophic ocean areas (**Paper IV and V**)

3. SUMMARY OF RESULTS

This thesis is organized using three methodological approaches by increasing the 'size of the tool', from microcosm experiments, through mesocosm experiment, and up to comparative analysis to attain the general objective. In addition, an idealized conceptual model is used to predict the different nutrient pathways in the microbial food web. The results obtained here begin with the utilization of labile organic carbon and mineral nutrients for bacteria and phytoplankton, including bacteria-phytoplankton interactions as competition, to finally reach global relationships in the control of bacterial growth in different ocean basins. Using this 'gradient' from small scale to large scale approaches, this thesis seeks to extrapolate and improve our understanding on the role of heterotrophic bacteria in biogeochemical cycles.

Papers I and II evaluates the bacterial growth response in ecosystems with different trophic status (i.e. chla concentration, dominant phytoplankton community) and bio-oceanographic conditions. Labile organic carbon (as main limiting factor) and mineral nutrients (co-limiting factor) can control bacterial growth during pre-phytoplankton bloom conditions (**Paper I**). The potential 'fresh' DOM from copepods activity can also play a major role controlling bacterial growth and carbon fluxes through the microbial food web in a coastal embayment and in a oceanic site (**Paper II**).

Paper III investigates the availability of glucose for bacteria, and of phosphate for phytoplankton and bacteria using two mesocosm experiments in Arctic coastal waters. Initial concentrations of ammonium, nitrate, phosphate, total organic carbon (TOC), chlorophyll a, bacterial abundance and turnover times of glucose and phosphate were similar between the two experiments. Despite the similar initial conditions TOC accumulation rates were different between both experiments. In the 2007 experiments, TOC accumulation rate reduced despite

increase of glucose addition when organic-C to mineral nutrients supply ratio was below the Redfield ratio, while TOC accumulation rate increased with increase of glucose addition when the supply ratio was above the Redfield ratio. In the 2008 experiment, TOC accumulation rate increased with the increase of the glucose addition despite the nitrogen source added (ammonium vs. nitrate). The low availability of mineral nutrients reduced the bacterial capability to consume degradable organic carbon in the Arctic coastal waters.

Paper IV describes through a idealized conceptual food web model how limiting nutrients are transferred from the dissolved form through the microbial food web to mesozooplankton. A review of five nutrients addition experiments, and comparing with other published studies, shows that the main system response in all cases seems possible to explain within the framework of the simple model. However, there are different systems attributes, such as flexibility stoichiometry and predatory losses, that can affect the pathway and speed of nutrients transfer in each experiment.

Paper V analyzes a new empirical dataset for bacterial production, biomass and growth rate in different ocean basins and the effect of the trophic status (i.e. chla concentration) on the relationship between bacterial production and biomass. The slope and intercept of the relationship is strongly affected by chla concentration and predator's abundance showing an increase for both parameters from oligotrophic to eutrophic ecosystems. A similar increase was possible to estimated exploring a density-dependent logistic growth model, where a negative relationship between BGR and bacterial abundance suggested that bacteria are resource limited in eutrophic systems. At global scale, from oligotrophic to eutrophic systems bacterial growth seems to be predator limited (top-down control) and resource-limited (bottom-up control) respectively.

4. DISCUSSION

4.1 Simplicity versus complexity in microbial food webs.

The analysis of the microbial food webs is not only restricted to a qualitative level. As an example the use of box models is the great importance to obtain a quantitative level including predictive power. In that sense, the construction of a model that incorporate all the important types of plankton may seem the obvious step further. The debate concerning the feasibility of multiple plankton functional types (PFTs) lead to the discussion of what is needed and what is possible to include (Anderson 2005, Flynn 2005, Le Quéré et al. 2005, Thingstad et al. 2010, **Paper IV**). **Paper IV** tries to explore this problem evaluating if is possible to start with simple conceptual models with feasible analysis and experimental verification, and add new details such as compartments, processes and/or pathways in steps (detailed analysis in Thingstad et al. 2010). As an example, is possible to observe the simplified version of the linear pelagic food chain (nutrients-phytoplankton-zooplankton-fish), and then exchange the phytoplankton link with a microbe link, creating a more complex microbial food web (Fig. 1). The three-pathway model shown in Fig. 1 with a (1) "bacterial", a (2) "autotrophic flagellates", and a (3) "diatom" entry point for the mineral nutrients seems a modest step up in complexity from the linear pelagic food chain. Each of these osmotroph PFTs has its separate phagotroph predator, forming three parallel "vertical" food chains (Fig. 1). The phagotrophs are connected through the "horizontal" carnivorous food chain from "heterotrophic flagellates" via "ciliates" to "copepods".

As mentioned above, a further step in complexity could be the addition of processes and/or pathways in a model perturbed by nutrient additions (e.g. **Paper III and IV**). Consider an experiment characterized by mineral nutrient limited phytoplankton growth rate, when limiting nutrients are added, this will alleviate growth rate limitation, lasting for a period

depending on the ratio between existing osmotroph biomass and the dose supplied. As the nutrients are assimilated and converted into new osmotrophs, the system somewhat paradoxically shifts to increased growth rate limitation since the biomass of osmotroph competitors has increased, but not the rate of recycling. In this state, bacteria will therefore experience high competition pressure for mineral nutrients, driving the system towards mineral nutrient limited bacterial growth. If mineral nutrient stressed phytoplankton excretes organic-C available to bacteria, this will drive the system further towards bacterial mineral nutrient limitation. With high osmotroph biomass, a trophic succession to the phagotrophs would be expected. The effect of this transfer up the "vertical" food chains to the phagotrophs in Fig.1, would be reduced competition as well as increased recycling, and therefore a reduced mineral nutrient stress for the remaining osmotrophs and an increased potential for bacterial consumption of labile dissolved organic carbon (L-DOC). The timing of these shifts in nutrient limitation conditions thus depends on the characteristic time scales for the numerical response in the different phagotrophs. Since is possible to expect a faster numerical response in ciliates than in copepods, this model predicts a phytoplankton bloom in a system dominated by diatoms (Pathway 3) to last longer and reach higher levels than a flagellate-dominated bloom (Pathway 2). From the previous arguments, the consequence is also that the model predicts a prolonged period with mineral nutrient limited bacterial growth if diatoms are present.

Other common complexity step that can be included are: ranges of phytoplankton size classes, genetic diversity in the prokaryotes (Venter et al. 2004) and protist (López-García et al. 2001) communities and combined strategies of heterotrophy, autotrophy and mixotrophy between others. But to focus on how the balance between pathways of nutrients changes with biogeochemical factors such as: total content of a common limiting element, whether bacterial growth is limited by organic carbon or by mineral nutrients, and organism properties as

nutrient uptake affinities in osmotrophs, clearance rates in phagotrophs and yield coefficient would probably lead to a better prediction of experiment outcome (**Paper IV**).

Based on the previous conceptual model (Fig. 1), different tools can be apply to clarify the interaction between marine microorganisms (osmotrophs and phagotrophs) starting from experimental (natural or near-natural) approaches (**Paper I, II, III**) through the idealization of conceptual models (**Paper IV**) and toward the construction of datasets and comparative analysis (**Paper V**). Thus, different time-space scales and questions can be approached using one or another tool. Microcosms experiments (e.g. chemostat laboratory) or 'bottle experiments', can explain interaction between prey and predators or individual utilization of substrates in the scale of hours to days. Mesocosm experiments, leads the possibility to approach community interactions in larger time scales as weeks. And in longer temporal and space scale, comparative analysis leads to answer global patterns, defining relationships between two ecosystems properties or processes (Table 1).

Table 1. Experimental and comparative analysis approaches. Temporal scales and possible microbes' interaction studies.

Approach	Temporal scales	Space scale	Aim of the studies examples
Microcosm experiments	Hours, days	Usually below 5-10 liters	Prey-predator interactions Uptake of substrates Respiration of substrates
Mesocosm experiments	Days, weeks	Usually above 100 liters	Community interactions Effect of environmental factor over community structure and function
Comparative analysis	Short-, long time-series. Inter-annual or decadal changes	From specific sites to large regions (e.g. ocean basins)	Relationships between ecosystem properties or processes Global patterns

The application of these three different approaches can test different microbial processes and interactions. Close interactions and the effect of carbon and mineral nutrients can be tested using 'bottle experiments', while the effect over the microbial community can be observed

under a mesocosm experiments. A macro-ecological approach by the use of comparative analysis will search for statistical patterns between different relationships that can explain, for example, the control of bacterial growth rates at global scale and in specific ocean basins.

4.2 Applying an idealized conceptual model in different manipulated experiments.

The seasonal variability and oceanographic conditions are fundamental features that trigger phytoplankton blooms and therefore different stage of limitation for bacterial growth. In marine environments is possible to find different scenarios depending on the structure of the phytoplankton community, mineral nutrients and labile organic carbon concentrations. A possible scenario in oceanic and coastal environments would be a situation of mineral nutrient replete conditions with low dissolved organic matter concentration (**Paper I**). In an open ocean area with a dominant nano-phytoplanktonic community, mineral nutrient are available for bacteria and phytoplankton utilization leading to bacterial growth limitation by carbon. Under this condition, heterotrophic bacteria may use all labile organic carbon preventing its accumulation in the water column (**Paper I**). Other environments such as coastal areas and embayments are usually dominated by diatoms and large phytoplanktonic cells. The change in the dominant phytoplankton community and its interaction with their predators (copepods, Fig. 1) could increase the organic matter concentration and its liability by processes such as sloppy feeding, excretion and leaching from faeces (Nagata 2000). A different scenario with growth limited by mineral nutrients could be suggested under the conditions described above (**Paper II**).

A more complex situation is possible to observe by adding mineral nutrients (Si, ammonium and nitrate) in manipulated mesocosm experiments at different supply ratios of glucose-C to

mineral nutrients. Different phytoplankton communities will dominate depending on the nutrient added and different responses in the accumulation of TOC will be observe with the availability of glucose for bacteria and phosphate for phytoplankton and bacteria (**Paper III**). It has been observed that silicate additions lead to a dominant diatom community, but differences in the type of diatom bloom can be attributed to the nitrogen source added. Nitrate can induce chain forming diatoms (Havskum et al. 2003), while ammonium can induce small solitary diatom (Thingstad et al. 2008, **Paper III**). The resulting phytoplankton community in silicate deplete system leads to a flagellate bloom expecting a faster response in ciliates (pathway 2, Fig. 1) that in copepods abundance preying over diatoms (pathway 3, Fig. 3). The dominance of small diatoms (e.g. cells < 10µm, Paper III) allow the grazing of the same predator as those grazing on autotrophic flagellates (Fig. 1) reducing the differences in the dynamics of the diatom and flagellate pathways.

The ability to predict these differences in the transfer of nutrient through the food web requires an explanation for the mechanisms leading to small versus large diatoms. Stolte and Riegman (1995) assume that diatoms can store nitrate but not ammonium in the vacuole. Since the vacuole:citoplasm ratio increases with cell size, large diatoms will have a competitive advantage in an environment pulsed with nitrate, but not with ammonium. Contrary to this prediction, our test between ammonium and nitrate additions (see 2008 experiments in **Paper III**) induced only a phytoplankton community dominated by flagellates (1-10 µm). This indicates that nutrients entered the food web through the autotrophic flagellate pathway, with no net growth of bacteria nor diatom, despite the presence of excess Si and independent of the presence or absence of glucose. One possible explanation could be outlined from the predation pressure in the horizontal grazing food chain in Fig. 1. Biomass of

bacteria and diatoms could be controlled by dominant communities of heterotrophic flagellates and copepods respectively (Paper IV).

4.3 Processes involved in the determination of bacterial growth rate and efficiency.

Rates of change of bacterial populations in marine environments are usually underestimated of the actual growth rate because the effect of simultaneous removal of prey cells by grazers (Ducklow & Hill 1985, Ducklow 2000, **Paper I**, **Paper II**) and bacterial lysis by viruses (Weinbauer & Peduzzi 1995, Furhman 2000). Both processes can reduce or balance bacterial growth rates giving the appearance that the bacteria are not growing, similar for phytoplankton grazed by microzooplankton (Landry & Hassett 1982). If unambiguously estimation of bacterial growth rate can be obtained, and relate them to other variables as chla and temperature, then the derive growth rate can be used to estimate bacterial production from equation (1). In manipulated experiments, grazing and viral lysis can be minimized or eliminated completely by dilution with filtered water (usually below 0.8 and 0.2 µm pore size, respectively. **Paper I**). In these experiments, bacterial abundance increases over time, which can provides an estimate of bacterial production and specific growth rates (**Paper I**). The observation of changes in cell numbers or biomass over time is the most direct but perhaps not the easiest way to measure bacterial growth.

It has been establish that bacteria can respond to fluctuating nutrient availability by regulating the number of active transporters used to acquire substrate. Both organic and inorganic substrates concentrations can present very low concentrations (nanomolar to micromolar ranges) in marine environments, suggesting that bacteria growing in low nutrient seawater would favor allocation of cellular energy to acquiring resources at the expenses of growth (Church 2008). In contrast, bacteria growing under higher concentration of substrates might

maximize growth while decreasing affinity (α) for specific substrates, and bacteria growing under limiting nutrients might increase its affinity for substrates while growth might not be limited by the substrate availability (**Paper III**).

The ability of directly measure two fates of a given substrate uptake (e.g. glucose, amino acids, etc) into biomass and respiration offer the possibility of estimating BGE, but only from the incorporation of single added substrates (e.g. Crawford et al. 1974). This approach can be very sensitive and also allowed to estimated BGE in the most unproductive aquatic systems. But, the single model compounds may not be representative of the total range of substrates used by bacteria in marine environments. In addition, using short incubation times the intracellular carbon pools often do not attain equilibrium, with the result that respiration is greatly underestimated and BGE grossly overestimated. Thus, the combination of the short incubations and simple substrates produced BGE data with high values, ranging from 0.3 to 0.8. The use of single labeled compounds has been replaced by measuring BGE utilizing the *in situ* pool of organic matter (see review in del Giorgio & Cole 1998, **Paper I**). Thus, measurements of bacterial production (mainly using thymidine or leucine incorporation) coupled with sensitive methods to estimate bacterial oxygen consumption or DOC utilization, allowed BGE to be estimated without depending on the use of single substrates, and also allowed bigger set of data from *in situ* estimations. Still measurements of BGE can seldom be made at real time scale, and in marine systems the metabolic rates are often extremely low.

A compilation of more than 200 direct measurements of BGE from a variety of natural marine ecosystems showed a large range in BGE, ranging from <0.01 to >0.6, but most values are clustered in the 0.05 to 0.3 range (del Giorgio & Cole 1998). The average values for open-ocean areas (0.15 ± 0.12 SD) are lower than the average value for coastal areas (0.27 ± 0.18 SD). Similar range of BGE was estimated in the Norwegian Sea with *in situ* average values of

0.2 (**Paper I**). These differences in BGE among different marine areas are mainly driven by changes in bacterial production and less variable bacterial respiration among different marine areas.

As a general trend, there is a broad positive relationship between BGE and BGR, mainly because BGR tends to increase on average along gradients of primary production (Cole et al 1988, White et al. 1991). But over small scales the relationship between BGE and BGR does not always hold (del Giorgio & Cole 1998). Thus, at this scale there is not general relationship between BGE and BGR in natural bacterial communities, where the data suggest that BGE may covary with BGR (**Paper I**).

It has often been assumed that BP is a good index of organic matter supply to heterotrophic bacteria (Cole et al. 1988), thus, the hypothesis that bacteria maximize utilization rather than efficiency (BGE suppose to decline towards more oligotrophic areas, where presumably the supply of carbon and energy is slower) can be important to interpreted the current massive measurements of BP in the oceans. Maximizing the rate of utilization would imply that total bacterial carbon consumption (BP + BR) reflects the total amount of organic matter available for bacteria. Although the sum of BP and BR is positively correlated with primary production, it has a much smaller range than BP alone, suggesting that the total amount of organic matter available for bacteria is less variable than previous recognized. Because of the differences in BGE along gradients of productivity, the fate of the C utilized by bacteria varies greatly. For example, the average total carbon flux through bacterioplankton (BP + BR) can differed by three fold between open ocean systems and productive coastal areas (del Giorgio & Cole 1998). Thus, the response of the bacterial assemblages to the different concentration and quality of carbon sources, produced mainly by phytoplankton or zooplankton, can differ between coastal and oceanic areas (**Paper II**). Potential C losses by copepods feeding

activities might be not important in fueling BGR in productive coastal areas (**Paper II**), but the release of available C from phytoplankton would control growth and production (Nagata 2000). The phytoplankton community composition might also regulate bacterial growth observing C-limited bacterial growth in areas with small cell composition (**Paper I**) and bacterial growth control by other sources (e.g. mineral nutrients, predators) in coastal areas where large phytoplankton is dominant (**Paper II**).

An extra problem affecting the estimations of bacterial growth and efficiency is the presence of non-growing cells, producing heterogeneity of population growth rates. If thymidine isotope were used by definition would address the dividing cells. If only part of the total cell population were actively incorporating isotope, the incorporation rates would increase faster than the total cell count (Ducklow 2000). The use of flow cytometry allow us to detected directly bacteria subpopulations (DNA content). In oceanic environments, low nucleic acid (LNA) bacteria are dominant when substrate supply rate are slow (Gasol et al. 1995), while the contrary can be observed for high nucleic acid (HNA) bacteria when substrates (mineral nutrients and labile carbon) are available, showing high variable results (**Paper I**).

4.4 Factors affecting the bacterial consumption of carbon & mineral nutrients.

There is limited knowledge about the processes that control the cycling of DOM and on the different types of DOM compounds in seawater. Thus, the complexity of natural substrates combined with the dilute concentrations makes evaluating the turnover, utilization of individual substrates and subsequence bacterial growth difficult. To approach this problem, it have been necessary to assume first the open ocean as a nutrient-limited environment, where bacterial growth can depends mainly on two processes: (1) the rate that cells transport and assimilate growth-limiting nutrients, and (2) the rate that cells metabolize intracellular substrates. Thus, under steady state conditions, it is assumed that nutrient-limited bacterial

growth depends on the transport rate of the growth limiting nutrients. It is also possible to assume that the dominant groups of pelagic bacteria posses multiple high-affinity transport systems that enable them to simultaneously transport several types of nutrient substrates. Thus, heterotrophic bacteria are able to utilize very low concentrations of inorganic and organic solutes in nutrient deplete oceanic areas. The specific affinity, defined as the volume of water cleared for substrates per unit of biomass and per unit of time (analogous to the clearance rate of phagotrophic organisms), can be a good index to measure competitive ability between osmotrophs. At the same time is a useful parameter to evaluate the substrate pool available for microorganisms (Thingstad & Rassoulzadegan 1999).

The relationship between specific affinity (α) and the Michaelis-Menten parameters maximum specific uptake (V_{max}) and half saturation constant (Km) is illustrated in Fig. 3. Specific uptake rate (V) is V/B, where B is the biomass; hence V_{max} is V_{max}/B.

The specific affinity (α) can be described through the equation:

$$\alpha = \frac{V_{max}}{Km \; B} \tag{3}$$

The specific uptake rate (V_{max}) and specific affinity (α) describes how efficient microbes take up substrates at high and low substrate concentrations, respectively (index to measure competitive ability). **Paper III** shows an example of how affinity can change in a mesocosm experiment when mineral nutrients are depleted in a gradient of glucose and silicate. Thus, low values of α reflect lower substrate affinity (i.e. maximal growth is achieved at elevate substrate concentration), while high values of α indicate an increase in substrate affinity (maximal growth occurs at low nutrient concentration).
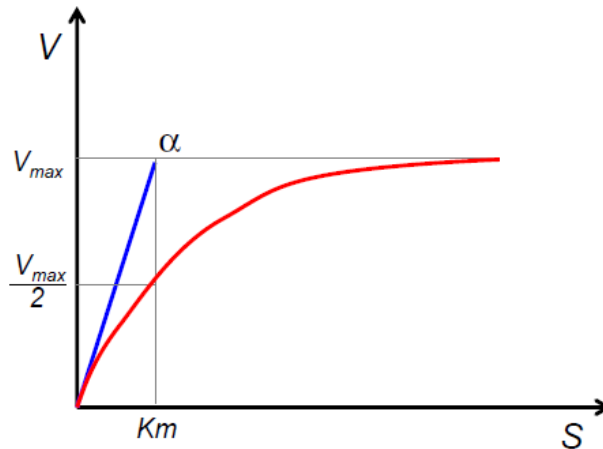
Figure 3. Relationship between Michaelis-Menten parameters and affinity. Maximum specific affinity (α) corresponds to the constant part of the slope of the Michaelis-Menten curve (blue line) where the substrate concentration (S) approaches zero (grey area). The affinity constant defines the maximum uptake capacity for the organisms.

The utilization of energy towards biosynthesis may control competition and also population succession amongst the bacterioplankton and phytoplankton. Thus, it is possible to hypothesize the kinetic response of two osmotroph populations to variable concentration of a single limiting resource (Fig. 4). In first case (a), the two population have identical maximal growth rates but population A has a greater affinity ($\alpha_A > \alpha_B$ and $Km_A < Km_B$) for the substrate than population B, and at low substrate concentration, population A would out compete B for the limiting resource. In the second case (b), populations A and B have similar substrates affinities ($\alpha_A = \alpha_B$), but different Km and μ_{max} values ($Km_A < Km_B$, $\mu max_B > \mu max_A$). Under this conditions, population B would increase as resources become more available. And in third case (c), both populations differ in μmax and substrate affinity. Thus, at low resource availability, population A will dominate, with population B out-competing A along a gradient in increasing resource availability. Thus, the principle of competitive exclusion (Hardin 1960) would predict that in equilibrium, two species competing for the

same resource could not stably coexist, meaning a different idea from the one expressed in the paradox of the plankton (Hutchinson 1961)
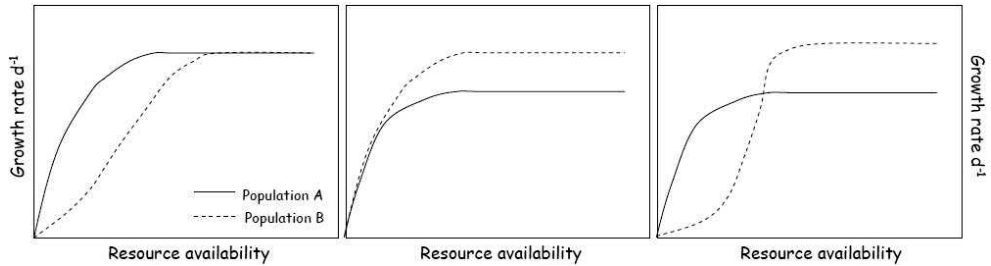

Figure 4. Hypothetical kinetic response of two bacterial populations (A and B) competing for limiting resources (Modified from Lalli & Parsons 1993)

From the analysis above, it can be possible to explain interaction such as competition between bacteria and phytoplankton when resources availability is limited, testing if organic C dynamics are controlled by competition between osmotrophs (bacteria – phytoplankton) for mineral nutrients and also by phytoplankton community composition (**Paper III**). Thus, an important question to approach would be, what portion or inorganic phosphorus and nitrogen uptake is due by heterotrophic bacteria and how this uptake is control the C utilization by heterotrophic bacteria.

4.4.1 Phosphate uptake.

A large and variable fraction of phosphate uptake can be due by heterotrophic bacteria (Kirchman 1994). These estimations are usually considered from the amount of radiolabeled PO_4^{3-} taken up by the small size fractions (i.e. <0.8 µm or <1.0 µm) or sometimes called 'bacteria' size fraction (picoplankton) (Fig. 5). A main source of error is the uptake over estimation mainly because in this size fraction, part of the uptake may be due by phytoplankton such as cyanobacteria (e.i. *Synechoccocus* sp., *Phlorococcus* sp.). Only few

studies corrected for phytoplankton uptake, reporting a PO_4^{3-} uptake fraction of 24–46% attributable to heterotrophic bacteria (Table 2). Kirchman (1994, 2000) approach these percentages calculating the maximum relative phosphate uptake from the ratio of bacterial production to primary production (BP:PP), ratio that is usually expressed in carbon units, corrected for C:P ratios of bacteria versus phytoplankton. Including the great variability in time and space, it make difficult to choose an average value for BP:PP and a single bacterial C:P ratio. Thus, assuming a BP:PP ratio of 0.2 and an average value of 53 for bacterial C:P and 106 for phytoplankton C:P, the maximum expected uptake of PO_4^{3-} would be about 40% of total uptake (Kirchman 2000). The calculated maximum uptake of phosphate is about equal to or less than the measured relative uptake of phosphate by heterotrophic bacteria, implying that bacteria obtain much of their P from phosphate and not from organic P. Even the importance of these measurements, only few studies have compared phosphate uptake and biomass production directly (e.g. Fuhrman & Azam 1982, **Paper III**). Thus, measuring both, phosphate utilization (e.g. phosphate turnover) and *in situ* phosphate concentration is possible to suggest the limiting stage of the microbial community (**Paper III**).
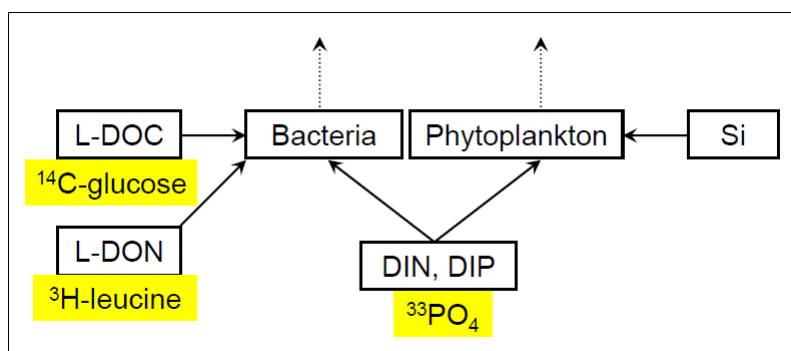

Figure 5. Example of isotopes used in the determination of mineral nutrient and organic carbon uptake by heterotrophic bacteria and phytoplankton. L-DOC: labile dissolved organic carbon; L-DON: labile dissolved organic nitrogen, DIN: dissolved inorganic nitrogen; DIP: dissolved inorganic phosphate; Si: silicate. Similar experiment set-up was used in **Paper III**.

4.4.2 Ammonium and nitrate uptake.

Like phosphate, ammonium uptake by heterotrophic bacteria varies greatly (Table 2). The

overall median estimated by Kirchman (1994) is about 40% which can be two-fold higher

than the maximum expected percentage, assuming the BP:PP ratio of 0.2 and C:N ratio of 6

and 4.2 for phytoplankton and bacteria, respectively (Kirchman 1994). Like phosphate uptake,

estimations of DIN (i.e. ammonium, nitrate and nitrite) uptake may be compromised by

phytoplankton uptake in the picoplankton size fraction. Frequently, the stable isotope ^{15}N

have been used to estimate DIN uptake, but the use of $^{33}PO_4$ is also of frequent use to estimate

phosphate turnover and affinity (**Paper III**).

Natural assemblages of heterotrophic bacteria have not been thought to take much nitrate

because of the energetic cost. Use of nitrate requires five NADHs compared to one for

ammonium (Vallino et al 1996). Even when few studies exist to represent the global ocean, it

seems that bacteria use more ammonium than nitrate, overall, bacteria account for 42 and

16% of ammonia and nitrate uptake, respectively (Table 2). However, these values are based

in very different studies of highly diverse marine systems

Thus, in general, it is worthwhile to focusing on ammonium uptake, mainly because this

compound is one of the main N sources supporting bacterial growth in marine environments

together with amino acids and nitrate. There is a not clear consensus about the preferences of

bacteria for ammonia or amino acids (e.g. Kirchman et al. 1989, Russel & Cook 1995, Vallino

et al. 1996), and an obvious incomplete picture of which compounds are controlling bacterial

growth rates mainly because of the difficulties to examine uptake of all possible DOM

components. As a general trend, comparisons of average ammonium uptake with bacterial

production indicates that a large fraction of bacterial growth can be supported by ammonium

Table 2. Summary of studies measuring ammonium (NH_4^+), nitrate (NO_3^-) and phosphate (PO_4^{3-}) uptake by heterotrophic bacteria in marine waters. Values expressed as percentage of the total uptake by bacteria.

Area	NH_4^+	NO_3^-	PO_4^{-3}	Comments	Reference
North Atlantic	22-39	4-14		Spring bloom	Kirchman et al. 1994
	8	27		Coast to open ocean transect	Harrison & Wood, 1988
			24-46	Corrected for phytoplankton	Nalewajko & Lee 1983
South Pacific Peruvian coast	50-75			$^{14}CO_2$ into proteins	Laws et al. 1985
			5	Pre-incubation, size fractionation	Harrison 1983
Subarctic Pacific	31	32		Starvation P, 4 months average	Kirchman & Wheeler, 1998
Fresh and marine waters			60		Kirchman 1994

4.4.3 Organic carbon uptake.

Concentration of DOC in marine environments typically range between 60 and 90 µM and decline to about 40 µM in the deep sea (Hansell 2002). This DOC can be broadly defined based on their turnover times as refractory, semi-labile and labile. The less abundant pool of DOC includes the labile DOC, with utilization times ranging from minutes to days and very low concentrations. Most of the studies on limitation of bacterial growth by DOC have focused on specific compounds experiments such as utilization of glucose (Havskum et al. 2003) and some few in the utilization of pyruvate (Al-Sarawi et al 2008). In comparative studies of bacterial growth and DOM degradation (e.g. Carlson et al. 1998) was found that 89% of the total organic matter produced during the summer growing season was retain as particulate carbon, and the small DOM fraction was rapidly consumed by bacteria. In productive areas still remains unclear why the DOC produced under this ecosystem supposed to be more biologically reactive than the DOC produced in oligotrophic areas with low nutrient concentrations. A possible explanation is that the type of substrates available to support bacterial growth have a great nutrition value (or lower C:N:P ratios) in high-nutrients marine environments than those substrates available in low-nutrient environments (Church 2008). In addition, there is experimental evidence that the supply of mineral nutrients can

facilitate the use of semi-labile DOC (Zweifel et al. 1993, Thingstad et al 1997, 2008, **Paper II**).

There are several examples where glucose and sometimes dissolved free amino acids (DFAA) supply a major fraction of the carbon required to support BP and growth. Rich et al. (1997) found that despite high concentrations of both glucose and DFAA in the Arctic Ocean, glucose alone met upwards 100% of the BP. Similar response was found in **Paper II** were glucose alone increase > 5 folds BP and a combination of glucose and mineral nutrients enhance BGR in the Norwegian Sea. In the Equatorial Pacific, glucose alone could support 15 – 47% of the production and a large fraction of the respiratory demands (Rich et al. 1997). And in coastal and oceanic areas off northern Chile (**Paper I**) labile DOC released from copepods activity always enhances BGR and bacterial carbon demand.

Thus, observing the effect of organic carbon and mineral nutrients on the microbial food web dynamic; when mineral nutrients are sufficiently high, diatoms usually are dominant to take up nutrients compared to smaller phytoplankton and bacteria (Havskum et al. 2003, Tanaka et al. 2008). If the addition of labile DOC is smaller than Redfield ratio, the dominant-diatom community can out compete heterotrophic bacteria and consume more phosphate, established high biomass, showing higher organic C production and slower response of nutrient transfer compared to a faster response in a flagellate-dominant community (Thingstad et al. 2008, **Paper III**, **Paper IV**). With increased addition of labile DOC, or higher carbon:nutrient Redfield ratio, the above differences can became small.

A critical factor controlling the dynamic of organic C is the dominant phytoplankton community observed depending on the availability of nutrients such as ammonium, nitrate

and silicate deplete or replete conditions (Thingstad et al. 2008, **Paper II**, **Paper III**), and the availability of labile organic carbon (**Paper I**, **Paper II**).

4.5 Comparative analyses and its application in marine microbial processes.

In marine microbial ecology, different doubts in comparative analysis are related with conversion factors and the use of different methodologies. This approach lead the assumption of confident determinations of bacterial growth and losses, and leave a large discussion on the different conversion factors involved in BB and BP estimations, plus the restriction and methodological problems connected to grazing rates estimation on bacteria.

Several studies do not include empirical conversion factors, assuming theoretical values from previous data in similar environments. Thus, underestimation and overestimation of BP and BB is a common error factor in microbial ecology studies. Another example is the common use of thymidine (TdR) or leucine (Leu) isotopes to estimate bacterial production and the use of mixed measurements in comparative analysis. Both approaches are different in definition. Thymidine based measurements (Fuhrman and Azam 1982) estimate the productivity rate of actively growing bacterial cells, as a proxy of DNA synthesis, and leucine measurements (Simon and Azam, 1989) estimates the incorporation of leucine into bacterial protein as a proxy for formation of new bacterial biomass production, which measure separate though related physiological processes. Thymidine and leucine incorporation rates do co-vary over a variety of time and space scale (Kirchman 1992, **Paper V**). Variations in Leu:TdR ratios can be substantial and can change with growth rates and the physiological state of bacterial assemblages. This ratio has a long variability in the North Atlantic with values from 2.6 – 116.3 in the upper 200 meters (Ducklow 2000). In addition, Leu:TdR ratio can varies from oligotrophic to eutrophic regions (**Paper V**). Several studies address relative changes in

Leu:TdR ratios, but no one to date has been able to explain quantitatively the significance of the values of the ratio for a given sample or for a given regional ratio.

One way to visualize the regulation of bacterial by bottom-up and top-down control is to consider how biomass and growth rates change along a resource gradient at low and high levels of predation (Fig. 6). And further, a simple approach that allows us to test a large set of data for different ocean basins is the relationship of bacterial biomass and production and the analysis of the variation in the slope and the intercept of this relationship.
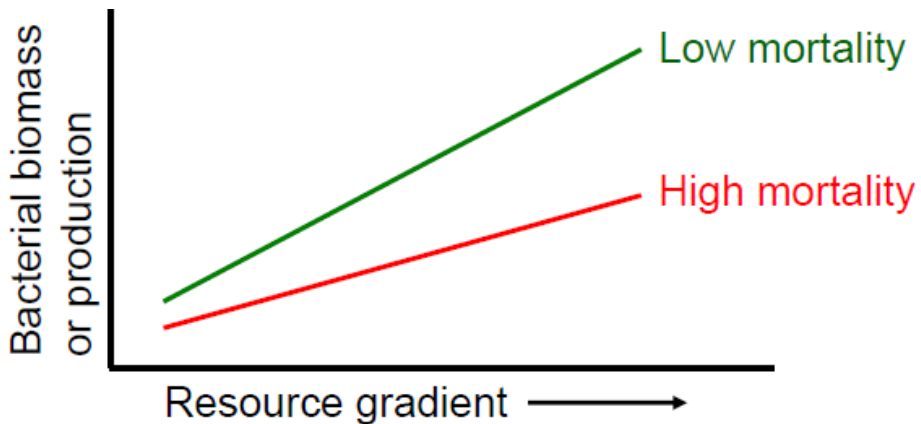

Figure 6. Hypothetical response of bacterial biomass and productivity across a gradient of increasing resources assuming that biomass and productivity are coupled. From Pace & Cole 1994)

These studies can provide strong evidence for the importance of resources to bacteria but not to directly address the problem of bottom-up and top-down control. Billen et al. (1990) argued that the relationship between BP and BB could be used to evaluate bottom-up and top-down control. As it was discussed above, since bacterial resources are difficult to measure, BP can serves as a surrogate measure of resource. Thus, variability in BP reflects variability in

resource inputs. From Figure 6 and from the regression of BB as a function of BP, should have a steep slope if biomass is determined by resources, and alternatively, there should be a shallow slope (or no relationship) if other factors such predation or viral lysis (Fig. 2) are more important. Such analysis does not consider two factors. First, there is no distinguish between predators and virus effect (**Paper V**), and second, in the situation where predation on bacteria is a major mechanism of resource recycling, the increase in mortality might lead to resource regeneration by consumers, resulting in BB uncoupled from increases in BP.

These relationships between bacterial productivity and biomass imply that large-scale differences in resource supply are a crucial determinant of bacterial biomass. However, these relationships are not as strong when small scales are considered. Thus, weak relationships imply that bottom-up control may be less significant at local scales and may vary seasonally within sites (Ducklow 1992).

A different perspective is provided when data on bacterial abundance and heterotrophic flagellates are summarized (e.g. Sanders et al 1992, **Paper V**). In several cases, like eutrophic environments, flagellate grazing can be insufficient to regulate bacteria and alternatively in oligotrophic environments substrate supply controls bacterial abundance, but grazing by heterotrophic flagellates reduces bacteria below carrying capacity in more eutrophic systems (Sanders et al. 1992) and decrease the slope of the bacterial production and biomass relationship (**Paper V**).

5. Conclusions

The use of different approaches to answer the question of how bacterial growth is controlled in marine environments, can improve our understanding on different time and special scales of resolution, being possible to solve this common question from specific interaction and bacterial uptake of single compounds to global microbial trends. In that sense some specific conclusion can be drawn as follow:

1 — The structure of the dominant phytoplankton community can control (1) the labile organic carbon sources for heterotrophic bacteria and (2) the competition between osmotrophs for mineral nutrients.

2 — Labile organic carbon sources from different origens, for example, phytoplankton exudates in coastal areas or copepod activity in oligotrophic oceanic areas, affect the availability of substrate for bacterial growth.

3 — Carbon and mineral nutrient limitation can control bacterial growth depending on the structure of the phytoplankton community. Small phytoplantonic cells may keep mineral nutrients available for bacteria and may not produce labile carbon substrates from exudates. Opposite, large chain diatoms may outcompete bacteria for mineral nutrients but may produce labile organic carbon from exudates and zooplanton grazing.

4 — Changes in the supply ratios of labile organic carbon, silicate and nitrogen (ammonium and nitrate) additions, as well as phytoplankton community composition would modify the main pathway of nutrient transfer in the food web.

5— Idealized models are able to explain just qualitatively the responses of the microbial food web such as the control of bacterial growth and the main pathways of nutrients. Understanding the properties of these 'first order' models seems required to establish a fundament upon more elaborated models can be built.

6 — Is possible to resolve until certain level the impact of two modes of control of bacteria in plankton (substrate availability and predator activity), using empirical data. Comparative analysis studies generally confirm the important of resource regulation (bottom-up control) of bacteria at least at large scales, meaning that at higher bacterial biomass is possible to estimate lower bacterial growth rate.

6. Future Perspectives

The need to connect metabolic rates in an individual experiment could solve the gaps in the understanding of microbial activity and interactions. For example, it seems obvious the development of instantaneous and coupled measurements of bacterial respiration and production to further observe the effiency of bacterial growth in marine environments. The observation of bacterial growth rates and efficiency can allow us to understand the factors controlling bacterial activity and carbon sequestration in the ocean. Questions on precision and accuracy are important in the measurements of bacterial growth and mortality, for that reason these studies must be interpreted cautiosly and perhaps rephrase the questions trying to resolve if bacterial losses overcome bacterial growth in contrasting marine environments.

The ambition to predict the pathway taken by nutrients in a given experiment is not fulfilled with a 1st-order model alone. More elaborate idealized models can be built, potentially minimizing some of the extra variables associated with more complex ecosystem models. Mechanisms such as flexible stoichiometry, predator control and the interaction between both needed to be evaluated to explain the observed variability in nutrient pathways and can be suggested for inclusion in 2dn-order food web model.

Even when 'easy to measure' variables (e.g. bacterial abundance, chla, BGR, etc) are always need it to underatand the biology of the plankton, it has not been reached the real effect of predation and mortality impact. Protozoans might not control bacterial abundance but can possible control bacterial community structure and activity (i.e. active bacteria such as HNA bacteria), thus, more attention will be need it on the non-stable bacterial abundance that has changes in the percentages of active bacteria, changes in phylogenetic composition and changes in size structure. A comprehensive analysis of parameters such as bacterial growth

rate in different marine regions and the elaboration of different hypothesis across a gradient of productivity are still in discussion to improve the understanding on the role of bacteria in biogeochemical cycles at global scale.

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