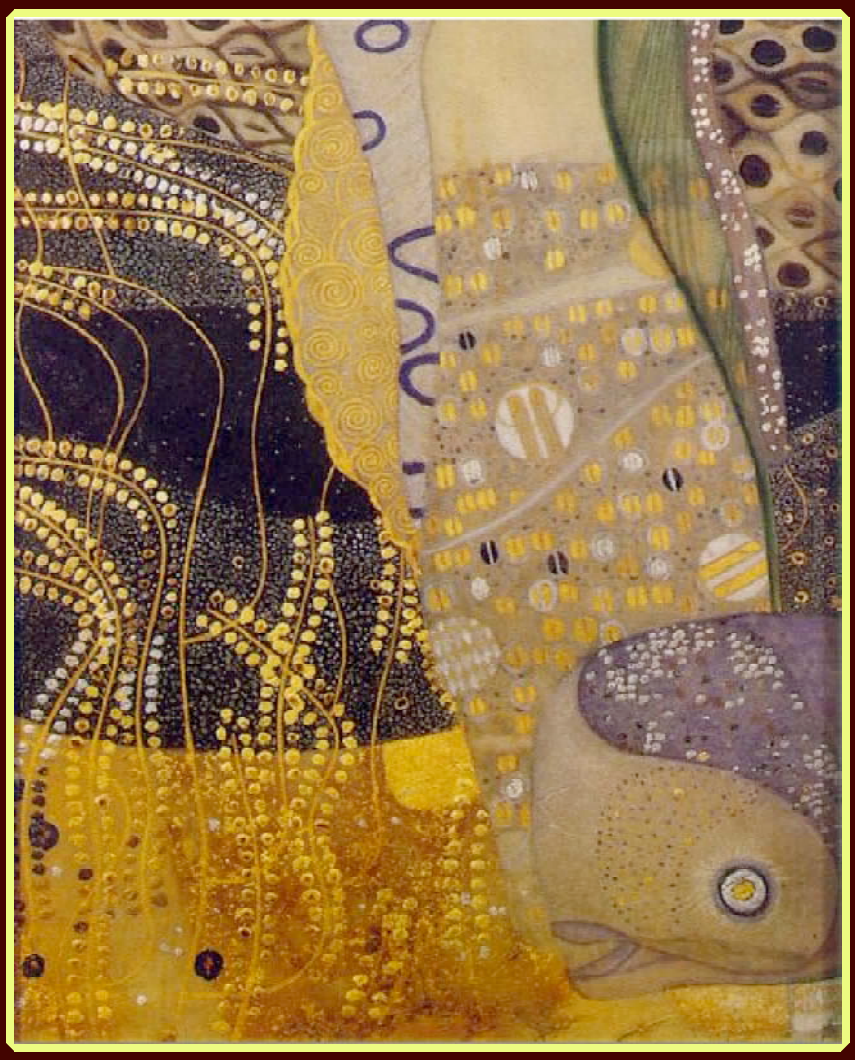

Links between bacterial diversity and carbon cycling in the sea

Laura Alonso Sáez

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Institut de Ciències del Mar (CSIC)

Universitat Politècnica de Catalunya

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Laura Alonso Sáez

Vist-i-plau del director de la tesi

Dr. Josep M. Gasol
Investigador-CSIC

“Se trataba de encontrar un lenguaje
que no fuera literario”
(Julio Cortázar, *Rayuela*)

A mis padres
y a mi hermano, Israel

Cover: Marine food web according to Gustav Klimt
"*Water serpents*", fragment

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Resumen

A pesar del gran avance en nuestro conocimiento sobre la diversidad bacteriana marina y el papel de las bacterias en el ciclo de carbono oceánico, aún se sabe muy poco sobre la relación entre estas dos importantes características en condiciones naturales. El estudio de esta relación se ha visto limitado por la dificultad para obtener cultivos puros de bacterias representativas del medio marino, lo que nos permitiría disponer de información sobre sus capacidades metabólicas. El reciente desarrollo de técnicas de resolución individual ha permitido el estudio de la actividad de poblaciones bacterianas in situ, y recientemente, hemos comenzado a conocer cual es el papel de grupos específicos de bacterias marinas en el ciclo de carbono. En esta tesis, se estudia la relación entre diversidad bacteriana y función a lo largo de dos gradientes en aguas marinas: un gradiente temporal (estudio estacional en una estación costera del Mediterráneo), y un gradiente espacial que cubre diferentes estados tróficos en aguas oceánicas (desde el afloramiento de Cabo Blanco hasta el giro subtropical del Atlántico Norte). Primeramente, se ha descrito exhaustivamente la estructura de la comunidad bacteriana en ambos lugares de muestreo, utilizando simultáneamente diversas técnicas genéticas como bibliotecas génicas, hibridación in situ con sondas fluorescentes y "*fingerprint*" de genes que codifican el RNA ribosómico 16S. Dado que los estudios se concentran en aguas superficiales, las Archaeas son poco representativas y nos concentramos en las bacterias. También se ha estudiado exhaustivamente el uso bacteriano de carbono a lo largo de ambos gradientes, con especial énfasis en la identificación de las limitaciones en las metodologías utilizadas. Tras estos estudios descriptivos, se llevaron a cabo estudios comparativos en los que los cambios en la estructura de la comunidad bacteriana fueron sistemáticamente comparados a los cambios en las medidas de procesamiento de carbono, incluyendo producción y respiración bacteriana. Este tipo estudios no se habían realizado anteriormente en muestras naturales marinas. Encontramos diversas similitudes entre los dos sistemas estudiados en cuanto a la estructura de la comunidad bacteriana, como el aumento en la proporción de bacterias del grupo Roseobacter en aguas ricas en nutrientes, y la predominancia del grupo SAR11 en aguas menos productivas. Sin embargo, mediante el uso de técnicas estadísticas multivariantes y el test de Mantel, no se pudo probar una fuerte relación entre diversidad y función en ninguno de los gradientes. Esta ausencia de relación podría deberse a varias razones, incluyendo el hecho de que las medidas utilizadas para describir la función en el ciclo de carbono (producción de biomasa bacteriana y respiración) son tan generales y cruciales para el mantenimiento del ecosistema que son llevadas a cabo de forma redundante por los distintos grupos bacterianos. Sin embargo, en un segundo estudio mucho mas específico, se empleó una técnica de resolución individual (microautoradiografía combinada con hibridación in situ con sondas fluorescentes) y se mostró que distintas poblaciones bacterianas llevan a cabo distintos patrones de uso de carbono a lo largo del estudio estacional en la Bahía de Blanes, lo que sugiere que los grupos bacterianos no son equivalentes en la utilización de carbono. Finalmente, estudiamos el efecto de una variable ambiental, la luz ultravioleta y la radiación PAR, y encontramos que inhibía o estimulaba diferencialmente la actividad especifica de varios grupos bacterianos, y por tanto su papel en el procesamiento de carbono en el medio planctónico marino.

Resum

El nostre coneixement sobre la diversitat bacteriana al mar i el del paper que els bacteris tenen en el cicle del carboni han avançat molt en les darreres dècades, tot i això, es coneix ben poca cosa de la relació entre aquests dues conceptes. L'estudi de la relació entre diversitat i funcionament de l'ecosistema es veu limitat per les dificultats d'obtenir cultius purs de bacteris marins representatius, que podrien oferir informació sobre les seves capacitats metabòliques. El desenvolupament recent de tècniques d'anàlisi a nivell individual ha permès l'estudi de l'activitat in situ de les poblacions marines, permetent avenços en aquesta relació diversitat-funció. Comencem a saber quin és el paper específic de grups concrets de bacteris en la circulació de carboni a l'oceà. En aquest treball, hem analitzat la relació entre diversitat bacteriana i funcionament de l'ecosistema al llarg de dos gradients en medi marí: un gradient temporal (estudi estacional en una estació costanera mediterrània), i un gradient espacial que comprèn diferents estats tròfics en aigües oceàniques (prop de l'aflorament de Cap Blanc al gir subtropical de l'Atlàntic Nord). Primer descrivim exhaustivament l'estructura de la comunitat de bacteris als dos llocs, mitjançant l'elaboració simultània de biblioteques de clons, hibridació in situ amb sondes fluorescents i emprentes genètiques dels gens que codifiquen per l'rRNA 16S. Com que tots dos estudis es centren en comunitats bacterianes d'aigües superficials, els Arqueus són poc representatius i ens hem centrat en els bacteris. També hem descrit exhaustivament la utilització de carboni per part dels bacteris als dos indrets, prenent especial cura amb identificar i restringir les limitacions en la metodologia utilitzada per aquestes mesures. Després hem fet estudis comparatius en els quals els canvis en l'estructura de la comunitat bacteriana s'han comparat sistemàticament amb els canvis en un seguit de mesures del processament de carboni per part dels microorganismes, incloent-hi la respiració i la producció bacteriana, car aquesta mena de comparacions són infreqüents a la literatura. Els dos sistemes estudiants comparteixen algunes característiques, com ara les abundàncies creixents de bacteris del grup de Roseobacter en aigües plenes de nutrients, i la predominança de bacteris del grup SAR11 a les aigües menys productives. Tanmateix, i amb la utilització de tècniques estadístiques multivariants i testos de Mantel, no hem pogut provar cap relació estreta entre diversitat bacteriana i funcionament a cap dels dos gradients. Aquesta mancança de relació pot ser deguda a diverses raons, entre elles el fet que les mesures a l'engròs per descriure la funció dels bacteris en el cicle del carboni (producció de biomassa o respiració bacterianes) són tan generals i tan importants per al manteniment de l'ecosistema que són realitzades de forma redundant per diferents grups bacterians. De tota manera, un estudi més concret, utilitzant una aproximació a nivell de cèl·lules individuals (microautoradiografia combinada amb hibridació in situ amb sondes fluorescents) ha mostrat que diferents poblacions bacterianes tenen diferents patrons d'utilització de carboni al llarg de l'estudi estacional de la Badia de Blanes, la qual cosa suggereix que els diferents grups bacterians no utilitzen de la mateixa manera el carboni. Finalment, hem estudiat l'efecte inhibidor o estimulador d'una variable ambiental, la radiació PAR i la UV, sobre els diferents grups bacterians. Hem pogut mostrar com aquestes condicions ambientals afectaven de forma diferent l'activitat dels diferents grups bacterians i, per tant, el seu paper en el processament de carboni en el medi planctònic marí.

Summary

Although our knowledge about bacterial diversity in marine waters and that about the role of bacteria in the oceanic carbon cycling have greatly advanced in the last decades, very little is known about the linkage between those important characteristics, particularly under *in situ* conditions. The study of such relationship is hampered by the difficulties in obtaining pure cultures from representative marine bacteria, which would provide information about their metabolic capacities. The recent development of single-cell techniques has allowed studying the activity of marine populations *in situ*, allowing advances on this topic. We are starting now to know what is the specific role of specific groups of bacteria in carbon cycling, and the relationship between bacterial community assemblage and the global magnitude and patterns of carbon cycling in the ocean. In this study, we assessed the linkage between bacterial diversity and function along two gradients in marine waters: a temporal gradient (a seasonal study in a coastal Mediterranean station), and a spatial gradient covering different trophic states in oceanic waters (from the Cape Blanc upwelling to the N Atlantic subtropical gyre). We first described exhaustively bacterial assemblage structure at both sites, with the simultaneous use of clone libraries, fluorescence in situ hybridization with specific probes and fingerprinting of genes encoding the 16S rRNA. Since both studies were focused on surface bacterial assemblages, *Archaea* were minor components and we focused on *Bacteria*. We also exhaustively described the use of carbon by bacteria at both sites, taking special care in identifying and constraining the limitations in the methodologies used for that purpose. Afterwards, we performed comparative studies in which changes in bacterial assemblage structure were systematically compared to changes in a range of *in situ* measurements of bacterial carbon processing, including bacterial respiration and production, since this type of comparisons in marine field studies are absent from the literature. Several similarities were found between both systems studied, in terms of bacterial community composition, such as the increasing abundances of the Roseobacter group in high-nutrients waters, and the predominance of SAR11 bacteria in less productive waters. However, and with the use of multivariate statistics and Mantel tests, a tight linkage between bacterial diversity and function could not be proved in neither of the gradients. This lack of pattern could be due to several reasons, including the fact that the bulk measurements that describe carbon function (bacterial biomass production or respiration) are so general and important for ecosystem maintenance that they are redundantly performed by different bacterial groups. However, a more specific study using a single-cell level approach (microautoradiography combined to fluorescent in situ hybridization) showed that different bacterial populations had significantly distinct patterns of carbon use along the seasonal study in Blanes Bay, which suggests that different bacterial groups are not equal in terms of carbon utilization. Finally, we studied the effect of a variable environmental factor, such as UV and PAR radiation, as it inhibited or stimulated the different bacterial groups. Environmental conditions were shown to affect differently the activity of specific bacterial groups and, thus, their role in the processing of carbon in the marine planktonic environment.

Introduction

General introduction

"If microorganisms are major consumers in the sea, we need to know what kinds are the metabolically important ones and how they fit into the food web."

Pomeroy, 1974 "The Ocean's food web, a changing paradigm".

Bacteria: a key component of the carbon cycle in the sea

The role that bacteria play in the oceanic carbon cycle was unveiled after the postulation of the so-called "microbial *loop*" hypothesis (Pomeroy 1974, Williams 1981, Azam et al. 1983). Although their role as remineralisers of organic matter and as nutrient recyclers had been recognized early on, bacteria were initially severely underestimated in terms of quantitative biomass, and considered to be mostly in a dormant state. First estimates of bacterial abundance were based on the number of isolates able to grow on agar plates (e.g. Zobell 1946). This technique was later shown to underestimate the real abundances of bacteria by several orders of magnitude (Jannasch & Jones 1959), which started to be measured in the 10^5-10^6 range after the development of epifluorescence microscope techniques (Francisco et al. 1973, Hobbie et al. 1977, Zimmerman 1977, Porter & Feig 1980). Such discrepancy between plate and microscopic counts was later named the "Great plate count anomaly" (Staley & Konopka 1985), and more recent estimates showed that marine prokaryotes contain more than half of the carbon stored within plant biomass at a global scale (Whitman et al. 1998).

The finding that these very abundant microorganisms were not dormant, but instead actively growing (Hagströmet al. 1979, Fuhrman & Azam 1980) through the consumption of DOC released by primary producers, changed the view of carbon dynamics in the sea. Once values of bacterial growth rates had been obtained, new hypotheses arose. For example, if bacteria were actively growing and the standing stock remained relatively stable, then there should be some type of bacterial biomass loss factor. Crustaceans and rotifers (e.g. Pedrós-Alió & Brock 1983), ciliates (e.g. Børsheim 1984) and heterotrophic flagellates (Fenchel 1982) were shown to graze upon bacteria and potentially control their abundance, forming the essentials of the *microbial loop* concept (Azam et al. 1983, Fig 1). It consists of a microbial carbon and energy pathway alternative to the traditional food chain the ocean, in which prokaryotes play a key role remineralizing the large oceanic dissolved organic carbon (DOC) pool (through

bacterial respiration, BR), and making available a significant part of this DOC to higher trophic levels (through bacterial secondary production).

First approaches in the measurement of bacterial carbon processing concentrated on the determination of bacterial heterotrophic production (BHP), the synthesis of new biomass by bacteria. The use of radioactive precursors of RNA (Karl 1979), DNA (Fuhrman & Azam 1980) and proteins (Kirchman et al. 1985) allowed a rapidly growing knowledge of the magnitude and distribution of BHP over the oceans (Ducklow & Carlson 1992). From the very first estimates it was shown that bacterial carbon production could represent a large percentage of the primary production of the ocean (10-50%, Fuhrman & Azam 1980, Cole et al 1988), and encouraged an exhaustive study of their role in the carbon cycle.

Despite the large database of BHP determinations, measurements of BR were neglected for a long time and assumptions about bacterial growth efficiency (BGE, i.e. the amount of substrate incorporated scaled to the total substrate consumption) were made based on the efficiency in the incorporation of single organic compounds. Such measurements have later been shown to drastically overestimate the efficiency of the incorporation of ambient DOC, and have pointed to the relevance of obtaining in situ measurements of BR and BGE. Refined estimates of bacterial carbon demand (i.e. the addition of BHP and BR) lead support to the view that a great part of the ocean is net heterotrophic for most of the time, since bacteria remineralize more carbon than is produced in situ (del Giorgio et al. 1997, Duarte & Agustí 1999). Although this view has been challenged in some studies (Williams 1998), it underlined the importance of carbon remineralization by bacteria in balancing global carbon budgets, and leaded to an increasing recognition of the need for accurate estimates of the spatial and temporal variability of bacterial carbon flux (Janhke & Craven 1995, Karl et al. 2003).

Our knowledge of the role of bacteria in the oceanic carbon flux and the trophic interactions involving microorganisms has considerably advanced in the last years (Azam 1998, Fenchel et al. 2001). For example, phages (i.e. viruses infecting bacteria) have shown to significantly affect DOM turnover by lysing cells (Berg et al. 1989, Proctor & Fuhrman 1990, Bratbak et al 1992) and decrease the efficiency of bacterial carbon use (Fuhrman 1999), and new forms of phototrophy (such as that based on proteorhodopsin, Béjà et al. 2000) can be widely distributed in marine bacteria. This suggests that further research in how bacterial activities affect carbon fluxes is needed, in order to integrate the role of bacteria in predictive biogeochemical models.

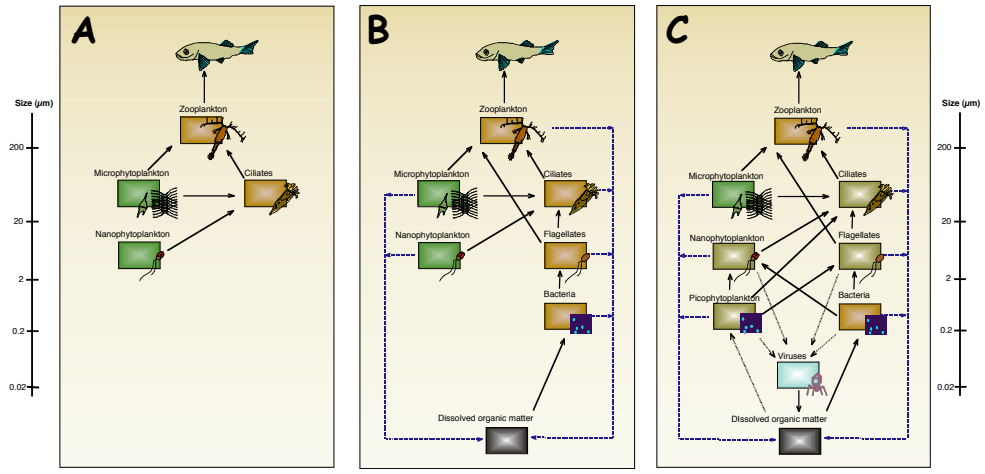

Fig 1- Marine food web including the classical view (a), the addition of the microbial loop (b), and the addition of the effect of viruses and new trophic interactions between the different levels (c). Fig. from Gasol et al (2005).

A current challenge in the assessment of the metabolic activity of marine bacteria is the measurement of the bacterial activity at the level of individual cells, by the application of single-cell techniques. Early measurements of bulk BHP or growth rates were in some cases accompanied by some of these methods, such as microautoradiography (Fuhrman & Azam 1980). This technique was among the first single-cell methods applied in aquatic ecosystems (Parsons & Strickland 1961, Wright & Hobbie 1965, Brock & Brock 1968, Hoppe 1976), to detect the numbers of bacteria that were actively taking up substrates, as indicated by radiolabeled tracers. Microscopical observation of the frequency of dividing-cells was also soon applied to marine samples, and correlated to the growth rates of isolates (Hagström et al. 1979). However, the relevance of the single-cell techniques was recognized after the development of specific stains and probes as indicators of bacterial activity, which revealed a wide range of metabolic states present among the natural bacterial assemblages (Fig. 2). Such physiological probes allow differentiation between states such as "dead" cells (e.g. cells without a nucleoid, Zweifel & Hagström 1995), membrane-damaged cells (e.g. Grégori et al. 2001), actively dividing cells (cells incorporating 5-bromo-2'-deoxyuridine –BrdU-, Urbach et al. 1999, Borneman 1999), or actively respiring cells (INT or CTC positive cells, Zimmermann et al. 1978, Rodríguez et al. 1992). Some of these techniques have been successfully combined with flow cytometry, allowing a rapid analysis of high numbers of samples (Gasol & del Giorgio 2000).

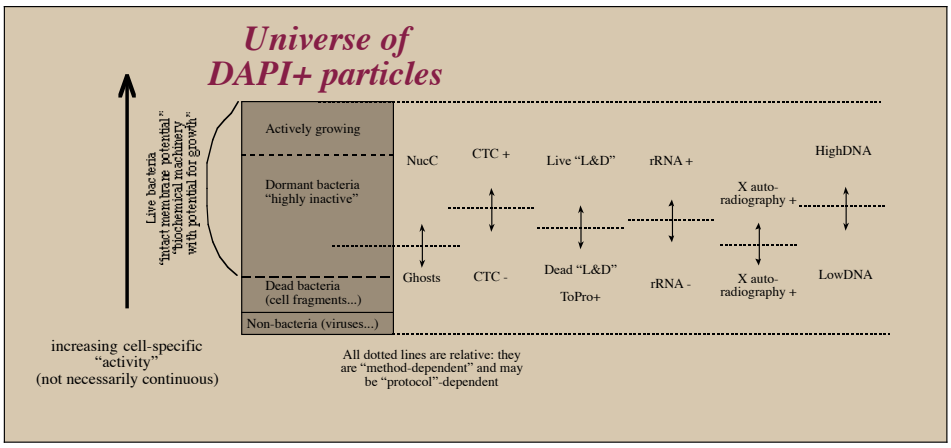

Fig 2- Summary of single cell-techniques and associated levels of activity in bacteria (Gasol, J.M., unpublished)

Results from these single-cell studies are sometimes controversial, since frequently suggest that a large part of the assemblage consists of dead or inactive cells. However, their application has undoubtedly introduced the view that bacteria cannot be regarded as a uniform assemblage, but rather as a continuum of physiological states (Smith & del Giorgio 2003). Lately, the combination of these single-cell techniques with phylogenetic probes is throwing light into one of the most important ecological questions: Which bacterial populations are metabolically active and, thus, drive the major part of the carbon flux in the sea?

Bacterial phylogenetic diversity in marine waters

Marine bacterial diversity was first studied through the examination of morphological and metabolic capabilities of bacteria isolated on agar plates. Later findings showed that this isolation approach produced a strong bias in the recovery of species, since it promotes the growth of bacteria stimulated by organic and nutrient enrichments, which are far from the usual environmental conditions found in oceanic waters. Parallel to the findings about the role of bacteria in the marine ecosystem, the development of culture-independent techniques allowed the assessment of the identity and assemblage structure of oceanic bacteria. These techniques are based on the extraction of some macromolecules, which can be used as genetic

markers to study phylogenetic relationships between species. For microbes, most studies have been based on the analysis of the gene encoding the SSU ribosomal RNA, due to its conserved function and universality among living species. Woese et al. (1977, 1987) were pioneers in the application of SSU rRNA methods for assessing microbial evolution, and Pace et al. (1986) introduced those techniques to microbial ecology studies.

The first results obtained from cloning and sequencing of environmental marine samples revealed that most groups of recovered bacteria did not correspond to cultured species and, thus, were previously unknown (Giovanonni et al. 1990, Schmidt et al. 1991). In a review of cloning results of *Bacteria* in marine waters carried out ten years later (Giovanonni & Rappé 2000), Alphaproteobacteria appeared as the most important group inhabiting the ocean, with three quantitatively relevant lineages: SAR11, Roseobacter and SAR116. A group of Gammaproteobacteria, SAR86, also appeared in substantial proportions in marine clone libraries, and other common groups in the photic oceanic layer were Actinobacteria and marine picophytoplankton (Fig 3). Later studies by fluorescence in situ hybridization (FISH) with specific probes showed that the group Bacteroidetes was also highly abundant and widespread in the ocean (Glöckner et al. 1999), and their low recovery in clone libraries was due to a mismatch against this group by usual primers used for cloning (Kirchman 2002).

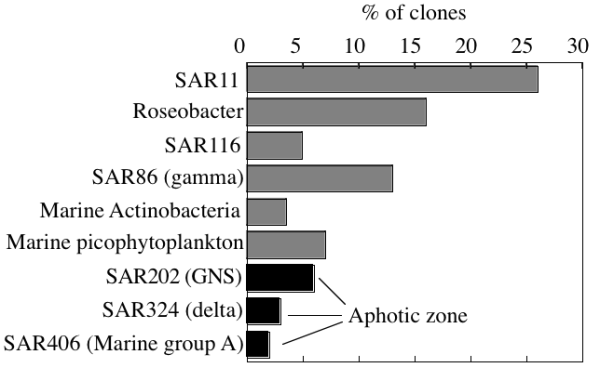

Fig 3- Frequency of the most common bacterial groups obtained by cloning and sequencing up to 1999, in a review of studies assessing planktonic marine samples (From Giovanonni & Rappé 2000)

Cloning and sequencing of environmental samples have undergone an exponential increase in recent years, and nowadays, the amount of environmental sequences largely surpasses those of cultured microorganisms (Rappé & Giovanonni 2003). Marine prokaryotes are distributed through a relatively few taxa within the kingdoms *Bacteria* and *Archaea* (Fig 4), with some of them entirely represented by uncultured members with unknown physiological characteristics. As an example, while several members of Roseobacter have been isolated from marine waters,

SAR86 remains still uncultured. SAR11, which is probably the most abundant bacterial group in the surface ocean (Morris et al. 2002), was only recently isolated after a great effort in the development of new culturing approaches (Rappé et al. 2002). Similarly, the first culture of a representative marine member of *Crenarchaea* was only isolated very recently (Könneke et al. 2002). Given the slow advances in the art of culturing of representative marine bacteria, we are currently in a situation in which growing knowledge about bacterial diversity is challenged by the difficulties in assessing the function of these organisms.

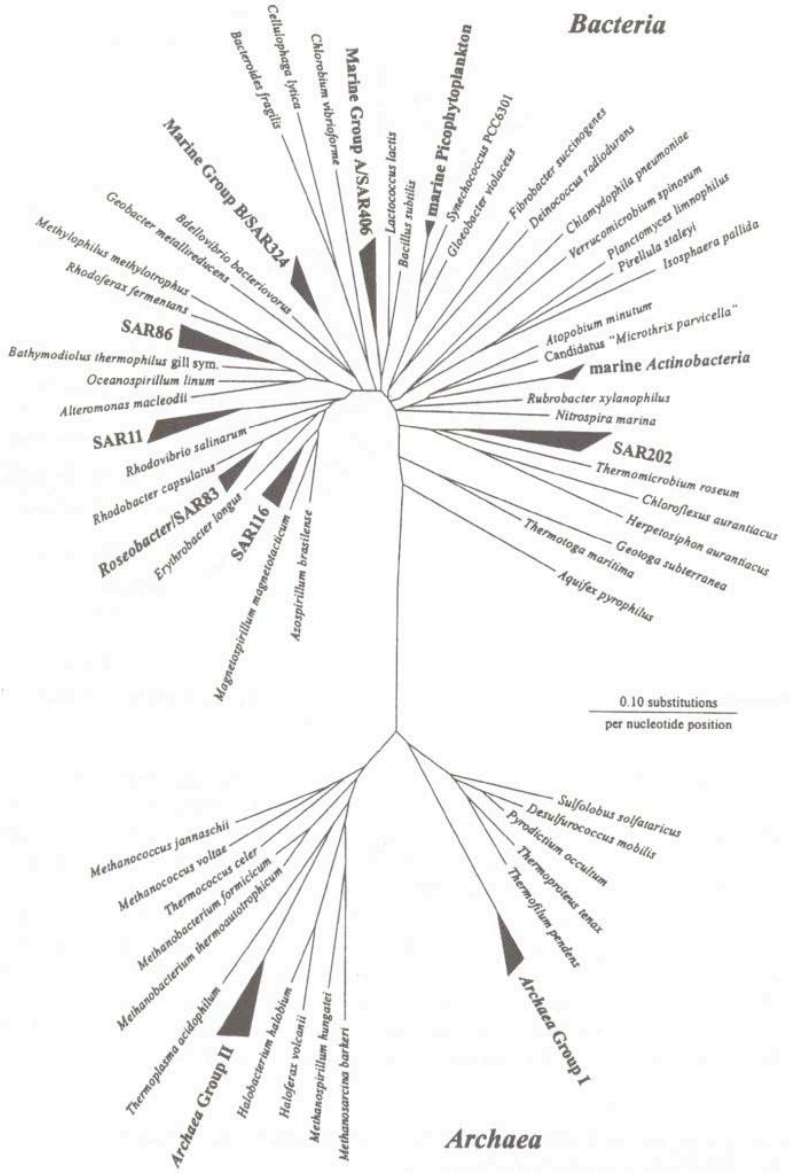

Fig 4- Phylogenetic tree displaying relationships among the most widespread SSU rRNA gene clusters from marine plankton (from Giovanonni & Rappé 2000).

One strategy to unveil ecological functions of bacterial populations is to analyze their patterns of distribution in the ocean along spatial gradients, such as along the vertical profile. As an example, two different populations (or "ecotypes") of *Prochlorococcus* were found at different depths, adapted to low light and high light conditions (Moore & Chisholm 1999). Besides cloning and sequencing, the application of fingerprinting techniques (denaturing gradient gel electrophoresis -DGGE-, terminal restriction fragment length polymorphism -T-RFLP-, and others) has considerably enlarged the knowledge about the changes in the composition of bacterial assemblages through environmental gradients. Furthermore, despite lacking isolates for most marine bacterial groups, the sequences of uncultured organisms have been used to design probes for specific groups, which allow their direct quantification *in situ*, through fluorescence *in situ* hybridization (FISH). The application of this technique to marine samples showed, for example, that the bacterial group Bacteroidetes was more abundant in marine waters than previously thought (Cottrell and Kirchman, 2000), and that marine *Archaea* were found in significant abundances in the mesopelagic zone of the ocean (Karner et al. 2001).

Knowledge about the ecological function of specific bacterial groups (or "bacterial functional groups") should accelerate the incorporation of bacterial assemblage structure into quantitative models of carbon cycling. For example, it has been shown that specific groups of zoo- and phytoplankton have different impacts on carbon flux (Legendre & LeFèvre 1995, Legendre & Rassoulzadegan 1995). However, whether functional groups of bacterioplankton exist is still unknown. This kind of knowledge would help divide the bacterioplankton "black box" into boxes of different biogeochemical significance, and improve the predictions of bacterial carbon processing, based on assemblage structure data.

What controls bacterial diversity in aquatic ecosystems?

From a bulk perspective, the standing stock (biomass) of the bacterial assemblage is controlled by the well-known processes collectively named top-down (e.g. grazing, viral attack) and bottom-up (environmental factors, nutrients, DOM availability). However, if the "black box" of bacteria is opened at the phylogenetic level, the impacts of bottom-up and top-down factors on bacterial assemblage structure are not that clear.

The effect of bottom up factors such as environmental variables (nutrients, salinity, pH) has been shown to influence bacterial assemblage composition in estuaries (Cottrell & Kirchman

2000, Bouvier & del Giorgio 2002), and freshwater environments (Gasol et al. 2002, Langenheder et al. 2003). In marine waters, bacterioplankton composition has been shown to change along temporal (seasonal cycles, Pinhassi & Hagström 2000, Morris et al. 2005, Schauer et al. 2003, Eilers et al. 2001, chapter III) and spatial scales (Pinhassi et al. 2003, Chapter II) including the vertical profile (Morris et al. 2004, Giovanonni et al. 1996, Wright et al. 1997) and latitudinal gradients (Selje et al. 2004, Pommier et al. 2005). This suggests that temperature, light, nutrients and the quantity and quality of DOM can modulate bacterial species composition in the ocean. Significant changes of bacterial assemblage structure have also been found along gradients of productivity (Yannarell & Tripplet 2004, Lindstrom 2000, Schafer et al. 2001, chapter II), but few field studies have attempted to document the relationship between primary productivity and bacterial diversity (Benlloch et al. 1995, Schäfer et al. 2001). Horner-Devine et al. (2003) assessed this topic with mesocosms mimicking small ponds, and suggested that different groups of bacteria (Bacteroidetes, Alpha-, and Betaproteobacteria) exhibited different responses (hump- versus U-shaped) along gradients of productivity.

The effect of top-down factors is also very significant in shaping bacterial assemblage composition. It is known that bacterial grazers (mainly flagellates) do not exert a uniform predation pressure on the bacterial assemblage (Jürgens & Matz 2002, Vázquez-Domínguez et al. 2005, Pernthaler et al. 1997, 2001). Additionally, several studies indicate that flagellates tend to graze upon the active component of the bacterioplankton assemblage (del Giorgio et al. 1996). If different bacterial groups have different levels of activity, then predation would be higher upon some specific groups of bacteria, and this could modulate the assemblage composition (Pernthaler 2005, Pernthaler & Amann 2005).

Viruses can also have important effects on bacterial assemblage composition, although their control is controversial. The fact that viral infection is highly specific and dependent on prey abundance lead to the postulation of the "killing the winner" hypothesis. This hypothesis suggests that viruses would control the most abundant (and successful) bacterial populations, preventing their dominance of the bacterial assemblage. Few studies have experimentally assessed such topic, and yielded controversial results (Weinbauer 2004, and pers. com.). In general, viruses are believed to be drivers of bacterial diversification, but the underlying mechanisms and the importance of lytic versus lysogenic phages remain unclear (Weinbauer & Rassoulzadegan 2004, Weinbauer 2004).

Can bacterial diversity control carbon processing and ecosystem functioning?

The role of diversity (i.e. all biotic variation from the level of genes to ecosystems across spatial, temporal, and biotic scales of organization) on ecosystem functioning (collective metabolic activities of organisms within a habitat consuming energy, moving matter between organic and inorganic pools, and controlling the flow of nutrients water and atmospheric gases) has long been studied, mostly from the perspective of ecosystem stability (Odum 1953). The term ecosystem resilience applies to the ability of an ecosystem to resist changes in environmental conditions and maintain an equilibrium. Naeem (2002) reviewed the general questions debated on this issue such as: are diversity and stability related? Are species unique or redundant in their contributions to ecosystem processes? Does niche complementarity lead to greater efficiency of resource use? Which are more informative, descriptive or experimental studies? Should species be grouped by their ecological properties rather than their taxonomic affiliations?

By far, most studies have focused on establishing a relationship between the effect of species richness (i.e. the number of species) and ecosystem functioning. Although several hypothesis have been postulated, three main relationships can be identified (Naeem et al. 2002, Fig. 5):

i) Species are primarily redundant, or partially substitutable. This implies that loss of species can be compensated for by other species, or the addition of new species does not add anything to the system.

ii) Species are primarily singular ("keystone species"), and thus make unique contributions to ecosystem functioning. Their loss or addition would cause detectable changes in functioning.

iii) Species impact is idiosyncratic or unpredictable and, therefore, makes different contributions to ecosystems depending on extrinsic and intrinsic factors. Idiosyncrasy does not mean that there is no effect of variation in diversity, not even that the response is unpredictable, but that the slope of the relationship is not monotonic.

It is noticeable, though, that using species richness as a simple measure of biotic diversity has no explicit explanatory power, since ecosystem level processes are affected by the functional characteristics of organisms, rather than by their taxonomic identity. Thus,

functional attributes of species and functional diversity must be considered if a mechanistic understanding of diversity effects is sought (Hooper et al. 2002).

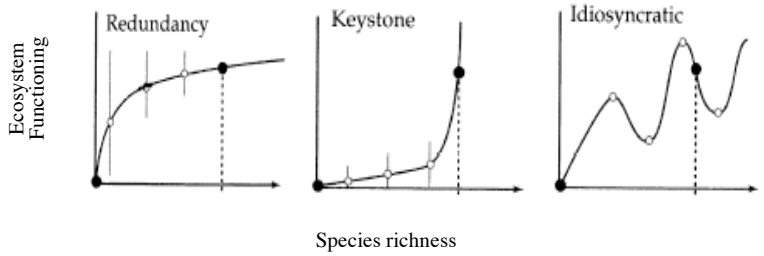

Fig 5- Relationship between species richness and ecosystem functioning (adapted from Naeem et al. 2002)

The large majority of the research on diversity and ecosystem functioning has focused on terrestrial habitats, while few studies have been carried out in aquatic ecosystems (Giller et al. 2004). Emmerson & Huxman (2002) re-examined published data on marine environments, mainly from benthic habitats, and found positive correlations between sediment species richness and ammonium flux, or between macrofaunal species richness and benthic secondary production. The study of how microbial and, especially, bacterial diversity is related to the functioning of ecosystems is one of the major challenges of current ecological research (Loreau et al. 2001), since bacteria carry out most of the biogeochemical transformations in all ecosystems.

Important limitations for the study of the relationship between prokaryotic diversity (i.e. species richness and evenness) and ecosystem functioning are the methodological difficulties to study bacterial diversity in marine waters, and the lack of a reliable and undisputed species concept (Rosselló-Mora & Amann 2001). The use of molecular fingerprinting methods has opened an avenue for this kind of research. Even if these techniques have lower phylogenetic resolution than clone libraries, they allow a reasonably straightforward comparison of the species composition in a large number of samples, and they provide the capability of following rapid changes in bacterial assemblage structure (Forney et al. 2004). Therefore, they provide the opportunity to investigate how bacterial community composition changes along environmental gradients or in response to a stress factor, and to correlate such differences to changes in carbon functioning.

Approaches for the study of the linkage between bacterial structure and function.

The linkages between bacterial diversity and ecosystem functioning have been studied from different perspectives. Basically, four approaches have been taken to assess this topic in aquatic ecosystems:

i) Correlation analyses between changes in bacterial diversity and bulk carbon processing.

The correlation of phylogeny and physiology in microbial organisms is becoming a crucial topic in our attempts to understand ecological systems (Giovanonni & Rappé 2000). Most studies in aquatic ecosystems have been based on transplant experiments, in which bacterial assemblages are grown under different conditions (different types of waters), and the effects of changes in bacterial assemblage structure (i.e. the relative abundance of species) on carbon processing are analyzed. Several studies in freshwater (Langenheder et al. 2005, Kirchman et al. 2004) and estuarine (del Giorgio & Bouvier 2002) environments have yielded contrasting results. Some studies suggest a weak coupling between bacterial assemblage composition and function, at least in broad-scale functions (e.g. biomass production or respiration, Langenheder et al. 2005, Kirchman et al. 2004). However, some experiments have shown functional changes associated with changes in assemblage composition (Kritzberg et al. 2006, Kirchman et al. 2004). Similar studies carried out under in situ conditions are very scarce, and have focused on the effect of richness (i.e. number of phylotypes in a sample) on carbon functions, such as respiration and production (Reinthaler et al. 2005, Winter et al. 2005, Arrieta et al. 2005). These studies have shown decreasing cell-specific BHP and BR with increasing bacterial richness, and Arrieta et al. (2005) suggested different relationships between species richness and leucine incorporation for Bacteria and Archaea.

In this thesis we focused on the effect of changes of in situ bacterial assemblage structure, instead of bacterial richness, on the carbon metabolism through natural gradients. This correlation approach, to our knowledge, has seldom been reported before in studies of marine bacterial assemblages (but see Fuhrman et al. 2006), and can provide valuable insights into the correlates of carbon processing. However, a drawback that has to be taken into account is that effects on carbon processing due to diversity cannot be readily separated from effects due to *in situ* covarying factors, such as environmental (temperature, DOC concentration, etc.) parameters.

ii) Study of the metabolism of isolated bacteria: Efforts on isolation of representative marine phylotypes.

The isolation of representative members of marine bacterial groups would allow to study of their physiological properties and metabolic capabilities. However, most phylotypes cultured by traditional techniques (isolation on agar plates) are not representative of environmentally dominant marine bacteria (Giovanonni & Rappé 2000). There is a strong recovery bias that now seems to be associated to the inability of oligotrophic marine bacteria (including the SAR11 cluster) to form colonies on agar surface, possibly as a strategy for optimizing the access to nutrients of individual cells (Simu & Hagström2004). New culturing techniques, such as dilution to extinction (Button et al. 1993), have been developed to retrieve marine oligotrophic bacteria. A remarkable result of the use of this method was the culturing of a member of the dominant bacterial group SAR11 (*Pelagibacter ubique*, Rappé et al. 2002), which has allowed characterizing the metabolic properties of a real oligotrophic marine bacterium that dominates the ocean.

iii) Combination of single-cell activity techniques with phylogenetic probes to study the role of specific populations.

Some culture-independent techniques have allowed studying the role that many bacteria, which cannot be successfully isolated, play in the environment. The study of rRNA instead of rDNA can provide some clues about the active fraction of a community (Moeseneder et al. 2001). This technique, however, can only indicate which bacteria have higher proportions of ribosomes and potential for being active, but it does not show whether they were indeed active, nor how active they were. The advent of single-cell techniques combined with phylogenetic probes has allowed in situ studies of the metabolic characteristics of bacterial groups. Besides the utility for knowing the ecology of the uncultured bacteria, these techniques are also highly informative for their cultured counterparts, since properties determined in the laboratory may not necessarily reflect the activities and physiology in the environment.

The two most important single-cell approaches are: cell sorting combined with phylogenetic analysis and MAR-FISH (microautoradiography combined with FISH). Cell sorting of specific cytometric populations (such as high and low-nucleic acid content cells, HNA and LNA) has been successfully combined with the analysis of their phylogenetic affiliation by FISH or PCR-based techniques, and with the addition of radioactive probes to measure group-specific activities (Servais et al. 2003, Lebaron et al. 2002, Zubkov et al. 2001, Fuchs et al. 2005). In

such studies, different populations of bacteria can be phylogenetically identified, and their activity (in bacterial production or in the uptake of selected compounds) can be quantified (Zubkov et al. 2001, Longnecker et al. 2005). As an example, Zubkov et al. (2001) observed that HNA-populations dominated by Roseobacter and Bacteroidetes showed higher specific methionine incorporation activities as compared to the LNA-population, dominated by the SAR86 group in the Celtic Sea. However, biomass specific activities were similar for both groups, and LNA cells exhibit higher growth-specific rates.

MARFISH has become a very powerful technique for studying different metabolic capacities of bacterial populations. Studies with this technique have indicated different uptake activities of selected compounds (such as glucose, amino acids or proteins) by different phylogenetic groups of bacteria, and have even provided estimates of their contribution to total bacterial production (Cottrell & Kirchman 2003, Herndl et al. 2005). Some significant results obtained by this technique are the high heterogeneity in the bacterial uptake patterns of different DOM compounds (Cottrell & Kirchman 2000), the high heterotrophic activity (amino acid uptake) of *Archaea* in marine waters (Ouverney & Fuhrman 2000, Teira et al. 2004) and, more recently, the autotrophic capabilities of this group (Herndl et al. 2005). A limitation of this technique is that it depends on the use of specific phylogenetic probes, which might be covering only a fraction of the bacterial groups in a sample, or that target organisms at a phylogenetic level different of which the metabolic characteristics should be analyzed.

iv) The genomics approach.

The genomic approach consists on the analysis of the complete genomes of isolated bacteria. By the knowledge of the information contained in their genes, their metabolic capabilities can be derived, although to what extent are those genes functional remains unknown. As a representative example, the genome sequencing of a member of the ubiquitous SAR11 group (candidatus *Pelagibacter ubique*), revealed that this organism can use either phototrophy or respiration to conserve energy (Giovanonni et al. 2005). However, an important drawback of this method is that it requires the a priori isolation of the bacterium, which can be difficult for marine oligotrophic bacteria, as discussed before.

Recently, a metagenomic approach has circumvented the culturing approach, by the massive sequencing of genomic DNA from the environment. Such approach can even be used to reconstruct the genomes of uncultured organisms (Handelsman 2004). If sequencing is performed guided with a phylogenetic anchor, which means that only genes containing a phylogenetic marker are sequenced, this technique allows the linkage of phylogeny with

functional genes. Béjà et al (2000) associated proteorhodopsines to the SAR86 group of uncultured bacteria, and Venter et al. (2004) carried out one of the largest metagenomics projects to date in the Sargasso Sea, and found a high number of genes encoding for rhodopsin-like photoreceptors. Those genes were linked to a wide range of bacterial phylogenetic groups, including some taxa that were not previously known to contain light-harvesting functions, such as the Bacteroidetes group.

Future perspectives in the approaches to link bacterial assemblage structure and function include functional metagenomics (which consists in the transcription and translation of the gene or genes of interest and analysis of the gene product) and proteomics. The use of microarrays will also be very helpful in order to study patterns of gene expression of bacterial assemblages. Stable isotope probing of nucleic acids, has been recently developed in order to determine the metabolic capabilities of the active components of natural communities (Radajewski et al. 2000). By this technique, genomic DNA is extracted after the incubation of the bacterial assemblage with ^{13}C or ^{12}C compounds, and isotope-enriched DNA is purified by equilibrium centrifugation in CsCl ethidium bromide density gradients. A strength of this method is that enriched DNA will contain the entire genome of each functionally active component of the community. However, important drawbacks are that it is necessary to add a large excess of labeled substrate and use long incubation times to maximize its uptake, thus potentially biasing the results. Targeting RNA instead of DNA may reduce the need for long incubations periods in future improvements of this method. Finally, substantial improvements could be done on current single cell techniques, such as MARFISH, including its coupling with in situ PCR methods (Hodson et al. 1995, Chen et al. 1999), and the application of quantitative methods such as track or grain density autoradiography (Davenport & Maguire 1984, Carney & Fahnenstiel 1987) to evaluate relative or actual substrate uptake rates within mixed communities.

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Aims and outline of the thesis

This thesis has addressed three general questions:

i) Is marine bacterial assemblage structure related to carbon functioning?
ii) What are the bacterial populations actively driving the carbon flux in marine waters?
iii) How is the in situ activity of specific marine bacterial populations affected by environmental factors?

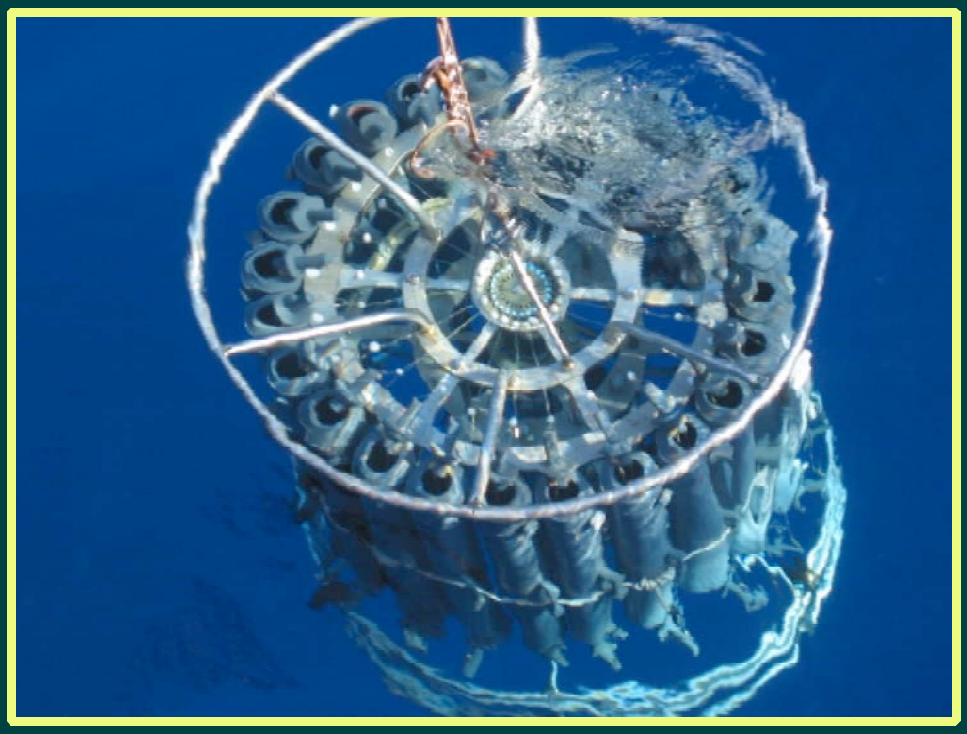
In order to assess the first question (i) we applied a correlation approach through natural gradients (spatial and temporal) in two different studies. Although a positive correlation does not imply causality, the main goal was to assess the link between the variability of bacterial assemblage structure and that of aggregate carbon processing properties at in situ conditions (without manipulations). In other words, we wanted to know whether in situ changes in bacterial assemblage structure were associated with changes in bacterial carbon processing, or whether similar bacterial communities could lead to different patterns of carbon processing.

Two studies were carried out in two different marine systems in order to assess this linkage. The first study was carried out along a spatial trophic gradient covering two transects in the NE Subtropical Atlantic Ocean, from the nutrient-rich NW African coast (including the Cape Blanc upwelling) to the oligotrophic North Atlantic gyre (**Chapters I and II**). The second study focused on the temporal succession of the bacterial assemblage in a coastal oligotrophic site from the NW Mediterranean. Monthly measurements of different bacterial carbon metabolic activities along with bacterial assemblage composition were carried out throughout a complete seasonal cycle, from March 2003 to March 2004 (**Chapters III and IV**).

The analysis of the general patterns of carbon metabolism and bacterial assemblage structure required a substantial effort in extensively measuring and understanding both parameters, since different approaches are used by different researchers, none of them free of ambiguities, and they have been very seldom combined in in situ studies of these characteristics. Specifically, **Chapter I** tried

to constrain and reduce the problems associated with the measurement of bacterial production (focusing on the empirical carbon-to-leucine conversion factors) and growth efficiency in oceanic waters. In the seasonal study in coastal waters, a great effort was made to exhaustively describe bacterial diversity and to compare different techniques to measure bacterial diversity and assemblage composition (**Chapter III**).

In order to assess the other general issues (ii and iii), we used a single-cell technique, which is MARFISH. Specifically, the identification of the active bacterial populations in carbon processing was assessed along the seasonal study in Blanes Bay, by analyzing the uptake of different substrates by distinct bacterial groups as it varied throughout the year (**Chapter V**). Finally, MARFISH was applied during a set of experiments with water from Blanes Bay in order to assess the effect of an environmental factor (UV and PAR radiation) on the specific single-cell activity of different bacterial groups (**Chapter VI**).



Chapter I

Large-scale variability in surface bacterial carbon demand and growth efficiency in the subtropical North East Atlantic Ocean

L. Alonso-Sáez, J. M. Gasol, J. Arístegui, J. C. Vilas, D. Vaqué, C. M. Duarte & S. Agustí
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Abstract

We present surface estimates of bacterial respiration (BR), bacterial heterotrophic production (BHP), and growth efficiency (BGE), and their relationship with nutrient availability, along a trophic gradient from coastal upwelling waters to the open-ocean waters of the eastern North Atlantic. BR generally ranged between 10 and 30 μg C l^{-1} d^{-1} and was relatively unaffected by nutrient enrichment. In contrast, BHP showed higher variability (more than one order-of-magnitude range) and was affected by carbon and/or phosphorus additions in different regions. Empirical bacterial carbon-to-leucine conversion factors (CFs) (range 0.02-1.29 kg C mol Leu^{-1}) decreased from the coast to the open ocean, largely influencing the BHP estimates in oligotrophic waters. We found high percentages of leucine respiration in oceanic waters (average 68% of leucine taken up by bacteria), possibly related to the low conversion factors found offshore. Empirical CFs were highly correlated to BGE (Pearson correlation coefficient r= 0.86, n= 12, p< 0.0004, log-log transformed), which varied between 1% in offshore waters and 56% in the upwelling. Empirical CFs could be critical not only for accurately constraining BHP, but possibly also for predicting BGE in oceanic waters.

Introduction

Planktonic heterotrophic bacteria are key players in the oceanic carbon cycle, recycling the large dissolved organic carbon reservoir (Hansell & Carlson 1998). However, methodological difficulties have limited a comprehensive understanding of bacterial carbon flux in the oligotrophic ocean. Even if bacterial respiration (BR) is the key parameter required to constrain both carbon remineralization and biogenic carbon export in the ocean (Rivkin & Legendre 2001), the available estimates of BR and bacterial growth efficiency (BGE) for oceanic waters are still very scarce. The precise measurement of these parameters is particularly important in the open ocean, where most of the carbon that bacteria process is respired, and BGE can be as low as 1% (del Giorgio & Cole 1998).

The accurate measurement of bacterial heterotrophic production (BHP) is also of great importance for the determination of bacterial growth rates and energetics in marine waters. BHP has been extensively measured over most oceanic regions (e.g., Ducklow & Carlson 1992), but the conversion factors required to convert the incorporation of leucine or thymidine to biomass accumulation are poorly constrained (Ducklow et al. 2002). The existing empirical

conversion factors are often very different from the widely used theoretical estimates, especially in oligotrophic waters. However, few studies have tried to assess what factors control their variability in natural systems and to what extent the use of standard theoretical factors can seriously affect BHP estimates.

In this study we examined the spatial variability in bacterial carbon flux in the NE Subtropical Atlantic across the transition zone between coastal waters, affected by the NW Africa Upwelling system, and the central open-ocean waters of the North Atlantic subtropical Gyre. BGE estimates had not yet been reported for this area. We assessed bacteria carbon cycling with two main objectives: (1) to constrain the range of variability of key parameters such as BR, BHP, and BGE and empirical carbon-to-leucine conversion factors and (2) to study the relationship between these metabolic processes and their in situ potential controlling factors.

Materials and methods

The study was conducted along two transects extending from the NW African coastal waters towards the open-ocean waters of the North Atlantic subtropical gyre (Fig 1), during two cruises on board the BIO Hespérides (COCA-I: 10 September- 03 October 2002, COCA-II: 20 May-10 June 2003).

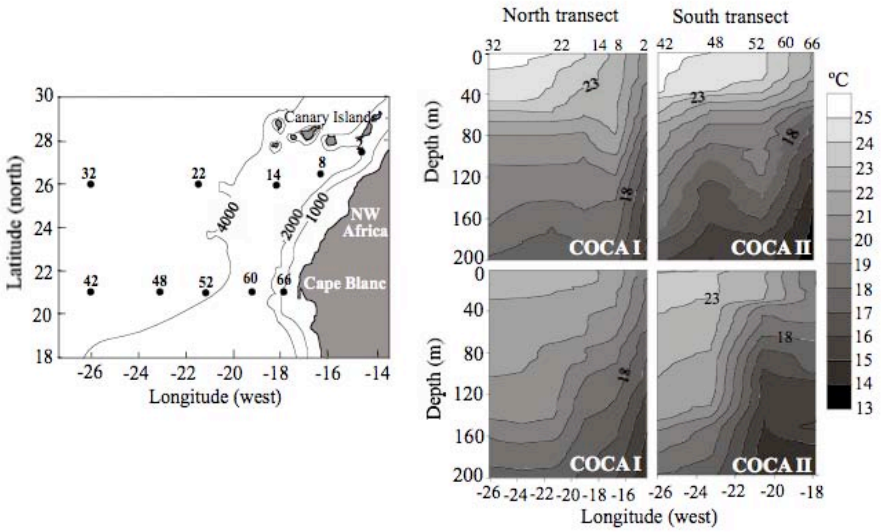

Fig 1- Map of the sampling area showing the stations No and location (left panel) and depth profiles of water temperature (ºC) along the North and South transects during the COCA I and COCA II cruises (right panel). Stations No indicate their position in the transects.

The main study was performed with samples collected from surface water (5 m) in 10 open water stations (depth>1000 m), during the COCA II cruise. Four or five additional depths were sampled throughout the euphotic zone (considered down to the depth reached by 1% of incident PAR light) at all stations for measurements of chlorophyll, bacterial abundance and bacterial heterotrophic production. During the COCA I cruise, the stations were sampled at a variable depth within the euphotic zone (25 to 85 m) for bacterial growth efficiency and resource limitation experiments. At each station temperature, salinity, and fluorescence were recorded using a CTD system (Idronaut MK-317 and Mark III-IOC) mounted on a General Oceanic rosette sampler equipped with 24 12-L Niskin bottles.

Chlorophyll, POC and PON. Samples (250 ml) were filtered through Whatman GF/F filters to determine the Chlorophyll *a* (Chl *a*) concentration. The filters were homogenized and kept refrigerated in the dark while pigments were extracted in 90% acetone for ca. 1 h. Fluorescence of the extracts was measured in a Turner Designs fluorometer.

Samples (2 l) for POC and PON were filtered through combusted (450°C, 12 hours) 25 mm Whatman GF/F filters. The filters were wrapped in combusted aluminum foil and frozen at –20°C until processed (few weeks later). In the laboratory, the filters were thawed and dried overnight at 65ºC in a desiccator with HCl fumes to remove carbonates, and finally dried overnight in a desiccator with silica gel. Previous to analysis, samples were packed into ultra-clean tin disks. The carbon analyses were performed on a Perkin Elmer-2400 CHN elemental analyzer, according to the JGOFS protocol (UNESCO, 1994).

Bacterial abundance and biomass-. Bacterial abundance was determined by flow cytometry. Samples (1.6 ml) were preserved with 1% paraformaldehyde + 0.05% glutaraldehyde (final concentration), let 10 min in the dark to fix, deep frozen in liquid nitrogen and stored at –80ºC. The samples were later thawed, stained with Syto13 (Molecular probes) at 2.5 μmol l^{-1} (diluted in DMSO) in the dark for a few minutes, and run through a Becton and Dickinson FACSCalibur cytometer with a laser emitting at 488 nm. Data were obtained in log mode until around 100000 events were acquired. Bacteria were detected by their signature in a plot of side scatter (SSC) versus FL1 (green fluorescence) as explained in Gasol & del Giorgio (2000). Picocyanobacteria were discriminated in a plot of FL1 versus FL3 (red fluorescence). When prochlorophyte fluorescence was very low, they were first enumerated in an unstained sample, and their abundance subtracted from the total bacteria determined with Syto13 staining. Bacterial biomass was estimated from bacterial abundance assuming a conservative conversion factor of 12 fg C $cell^{-1}$ (Fukuda et al. 1998)

Bacterial heterotrophic production- BHP was estimated from the rates of protein and DNA synthesis determined by the incorporation of tritiated leucine or thymidine respectively, into cold trichloroacetic acid (TCA). Leucine (Leu) and thymidine (TdR) were each added at saturating concentration (40 nmol l^{-1}) to four experimental replicates of 1.2 ml. Duplicate controls were established with the addition of 120 µl 50% TCA before the isotope addition. The Eppendorf tubes were incubated from 2 to 3 hours at temperatures as close as possible to the in situ in either water baths or temperature-controlled chambers. The incorporation was stopped with the addition of 120 µl of cold 50% TCA to the Eppendorf tubes and samples were kept frozen at –20ºC until processing. This was done following the centrifugation method of Smith & Azam (1992) with two runs (ca. 12000 rpm) and aspiration of the water. Finally, 1 ml of scintillation cocktail was added to the Eppendorf tubes, and they were counted after 24-48 h on a Beckman scintillation counter, with quenching correction using an external standard. The average coefficient of variation for the quadruplicate determinations was 15%.

We performed 13 experiments in order to determine the in situ carbon-to-Leu conversion factors (CFs) for surface water communities. The water-sample was gently filtered through 0.6 µm polycarbonate filters (Millipore, GTTP), then diluted (1:9) with 0.2 µm filtered (Acropack 1000, Pall) seawater, and incubated in 2 l acid-clean polycarbonate bottles in the dark. Size fractionation was needed in order to effectively remove all predators. Dilution was used to further promote bacterial growth in the time scale of the experiments. Subsamples were taken for Leu incorporation and BA measurements at every 12-24 hours until bacteria reached the stationary growth phase. Factors were computed with the cumulative method (Bjørnsen & Kuparinen 1991), which maximizes the use of the available data.

Bacterial respiration- BR was measured by following changes in dissolved oxygen during dark incubations of filtered water. The water was gently filtered with a peristaltic pump through glass fiber filters with approximate pore sizes of 0.6-0.8 µm and 14 cm of diameter (AP1514250 Millipore) to increase the filtering surface, reduce pressure, and avoid cell breakage. The efficiency of filtration was analyzed during the COCA I cruise in five stations. We found that, after filtration, we recovered on average (±SE) 78 ± 5% of total bacteria. Eight BOD bottles were carefully filled, and three replicate bottles were immediately fixed with Winkler reagents to determine the initial oxygen concentration. Four replicate bottles were incubated in the darkness at in situ temperature and fixed with Winkler reagents after approximately 24 hours. At each time step one additional replicate bottle was used to determine bacterial abundance and production as described above. The optimal incubation

time (24 h) was set from previous measurements in the COCA I cruise, when samples were taken at times zero, 12, 24, and 36 hours from filtered and unfiltered water of five stations: sta 2 and 8 (80 m), sta 32 (30 m), sta 48 (50 m) and sta 60 (40 m). Dissolved oxygen measurements were made by automated Winkler titrations based on colorimetric end-point detection (as described in Arístegui et al. 2005). The rate of respiration was determined by regressing O_2 against time for the 0-24 hours interval. We assumed a respiratory quotient of 0.88 (Williams & del Giorgio 2005).

Bacterial growth efficiency (BGE)- BGE was estimated as the ratio of bacterial production to production plus respiration in the filtered seawater (bacterial fraction). For that calculation, BR was estimated as described in the previous section in 24 h incubations, and bacterial net production was estimated in five different ways: a) in situ BHP based in Leu uptake using a theoretical CF (1.5 kg C mol Leu^{-1}, which assumes no isotope dilution), b) in situ BHP using the experimentally determined CF for each station, c) BHP at time zero of the incubation experiment (with the theoretical CF, 1.5 kg C mol Leu^{-1}), d) the integrated BHP in the 24 h incubation interval using the empirical CFs, and e) the change in bacterial biomass, estimated from the increase in cell numbers along the incubation and using a conservative factor of 12 fg C $cell^{-1}$ (Fukuda et al. 1998).

Nutrient limitation assays- During the first cruise (COCA I) we sampled five stations at a variable depth within the euphotic layer and the assays included experimental assessment of nutrient limitation of BHP and BR. For this purpose four gas-tight bilaminated plastic bags were completely filled with unfiltered seawater (approximately 15 l). Bubbles were carefully removed before starting the experiments. We established four treatments, inorganic additions (nitrate, ammonium and phosphate, 0.5 μ mol l^{-1} each), organic additions (glucose and acetate, 1 μmol l^{-1} each compound, 8 μmol C l^{-1} in total), inorganic plus organic additions, and an unamended control. The bags were incubated inside water baths adjusted to the in situ temperature. All treatments were sampled every day for BHP, BA, and oxygen concentration, and the data were integrated over the 96 h of incubation.

In the second cruise (COCA II) a simpler assay was designed, where only the effect on BHP was measured. Samples were taken from surface waters at all stations. Well cell plates (IWAKI) were filled with subsamples (10 ml) of unfiltered seawater. Nitrate plus ammonium (0.5 μmol l^{-1} final conc. each), phosphate (0.5 μmol l^{-1} final conc.), glucose plus acetate (1 μmol l^{-1} final conc. each compound), and a mixture of all of them (at the same concentrations) were added as enrichment treatments. Two subsamples were left unamended as replicated controls.

The plates were incubated at in situ temperature inside dark temperature-regulated chambers and we measured BHP after 24-48 hours.

Leucine respiration. Respiration and incorporation into biomass of uniformly labeled ^{14}C-Leu was measured in four replicated samples (4 ml) placed in 25 ml incubation Erlenmeyer flasks (Kimble/Kontes, Vineland, NJ ref 882360) and one control, which was initially fixed with TCA (5% final conc). We basically followed the protocol of Hobbie & Crawford (1969) with some modifications. L-[U-^{14}C-Leu] (Amersham, CF3183) was added (60 nmol l^{-1}) to the samples and the Erlenmeyer flasks were immediately closed with a rubber stopper fitted to a plastic well containing a piece of filter paper (Whatman n. 1 chromatographic paper). After 2-4 hours incubation at the in situ temperature, 200 µl of H_2SO_4 (2N) were injected with a syringe into the lateral arm of the Erlenmeyer flasks to stop the incubation. After 1 hour during which the Erlenmeyers were smoothly shaken several times to liberate the carbon dioxide, 200 µl of 2-phenylethylamine (P2641 SIGMA) was injected into the plastic well inside the Erlenmeyers until the filter paper was completely wet. We waited 30 minutes for fixation of the carbon dioxide onto the filter paper and opened the Erlenmeyer flasks. The filter papers were placed in a vial with Optisafe HiSafe-2 cocktail to estimate the respired fraction. Triplicate samples of 1.2 ml were taken from each Erlenmeyer to measure the incorporated fraction. We added 120 µl of 50% TCA to the subsamples and processed them with the centrifugation method described above for BHP.

Protein turnover. We followed the Kirchman et al. (1986) pulse-chase approach to determine whether there was turnover of intracellular proteins in the bacterial assemblages. Surface samples were treated with 100 mg l^{-1} cycloheximine to inhibit eukaryotes and to ensure that the decrease in radioactivity was due to protein turnover and not due to grazer activity on labeled ingested bacteria. We also run some parallel experiments in which water was filtered through 0.8 µm with a hand-held syringe and a 25 mm Nucleopore filter, but we saw no differences between both ways of removing the eukaryote activity. After preincubation with cycloheximine for ca. 20 min. ^{3}H-Leu was added at 40 nmol l^{-1} and samples taken every 15 min for ca. 120 min. Then a cold chase of 100 µmol l^{-1} unlabeled Leu was added to the samples, and radioactivity in the bacterial protein was measured with the protocol explained above every 30-60 min for the following 5-7 hours. Protein turnover is detected when there is a significant decrease in radioactivity after the addition of the unlabeled Leu. Results are expressed as % of the label recycled per hour.

Results

Regional oceanographic settings

The sampling area comprised 10 stations distributed along two zonal sections (5 stations at each section, Fig. 1). The Northern section (26ºN) extended offshore from the boundary of the coastal upwelling jet, near Cape Bojador, where an upwelling filament affected the station closest to the coast (Sta 2) in both cruises. Stations 8 and 14 were placed in the coastal transition zone between the coastal upwelling waters and the oceanic area. The southern section (21ºN) extended from the Cape Blanc coastal upwelling waters to the open ocean, crossing the Cape Vert Frontal Zone (CVFZ). During the COCA II cruise the upwelling core was located at 21°N and its influence reached as far away as station 52 (Fig. 1).

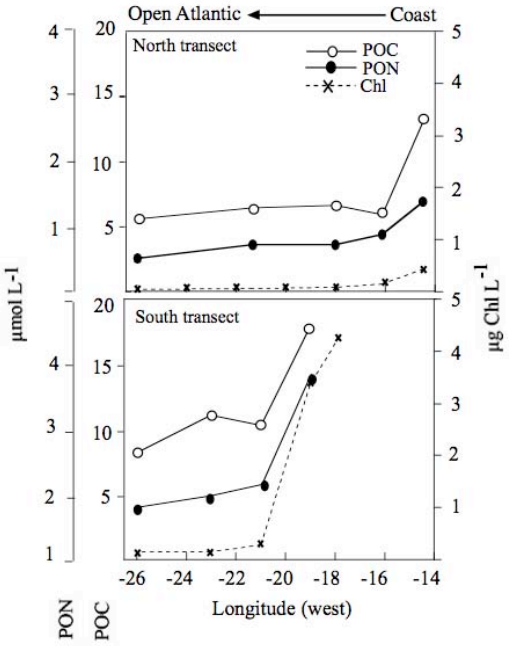

Fig 2- Measurements of POC, PON and Chl *a* at surface waters along both transects of the cruise COCA II.

The upwelling stations and the upwelling filament were generally characterized by higher concentrations of surface Chl *a*, POC and PON compared to the offshore stations (Fig. 2). During the COCA I cruise, the Cape Blanc upwelling center was placed at a more northern position and hence, its influence was restricted to the most coastal station (Fig. 1).

Table 1. In situ measurements of bacterial respiration (BR), leucine-based bacterial heterotrophic production (BHP) -with a theoretical conversion factor (CF) of 1.55 kg C mol Leu^{-1} and the empirical CFs determined for each station- and three BHP estimates obtained from the incubations and used to determine BGE: (1) Inital BHP (CF 1.55 kg C mol Leu^{-1}), (2) integrated BHP in 24 h of incubation calculated with empirical CFs, and (3) change in bacterial biomass (BBM), estimated from cell abundance and a conservative factor of 12 fg C cell^{-1} (Fukuda et al. 1998). BGE range was calculated using all BHP estimates and the BGE average was calculated using two BHP estimates (initial time and change in biomass, see text). Bacterial carbon demand (BCD) was estimated as BR + BHP in situ (with empirical CF). Zones are UPW: upwelling, NT: Northern transect, ST: Southern transect. [a] BHP with theoretical CF was used for the calculation since empirical CFs were not estimated.

Cruise	Zone	Stn	Depth	BR	Emp CF	BHP in situ		BHP (time 0)	Integ. BHP	ΔBBM.	BGE	BGE	BCD
						Theor. CF	Emp. CF	Theor. CF	Emp. CF	12 fg C cel^{-1}	range	avg	
			(m)	(μg C L^{-1} d^{-1})	(kg C mol Leu^{-1})	(μg C L^{-1} d^{-1})		(μg C L^{-1} d^{-1})	(μg C L^{-1} d^{-1})	(μg C L^{-1} d^{-1})	(%)	(%)	(μg C L^{-1} d^{-1})
Coca II	UPW	2	5	10.9	0.25	2.2	0.36	0.43	1.9	-	3 - 17	-	11.2
	NT	8	5	10.6	0.21	1.1	0.14	0.17	0.8	4.6	1 - 30	16	10.7
	NT	14	5	9.7	0.21	0.6	0.08	0.06	1.9	2.7	1 - 22	11	9.8
	NT	22	5	2.2	0.02	2.1	0.03	0.03	0.2	0 .02	1 - 49	1	2.2
	NT	32	5	11.3	0.16	5.9	0.63	0.27	1.6	1.4	2 - 34	7	11.9
	ST	42	5	17.7	0.10	5.1	0.32	0.48	0.9	4.0	2 - 23	11	18.0
	ST	48	5	22.2	0.17	3.3	0.37	0.96	2.5	4.5	2 - 17	11	22.6
	UPW	52	5	12.1	0.42	0.9	0.26	0.82	4.0	4.4	2 - 27	17	12.4
	UPW	60	5	30.8	1.29	2.6	2.15	43.1	42.3	34.3	7 - 58	56	32.9
	UPW	66	5	16.4	0.70	3.6	1.65	4.9	6.7	2.2	9 - 29	17	18.0
Coca I	UPW	2	80	63.9	-	0.04	-	0.73	-	3.6	< 1 - 5	3	63.9[a]
	NT	8	80	52.5	-	0.48	-	0.84	-	2.8	< 1 - 5	3	53.0[a]
	NT	32	30	45.4	0.14	0.51	0.05	1.92	7.3	2.4	<1 - 14	5	45.4
	ST	48	50	17.4	0.36	2.29	0.53	1.88	13.0	2.3	3 - 43	11	17.9
	ST	60	40	32.8	0.08	0.46	0.02	4.80	2.0	6.2	<1 - 16	14	32.8

Bacterial abundance and heterotrophic production.

During the COCA II cruise, surface bacterial abundance (BA) ranged from 0.41 to 5.65 x 10^6 bact ml^{-1}, being maximal at the stations closer to the Cape Blanc upwelling and lowest at the offshore stations (Fig. 3b). Both estimates of bacterial heterotrophic production (^{3}H-Leu and TdR uptake) were highly correlated through the euphotic zone (1 to 6 discrete depth samples per profile, Pearson correlation coefficient r= 0.89, n= 25, p<0.000001, log-log transformed), and ranged from 15 to 331 pmol Leu l^{-1} h^{-1} and 1 to 40 pmol TdR l^{-1} h^{-1}. The highest uptake rates were found at the offshore stations (159 and 139 pmol Leu l^{-1} h^{-1}, in stations 32 and 42 respectively, and 14 pmol TdR L^{-1} h^{-1} in both stations). On the other hand, the highest values of the empirical carbon-to-Leu CFs (0.7 and 1.29 kg C mol Leu^{-1}) were found at the stations closest to the upwelling with values closer to the theoretical CF of 1.55 kg C mol Leu^{-1} (Table 1). The CFs calculated in the offshore stations were lower, averaging (±SD) 0.14 ± 0.07 kg C mol Leu^{-1} (Table 1).

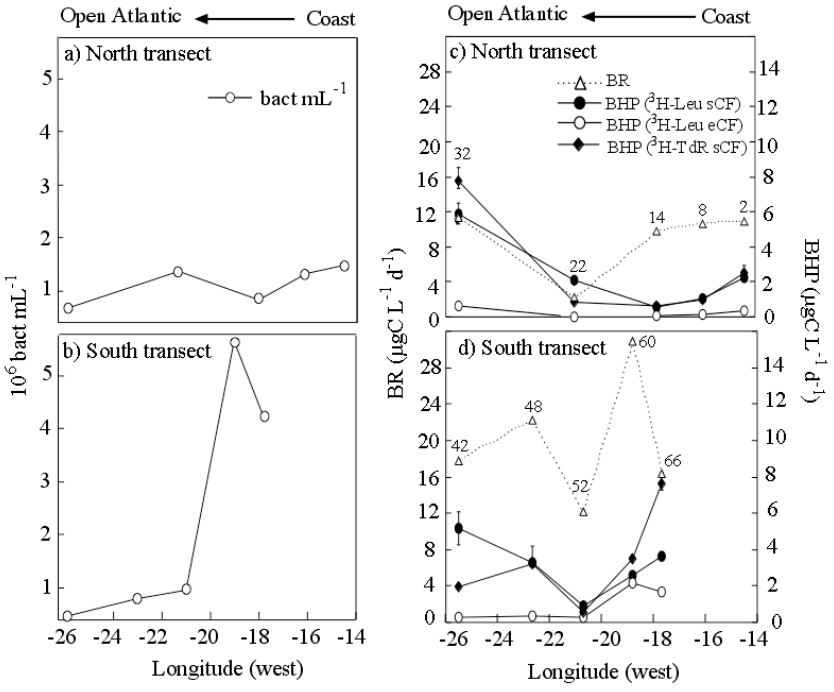

Fig 3- (a, b) Estimates of bacterial abundance and (c, d) measurements of bacterial respiration (BR) and bacterial heterotrophic production (BHP) estimated from leucine uptake (with standard, 1.55 kg C mol leu^{-1}, and empirical conversion factors) and thymidine uptake along the two transects (COCA II cruise). Stations' No are included above each corresponding data point in panels c) and d). eCF: empirical conversion factor, sCF: standard conversion factor.

Applying the empirical CFs, the BHP estimates were generally higher in the upwelling stations (with the exception of offshore station 32, in the north transect) and ranged between 0.03 and 2.15 μg C l^{-1} d^{-1} (Fig. 3). In contrast, using a theoretical factor of 1.55 kg C mol Leu^{-1}, the offshore stations at both transects yielded maximum BHP values (Fig. 3c,d), with estimates over 5 μg C l^{-1} d^{-1}. This difference also affected estimates of integrated BHP in the euphotic zone, which derived with a theoretical CF ranged between 24 and 581 mg C m^{-2} d^{-1}, while the range was much lower using the empirical CFs determined in the surface waters (1 to 127 mg C m^{-2} d^{-1}, Table 2). When we compared all the individual estimates in the euphotic zone, the values of BHP were only significantly higher in the upwelling area if empirical CFs were used (Anova $p < 0.05$, Tukey-Kramer posthoc test).

Table 2. Integrated values through the euphotic layer (1% PAR) of Chl *a*, bacterial biomass (BBM), bacterial heterotrophic production using the standard conversion factor (CF) 1.55 kg C mol Leu^{-1} (BHP^1) or the empirical CFs determined in each station at surface depth (BHP^2), and bacterial carbon demand during the COCA II cruise. For the BCD calculation we used the BR estimated for surface waters kept constant, and the BHP measurements with empirical CFs through the euphotic zone (range 3-6 discrete depths, average of 5). The depth corresponds to the limit of the euphotic layer.

Sta	Zone	Depth (m)	Integ. Chl *a* (mg Chl *a* m^{-2})	Integ. BBM (g C m^{-2})	Integ. BHP^1 (mg C m^{-2} d^{-1})	Integ. BHP^2 (mg C m^{-2} d^{-1})	Integ. BCD (g C m^{-2} d^{-1})
2	UPW	73	32	1.31	24	4	0.74
8	NT	96	28	1.08	325	44	1.00
14	NT	97	33	1.13	94	13	0.92
22	NT	116	27	1.60	103	1	0.25
32	NT	114	9	0.76	581	60	1.30
42	ST	100	30	0.59	379	24	1.70
48	ST	85	27	0.81	225	25	1.80
52	UPW	50	20	0.77	44	12	0.55
60	UPW	57	54	1.03	153	127	1.48
66	UPW	60	91	1.25	137	62	0.96

Bacterial respiration, growth efficiency, and carbon demand

During the COCA I cruise we analyzed respiration rates of whole unfiltered seawater and the filtered bacterial-size fraction in five stations. The respiration rate in filtered samples averaged (±SE) 78 ± 9% of the total microplankton respiration. The optimal incubation time for respiration measurements was also determined in these experiments. In general, BHP increased during the incubation, while the oxygen consumption rate was not significantly different from a constant decrease during the first 24 hours.

During the COCA II cruise, BR was quite constant in the northern transect (around 10 μg C l^{-1} d^{-1}, Fig. 3c) except for sta 22, which showed a lower respiration rate (Table 1). In the southern transect, BR was more variable and significantly higher, with an average value of 20 μg C l^{-1} d^{-1} (Fig. 3d). The surface BR estimates did not differ significantly between the upwelling and the offshore stations (ANOVA test, $p > 0.05$), although the limited data set (only a single measurement for each station) could probably affect the sensitivity of this analysis. BR was generally higher during the COCA I cruise than during the COCA II cruise, particularly in the North transect stations (Table 1).

Surface and integrated estimates of bacterial carbon demand (BCD) followed the same pattern of BR and were in general rather constant (3 fold range excluding station 22, Tables 1 and 2). Similarly, the surface BCD values were higher during the COCA I cruise (range 18 to 64 μg C l^{-1} d^{-1}) compared to COCA II (range 2 to 33 μg C l^{-1} d^{-1}, Table 1).

We calculated a range of BGE values for each station using the BR and five different estimates of BHP (see Methods section), which yielded quite different results (Table 1). The calculation of BGE can use the in situ values of BHP, the initial BHP of the BR experiment or the integrated BHP throughout the 24 h incubation. Furthermore, empirical or theoretical CFs can be used to convert Leu uptake into biomass production. Finally, net bacterial production can also be calculated from the increase in bacterial biomass during the BR determinations.

BHP determined at the initial time of the incubation (using the theoretical CF 1.55 kg C mol Leu^{-1}) was in the same range than the in situ measurements (i.e., with water directly obtained from the oceanographic bottle) only when the in situ measurements were calculated with the empirical CFs. A remarkable exception was station 60, which experienced a significant increase in both Leu and TdR-based BHP estimates after filtration of the seawater. Similar to in situ samples, Leu and TdR-based BHP estimates were highly correlated in these samples at time zero (Pearson $r = 0.90$, $n = 10$, $p < 0.0004$ log-log transformed, TdR results not shown).

BHP integrated during the 24 h incubations, or estimated from the change in BA assuming a conservative conversion factor for marine bacteria of 12 fg C $cell^{-1}$ (Fukuda et al. 1998), yielded significantly higher values of biomass production than the initial Leu-based BHP.

The resulting ranges of BGE are quite wide due to order-of-magnitude differences in the various approaches to calculate BHP. The median lower boundary of the range was 2% whereas the median upper boundary of the range was 28% across stations during the COCA II cruise (Table 1). We chose to average the BGEs obtained using the BHP at the initial time of the incubation and the change in biomass during the incubation as the most reliable estimation of BGE (see discussion). Calculated in this way, BGEs were lower for the oceanic stations during the COCA II cruise (range 1 to 11%) and higher towards the coast (range 11 to 17% with a higher value of 56% at the upwelling station 60). The values of BGEs in the five stations during the COCA I cruise were in the same range (3 to 14%).

Protein turnover and leucine respiration

The Leu-to-carbon CFs obtained (range 0.02 to 1.29 kg C mol^{-1}, Table 1) were low compared to the standard theoretical factor. Intracellular protein turnover and respiration of the added Leu (instead of incorporation) by bacteria were studied as possible explanations for these low empirical factors found offshore.

Table 3. Protein turnover rates calculated for the surface stations during the COCA II cruise with pulse-chase experiments. N: number of time points used to compute the rate of turnover. NS: no significant, $p > 0.05$. Zones as in Table 1.

Sta	Zone	N	Turnover rate (h^{-1})	SE	p	Turnover (% of incorporation h^{-1})
2	UPW	12	-0.078	0.028	0.03	6.51
8	NT	6	0.105*	0.024	0.01	No turnover
14	NT	6	0.041	0.017	NS	No turnover
22	NT	6	-0.053	0.011	0.01	3.56
32	NT	6	0.006	0.021	NS	No turnover
42	ST	6	-0.003	0.016	NS	No turnover
48	ST	12	-0.041	0.019	0.02	4.78
52	UPW	6	-0.002	0.009	NS	No turnover
60	UPW	5	-0.074	0.011	0.02	7.19
66	UPW	5	0.004	0.044	NS	No turnover

* Significant increase in label incorporated after addition of the cold-chase.

Protein turnover was measured following the exponential decrease in radioactivity after addition of a cold Leu chase. We only found significant protein turnover, detected when the exponential decrease in radioactivity is statistically significant, at four stations (Table 3). Even at those stations, the rate of protein turnover was low (3 to 7% of Leu incorporated per hour), indicating that bacteria in our sampling area were not recycling their proteins at a significant rate. Thus, protein turnover was not accounting for the low CFs found in offshore waters.

On the contrary, the proportion of the Leu taken up by the cells that was respired (in a time period of 3 h) was high, especially in offshore stations (Fig. 4). We found a gradient of Leu respiration from <20 to 40% in the upwelling area (of the total Leu taken up by bacteria), compared to 60 to 80% in offshore waters. We observed an inverse relationship between the percentage of Leu respired and the Leu CF along the gradient, although it was not statistically significant at the p= 0.05 level (Pearson r= -0.57, n= 10, p = 0.08).

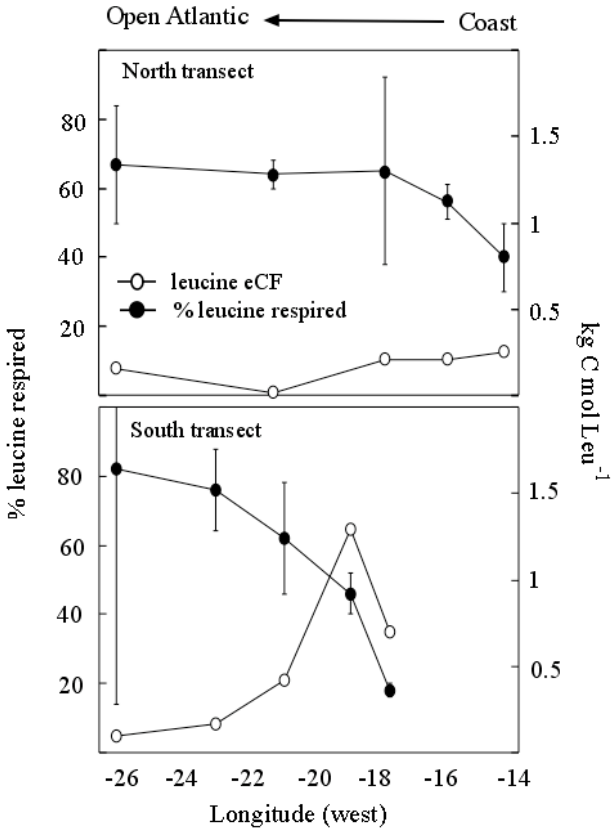

Fig 4. Percentage of leucine respiration over the total leucine: processing and empirically-derived carbon-to-leucine conversion factors (leucine eCF) in the Coca II cruise.

Nutrient limitation bioassays

Bacterial nutrient limitation was analyzed in both cruises and the responses are reported as BHP and BR ratios relative to the unamended controls (Tables 4 and 5). During the COCA I cruise, we found BHP limitation (with always a more marked effect on TdR measurements) by inorganic nutrients at all sampling stations except station 14, which showed a higher carbon limitation of BHP. BR was also stimulated by carbon at this station (Table 4). In the rest of the stations BR was unaffected by the addition of organic nor inorganic nutrients, although at station 42, BR increased by a factor of 2.4 with the addition of both resources simultaneously.

During the COCA II cruise, BHP was primarily stimulated by organic carbon additions in the stations closer to the coast (upwelling area and the coastal stations 8 and 14). BHP was unaffected by N additions, but P enrichment resulted in significantly higher BHP at the offshore station 22, in the Northern transect (Table 4). The oceanic stations in the south transect (stations 42 and 48) did not appear to be severely limited by neither organic nor inorganic nutrients. However, a strong response of BHP was found at the most oceanic station in the north transect (sta 32) when both organic and inorganic nutrients were added.

Discussion

The results presented here identified BHP as a very poorly constrained component of bacterial carbon budgets, largely due to discrepancies between empirical and theoretical CFs. The region studied included the North Atlantic subtropical gyre and the NW African coast upwelling, which differed greatly in dynamics as reflected in a decline in Chl *a*, inorganic nutrients and POC concentration offshore from the upwelling zone (Barton et al. 1998, Fig 2). Other parameters not specifically assessed in this study, such as bacterial biomass, also shows large uncertainties due to the variability of conversion factors, as discussed below.

BR estimates (range 2-64 μg C l^{-1} d^{-1}) did not decline offshore, and were comparable to values previously reported for oceanic waters such as the Eastern Atlantic Ocean (average 26 μg C l^{-1} d^{-1} taking 0.88 as respiratory quotient, Robinson et al. 2002), the Equatorial Atlantic (Moran et al. 2004) and the North Pacific subtropical gyre (average 8 μg C L^{-1} d^{-1} taking 0.88 as respiratory quotient, Williams et al. 2004). Higher BR estimates and also larger variability were found in the South transect compared to the North transect, matching a higher POC content in the surface waters at this latitude. This increase in POC is probably due to the influence of

the carbon-rich Cape Blanc upwelling waters, which can be advected hundreds of kms offshore, due to the combined effect of the upwelling filament and the CVFZ (see review in Arístegui et al. 2006). The higher BR during the COCA I cruise than the COCA II cruise was consistent with warmer surface temperatures and higher DOC concentrations observed during the COCA I cruise (Calleja, M. unpubl.).

Table 4. Ratio of integrated bacterial heterotrophic production (^{3}H-Leu and ^{3}H-TdR incorporation) and respiration (change in oxygen concentration, μmol l^{-1} O_2) after different enrichments scaled to unamended control measurements, over 96 hour-bioassays in the cruise COCA I. The stimulation diagnostic columns are qualitative interpretation of the results taking a minimum ratio of 2 for a positive response to the addition. C: carbon enrichment (glucose and acetate 1 μmol l^{-1} final conc. each), NP: nitrogen and phosphorus enrichment (ammonium, nitrate, and phosphate 0.5 μmol l^{-1} final conc. each).

Sta	Enrich.	Leu-BP	TdR-BP	BR (Δ O_2)	BHP stimulation	BR stimulation
8	C	0.88	1.06	1.44	Inorganic	No effect
	NP	1.44	2.23	1.57		
	CNP	1.42	2.21	1.57		
14	C	1.80	5.31	4.59	C	C
	NP	1.44	2.32	1.20	(Inorganic)	
	CNP	1.17	1.64	1.48		
32	C	1.12	2.13	1.00	Inorganic	No effect
	NP	1.64	9.95	1.11	(C)	
	CNP	1.92	12.34	1.10		
42	C	1.12	1.00	1.23	Inorganic	Co-stimulation
	NP	1.46	2.78	1.07		
	CNP	1.11	2.93	2.42		
52	C	1.09	1.04	1.10	Inorganic	No effect
	NP	1.16	2.63	0.89		
	CNP	1.31	2.30	1.00		

The BHP determinations showed the highest variability throughout the inshore-offshore gradient, within a factor of 10 using the theoretical CF, or close to 30 using the empirical CF and excluding station 22, which had an unusually low empirical CF. Leu and TdR incorporation rates were remarkably higher in offshore stations compared to the upwelling, a

pattern that has been reported in other studies (Barquero et al. 1998). However, the empirical CFs showed an opposite trend, thus the use of an standard CF for all stations yielded significantly higher BHP estimates in offshore waters compared to the use of empirical ones.

Table 5. Ratios of leucine-based bacterial production measurements after 24h incubation with additions of phosphate alone (P, 0.5 μmol l^{-1} final conc.), ammonium and nitrate alone (N, 0.5 μmol l^{-1} final conc. each), carbon alone (C, glucose and acetate 1 μmol l^{-1} final conc. each) and combined phosphate, nitrogen, and carbon enrichments (CNP) during the COCA II cruise. Values are relative to the unenriched controls. The diagnostic column is a qualitative interpretation of the results taking a minimum ratio of 2 for a positive response to the nutrient addition.

Sta	N	P	C	NPC	Diagnostic
8	0.87 ± 0.14	2.26 ± 0.18	32.59 ±1.74	11.29 ±0.62	C (P)
14	1.05 ± 0.14	1.25 ± 0.17	3.27 ±0.42	7.47 ±0.96	C
22	0.90 ± 0.36	4.46 ± 0.36	1.69 ±0.36	5.48 ±0.36	P
32	1.03 ± 0.13	1.33 ± 0.16	0.91 ±0.12	52.87 ±5.2	Colimitation
42	0.70 ± 0.10	1.03 ± 0.10	1.09 ±0.10	1.76 ±0.10	No limitation
48	0.81 ± 0.01	1.11 ± 0.01	0.93 ±0.01	1.04 ±0.01	No limitation
52	1.03 ± 0.04	0.92 ± 0.04	2.37 ±0.04	2.86 ±0.04	C
60	1.19 ± 0.06	1.03 ± 0.06	3.88 ±0.07	3.42 ±0.07	C
66	0.95 ± 0.04	1.04 ± 0.04	2.39 ±0.04	1.90 ±0.04	C

Surface BHP estimates in the oligotrophic, offshore stations derived using empirical CFs were similar to other estimates reported for the Equatorial Atlantic, where they used an empirical CF of 0.73 kg C mol Leu^{-1} (Moran et al. 2004). Our average (± SE) integrated BHP in the euphotic zone using the empirical CFs was 37 ± 13 mg C m^{-2} d^{-1}, comparable to the values reported by Agustí et al. (2001) for the same area in the Eastern North Atlantic using an empirical CF of 0.58 kg C mol Leu^{-1}, and the values reported by Barbosa et al. (2001) off the NW Iberian margin using an empirical CF of 0.48 kg C mol Leu^{-1}. In contrast, the use of the theoretical CF would yield extremely high integrated BHP estimates, over 300 mg C m^{-2} d^{-1} in our oceanic stations.

Meaning of the empirical conversion factors and implications of leucine processing- Simon & Azam (1989) calculated a theoretical carbon-to-Leu CF based on constant cellular ratios of protein/dry weight and carbon/dry weigth, and the measurement of an isotope dilution factor of 2 in coastal waters. Even if we assume no isotope dilution, the resulting CF

(1.55 kg C mol Leu^{-1}) is higher than most of the CF calculated for open waters in our study (range of 0.02-1.29 kg C mol Leu^{-1}) as well as in others (e.g. Carlson & Ducklow 1996, Zubkov et al. 2000, Ducklow et al. 1999). We found a clear tendency for CFs to decrease from inshore to offshore stations, similar to those reported by Pedrós-Alió et al. (1999) in the Mediterranean Sea, and Sherr et al. (2001) in the North East Pacific. These patterns along trophic gradients suggest that CF values are ecologically constrained and are not an artifact caused by the "enclosure effect" of re-growth incubations (Massana et al. 2001).

Despite the evidence that empirical CFs show a broad variability (e.g., 21 fold in Leu CF in the subarctic Pacific, Kirchman 1992) we are still far from understanding what controls these changes. For instance, Leu CF was unaffected by additions of organic compounds or ammonium in the subarctic Pacific (Kirchman 1992). Our results and previously reported CFs for oligotrophic, open-ocean waters, indicate that the theoretical CF can grossly overestimate BHP in oligotrophic waters. Isotope dilution has been repeatedly measured in different systems to constrain the upper limit of the empirical CF (Simon & Azam 1989, van Looij & Riemann 1993), but almost no studies have tried to assess the reasons for the low CF found in offshore waters. We examined two possible explanations for the low CFs from Leu incorporated into biomass production in oligotrophic waters:

i) That Leu is incorporated into the cellular protein but bacteria exhibit a significant protein turnover, leading to an overestimation of net incorporation. Proteins can be synthesized and continuously degraded (turnover) at high rates in selected growth conditions. If protein turnover occurs at a high rate, radioactivity can be incorporated into protein even if the net rate of protein synthesis is zero. Kirchman et al. (1986) estimated the protein turnover by marine bacteria in a salt marsh estuary and found rates between 0.012 to 0.311 h^{-1}. We performed the same type of experiments in our sampling region and found rates in their lower range between 0.04 and 0.08 h^{-1} (Table 2). Hence, bacterial protein turnover is slow in the oligotrophic oceanic waters studied, thereby unlikely to affect CFs.

ii) An alternative explanation for the low CFs could be non-specific incorporation of Leu into proteins and/or Leu catabolism. Although L-Leu has been usually shown to be incorporated only into the protein fraction (Kirchman et al. 1985) some discrepancies among macromolecular fractionation of labeled Leu or TdR in TCA precipitates have also been described (Torréton & Bouvy 1991). ^{3}H-Leu has been seen to precipitate in cold TCA even if it is not part of the protein fraction (Wicks & Robarts 1988, Hollibaugh & Wong 1992). These authors showed that an ethanol rinse could remove a high proportion of ^{3}H-Leu (and also

TdR) probably associated to cell membrane lipids as a storage mechanism. If Leu enters the cell but it is not used for protein biosynthesis the radioactivity measured inside the cells can overestimate the biomass production calculated with the assumptions of theoretical CFs.

Kirchman et al. (1985) found that, on average, 90% of the Leu taken up by bacteria was incorporated into protein and less than 20% was degraded to other amino acids in several aquatic systems. However, they also found that Leu degradation was much greater in less productive waters, reaching values of 49% of Leu degradation in two Gulf Stream samples. In studies measuring the uptake and respiration of specific ^{14}C- or ^{3}H- labeled amino acids, Leu is usually respired very slowly (Williams et al. 1976, Suttle et al. 1991), but higher percentages of Leu respiration (> 20%) have also been described in freshwater environments (Hobbie & Crawford 1969, Sepers 1981). Jørgensen (1992) found some of the highest reported values of Leu respiration (78-91%) in two eutrophic lakes.

We suggest that a high percentage of the ^{3}H-Leu that enters the cell is not used for protein synthesis in energy-limited systems, even if it can be detected as biomass production in the time scale of the Leu incorporation radio assay (hours). After incorporation into the cell, Leu could be respired. This process would lead to a lower carbon-to-leucine conversion factor than the theoretical one, which assumes that Leu is integrally incorporated into protein and used for biomass production.

In order to test this hypothesis we measured the respiration and assimilation of ^{14}C-labeled Leu along the productivity gradient. We found an increasing gradient of respiration of Leu offshore, opposite to the pattern of decreasing CFs offshore, which is consistent with the idea that Leu catabolism could be related to the low CFs. Interestingly, BHPs estimated from the incorporation of ^{3}H-[4,5]-Leu were significantly higher than those measured with uniformly labeled ^{14}C-Leu using the standard CF (1.55 kg C mol Leu^{-1}) for both estimates (Fig. 5). This suggests that ^{3}H-Leu incorporation rate could overestimate biomass production even in instantaneous measurements (time-scale of hours).

Since the concentration used for both substrates was saturating (40 nmol l^{-1} for ^{3}H- and 60 nmol l^{-1} for ^{14}C-leu) and the incubation time (2-4 hours) was similar, the differential molecular labeling is the most probable reason for the differences found. Jørgensen (1992) and Kuparinen & Tamminen (1984) found similar rates of incorporation of ^{3}H and ^{14}C-labeled Leu and glucose respectively. However, ^{3}H from different carbon position in substrates such as TdR (Hollibaugh 1994) or glutamic amino acid (Carlucci et al. 1986) have been shown to

follow different metabolic pathways (incorporation to macromolecules or catabolism). If [4,5-^{3}H] Leu (as it is usually used for routine BHP measurements) is degraded in the cell following the general catabolic pathway, the amino acid could initially be oxidized to CO_2 and acetil-CoA (that can be further oxidized in the Krebs cycle) without loosing most of the tritium signal. This could explain the differences between ^{3}H- and ^{14}C Leu-based BHP estimates, since the use of uniformly labeled ^{14}C-Leu could correct for Leu catabolism, as the label would disappear as $^{14}CO_2$. In agreement with this hypothesis, BHP estimates with ^{3}H-Leu were significantly higher than the ^{14}C-Leu estimates using the theoretical CF for both compounds (Fig. 5). On the contrary, BHPs calculated with ^{3}H-Leu using empirical CFs (that would correct for Leu catabolism) were in the range and positively correlated to the ^{14}C-Leu incorporation-based BHP estimates using the theoretical CF (Pearson r= 0.84, n= 10, p= 0.002).

Clearly, more research should be done focusing on the metabolic pathway followed by [4,5]-^{3}H-Leu into the cell in order to constrain present uncertainties on this measurement as an accurate estimator of BHP in oligotrophic systems. Further comparisons between the respiration of ^{14}C-Leu and [4,5]-^{3}H-Leu in oligotrophic systems, and the empirical CFs found for both compounds would help resolve this question. However, our results make clear that incorporation of [4,5]-^{3}H-Leu can overestimate carbon production unless corrected with empirical CFs, and remark the importance of estimating CFs in oceanic waters.

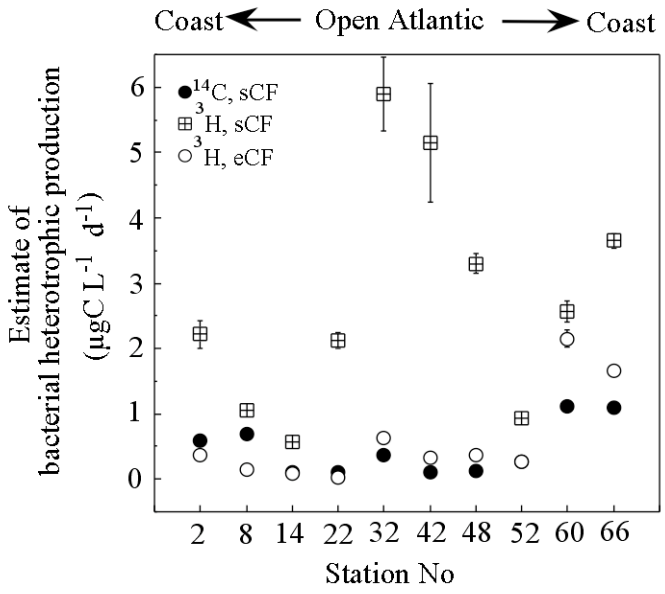

Fig 5. Bacterial heterotrophic production estimates based on carbon (^{14}C) or tritium (^{3}H) labeled Leu, and empirical or standard conversion factors. eCF: empirical conversion factor, sCF: standard conversion factor.

BGE estimation along the productivity gradient in NW Subtropical Atlantic waters.- Since most of the carbon that bacteria consume in oceanic waters is generally respired, the understanding of BGE variability would be crucial to predict bacterial carbon flux. However, BGE calculation is not trivial, given the different time scales required for the BR and BHP measurements. BHP can be measured almost instantaneously (minutes to hours) but direct estimates of BR require a minimal incubation time of 24 h in oceanic waters (cf. Arístegui et al. 2005).

There is a high diversity of approaches for BGE calculation in open ocean and coastal marine waters in the literature (Table 6), including different experimental settings (regrowth experiments or incubation of undiluted water), different methods for BHP and BR estimation and CFs. All of these differences can greatly affect the BGE estimates. As an example, Briand et al. (2004) found higher estimates of BGE using the increment of POC as BHP measurements compared to the use of TdR-based BHP in the same experiment, similarly to our results.

Besides empirical CFs for BHP measurements, CFs for bacterial biomass determination (i.e., carbon content per cell or volume) show a rather wide range, which can also affect the uncertainty in BGE estimates. In this work, the average biomass conversion factor calculated by Fukuda et al. (1988) for oceanic bacteria was used (i.e., 12 fg C $cell^{-1}$). This factor is lower than widely used factors in this type of studies (15-20 fg C $cell^{-1}$, Table 6), but still higher than other values derived in different studies based on low carbon-to-volume factors (Carlson et al. 1999), including samples from our oceanic region (7 fg C $cell^{-1}$, Zubkov et al. 2000). The use of this lower conversion factor (7 fg C $cell^{-1}$) for our bacterial biomass measurements would have produced, on average, 25% lower BGE estimates.

Different choices of BHP estimations produced an order-of-magnitude range of possible BGE values for any one sample (Table 1) and thus, it constitutes the factor of highest uncertainty for BGE calculation. Filtration had a moderate effect on bacterial abundance and respiration. On the contrary, Leu uptake rates were highly affected at the initial time of the incubation, showing significantly lower estimates compared to those of in situ. This effect has been related to the uncoupling between the members of the microbial food web (Obernosterer et al. 2003). The application of the low empirical CFs found for these initial measurements is probably not correct due to the significant changes in bacterial activity after filtration, and

would yield unrealistically low values of BHP. Hence, we decided to use the theoretical CF (1.55 kgC mol Leu^{-1}) for the BHP estimate at time zero, in an attempt of applying the most reliable estimate of BHP for the BGE determination. Since we had filtered our samples for the determination of BR, we considered that the in situ BHP (from unfiltered seawater) was not appropriate for the BGE estimate. We chose to use the average of the two most common ways of calculating BGE: the initial BHP and the change in bacterial biomass throughout the incubation. The result allowed us to conclude that BGE was higher in the upwelling area (average 30%) compared to offshore waters (average 9%).

Higher BGEs in upwelling waters are in agreement with the large-scale correlations between Chl *a* and BGE (del Giorgio & Cole 1998), since higher growth efficiencies can be expected when bacteria are growing on phytoplankton exudates. However, BGE was independent of DOC, DON concentrations or C:N ratio of the dissolved organic matter pool in our samples (Calleja, M. unpubl.).

Our BGE values in the offshore stations (average 9%) are comparable to the average of published BGE estimates for open waters (12%, see Table 6). A similar tendency for lower growth efficiencies in oligotrophic relative to eutrophic waters has been reported in freshwater and marine systems (del Giorgio & Cole 1998) and could be associated to the increased maintenance costs relative to total carbon uptake in low-nutrient waters (Biddanda et al. 2001).

Effect of nutrient availability on BGE.- It has been reported that BGE may be affected by substrate concentration and composition (Pomeroy & Wiebe 2001), and inorganic nutrient availability (Kroer 1993). Del Giorgio & Cole (1998) pointed that phosphorus and iron, but not nitrogen, could control BGE in marine systems.

BHP can be limited by organic carbon (Kirchman 1990), nitrogen or phosphorus (Pomeroy et al. 1995) in different oceanic regions. However, the factors controlling BHP could be very different from those that regulate BR (Smith & Kemp 1995) and very few studies have assessed the effect of nutrients on BR. We found that, independently of the factor limiting BHP, BR was generally unaffected by inorganic or organic additions, in contrast to the results of Pomeroy et al. (1995) and Obernosterer et al. (2003) who found BR to be primarily P-limited.

We found spatial and temporal variation in the type of BHP limitation. During the COCA I cruise, when the Cape Blanc upwelling waters affected a very reduced zone, most of the stations showed inorganic nutrient limitation of BHP (presumably phosphorus limitation, since

nitrogen alone never had any effect on BHP during the COCA II cruise). This agrees with other studies performed in the Atlantic Ocean where phosphorus limitation of bacteria has been encountered (Cotner et al. 1997, Rivkin & Anderson 1997).

Table 6. Review of BGE determination in marine waters, with special detail in the methods used for the determinations. [a] in situ mearurements , [b] 90% of oxygen consumption in the filtered treatment, [d] change in bacterial biomass, [c] initial measurement (time 0) from the unfiltered fraction, [f] initial measurement (time 0) from the filtered fraction, [g] unspecified time, [h] BGE calculated taking into account log phase and total incubation, [i] average BGE between two estimates with different carbon-to-volume factors.

Reference	Location	Type of experiment	BP method	BR / BCD method	Biomass factors (pg C μm^{-3}	fg C cell^{-1})	Leu factor (Kg C mol Leu^{-1})	TdR factor (cells mol TdR^{-1})	BGE range (%)	BGE avg (%)
Griffith et al. 1990	Georgia Bight nearshore	Non diluted	^{3}H-TdR[a]	ΔO_2[b]	-	20	-	1.18×10^{18}	-	6
	Georgia Bight shelf									2
Coffin et al. 1993	Santa Rosa Sound	Non diluted	ΔPOC[c]	ΔO_2	-	-	-	-	0.4 - 35	16
Kroer 1993	Santa Rosa Sound	Regrowth	ΔPOC	ΔDOC	-	-	-	-	26 - 33	30
	Gulf of Mexico									61
Biddanda et al. 1994	Louisiana shelf	Non diluted	^{3}H-Leu[d]	ΔO_2	-	20	2.3		38 - 55	45
	Lousiana slope								19 - 26	23
Toolan 2001	Massachusetts Bay	Non diluted	ΔPOC[c]	ΔCO_2	-	15	-	-	0 - 69	24
Gonzalez et al. 2003	Bay of Biscay	Non diluted	^{3}H-Leu[e]	ΔO_2	-	20	0.77	-	1 - 8	4
			^{3}H-Leu[f]						0.7 - 2	1
Robinson et al 2002	North Sea	Non diluted	ΔPOC[c]	ΔCO_2	-	-	-	-	18	18
Briand et al. 2004	New Caledonia	Non diluted	ΔPOC[c]	ΔO_2	-	20	-	-	1 - 42	21
			^{3}H-TdR	ΔO_2	-	-		2.9×10^{18}	1 - 20	10
Reinthaler and Herndl 2005	Southern North Sea	Non diluted	^{14}C-Leu[f]	ΔO_2	-	20	1.4	-	5 - 28	19
					Shelf waters			Total	0.4 - 69	20
Hansell et al. 1995	Sargasso Sea	Non diluted	^{3}H-TdR[g]	ΔCO_2	-	9	-	1.7×10^{18}	4 - 9	6
Cherrier et al. 1996	Eastern North Pacific	Non diluted	ΔPOC[c]	ΔDOC	-	19.6	-	-	1	1
Kahler et al. 1997	Southern Ocean	Non diluted	ΔPOC	ΔDOC	0.28	-	1.23	1.2×10^{18}	26 - 30	28
Sherry et al. 1999	Subarctic NE Pacific	Non diluted	^{3}H-TdR & ^{14}C-Leu	ΔO_2	-	20	2.2	2×10^{18}	4 - 31	12
Lemée et al. 2002	NW Mediterranean	Non diluted	^{3}H-LIR[e]	ΔO_2	-	15	1.5	-	1 - 43	11
Kirchman et al. 1991	North Atlantic	Regrowth	ΔPOC[c]	ΔDOC	0.14 -	0.5	-	-	1.6 - 9	5[i]
Carlson and Ducklow 1996	Sargasso Sea	Regrowth	ΔPOC[c,h]	ΔDOC	0.12	-	-	-	7 - 19	14
Carlson et al 1999	Southern Ocean	Non diluted	ΔPOC[j]	$\Delta CO_2/\Delta DOC$	0.10	-	-		9 – 38	20
					Open ocean			Total	1 - 43	12

During the COCA II cruise, when the upwelling affected a much larger area, the coastal (sta 8 and 14) and upwelling-affected stations (sta 2, 52, 60, and 66) showed carbon limitation. The high inorganic nutrient concentrations supplied by the upwelled waters could have switched bacterial inorganic nutrient limitation to carbon limitation. In other nutrient-rich areas, such as Georges Bank (Caron et al. 2000), the California upwelling (Kirchman et al. 2000), or the Southern Ocean (Church et al. 2000), BHP has also been reported to be carbon limited.

In the oceanic stations we found colimitation or no significant limitation by inorganic or organic treatments. The lack of evidence for resource limitation is consistent with the idea of a tightly coupled microbial food web, and the suggestion of grazer control of bacterioplankton (Ducklow & Carlson 1992; Gasol et al. 2002), which would provide the organic and inorganic nutrients needed for growth. In agreement with this hypothesis, the protozoan grazing effect was generally higher at the offshore stations (average 123% of BHP in stations 32, 42, 48), while it was lower in the upwelling area (average 70%, Vaqué et al. unpubl.).

BGE was highest at the coastal stations where carbon limitation (and no inorganic nutrient limitation) was found. This suggests that when supplied with inorganic nutrients, the organic carbon would be more efficiently converted into biomass. As an example, Sta 22, the only station where clear phosphorus limitation of BHP was found in the COCA II cruise, bacteria showed the lowest growth efficiency and empirical CF of all stations sampled. Similarly (although in an estuarine system, Chesapeake Bay), Smith & Kemp (2003) found higher BGE in a carbon-limited zone (Upper Bay, 39%), lower BGE in a phosphorus-limited zone (Lower Bay, 24%) and intermediate BGE in a resource sufficient zone (Mid Bay, 28%).

Relationship between empirical conversion factors and Leu respiration with BGE.-The lower BGE and higher percentages of Leu respiration in the more oligotrophic areas are consistent with the concept of energy-limited and growth-constrained bacteria. Bacteria in oligotrophic waters may need a high energy flux in the cell in order to maintain the energization of cell membranes and their uptake systems active to react to the low nutrient inflow (Ishida et al. 1986) or whenever environmental conditions change (del Giorgio & Cole 1998). Indeed, cell-specific maintenance requirements appear to be higher in oligotrophic areas with very low concentration of organic substrates and nutrients (del Giorgio & Cole 1998).

Leu respiration was very weakly correlated with total bacterial respiration and Leu incorporation efficiency was not significantly correlated to in situ BGE. Although the efficiency in the incorporation of single substrates has been assumed to be representative of in situ BGE in early studies, extrapolation from single compounds may lead to significant overestimation of natural BGE (del Giorgio & Cole 1998).

On the contrary, BGEs were highly correlated with empirical CFs (Pearson r= 0.86, n= 12, p< 0.0004, log-log transformed, Fig. 6) supporting the idea that both parameters reflect basically the same physiological processes. Since a theoretical CF (1.55 Kg C mol Leu^{-1}) was used for the BHP estimate in the calculation of the BGE, this relationship is not based on an autocorrelation. If low CFs were explained by Leu catabolism as we suggest, then CFs would be measuring the amount of substrate incorporated that is assimilated into biomass, similarly to BGE. If this relationship is confirmed in subsequent studies, BGEs could be predicted from empirical CFs for oceanic bacterial communities.

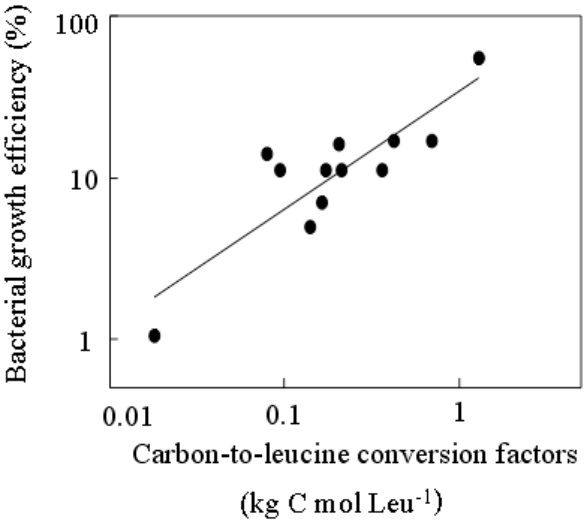

Fig 6. Relationship between the empirically derived leucine-to-carbon conversion factors and the bacterial growth efficiency for the data of the cruises COCA I and II.

Acknowledgements

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Chapter II

Bacterial assemblage structure and carbon metabolism along a productivity gradient in the North East Atlantic Ocean

L. Alonso-Sáez, J. Arístegui, J. Pinhassi, L. Gómez-Consarnau, J. M. González, D. Vaqué, S. Agustí & J. M. Gasol

Aquat. Microb. Ecol. (submitted)

Abstract

Bacterioplankton has the potential to significantly affect the cycling of organic matter in the ocean, but little is known about the linkage between bacterial assemblage structure and carbon metabolism. In this study we investigated whether changes in the phylogenetic composition of bacterioplankton were associated with changes in bacterial carbon processing (bacterial production, respiration and biomass) in the subtropical North East Atlantic Ocean. We found consistent differences in the composition of the bacterial assemblage, as revealed by DGGE and CARDFISH, along a gradient from the NW African upwelling to the oligotrophic subtropical Gyre. The percent contribution of Bacteroidetes, Roseobacter and Gammaproteobacteria groups significantly increased in more productive waters, while the SAR11 clade of the Alphaproteobacteria remained relatively constant (average 28% of DAPI stained cells) throughout the area. Changes in bacterial assemblage composition (from a DGGE fingerprint) were significantly correlated with changes in carbon processing variables through the area, although such linkage was not strong. The abundance of the groups Roseobacter and Gammaproteobacteria was highly correlated with the concentration of particulate organic carbon and chlorophyll *a* across the area, supporting the affinity of these groups to environmental enriched conditions. The abundance of Roseobacter was also correlated with bacterial heterotrophic production, suggesting their active participation in carbon processing.

Introduction

Great efforts in marine microbial ecology have focused on determining the role bacterioplankton play in the flux of carbon in the ocean, resulting in a good knowledge on the spatial variability of bacterial activity (e.g. Ducklow & Carlson 1992, Ducklow 2000). The advent of molecular biology techniques in the late 1980's allowed examination of the diversity of prokaryotes in oceanic waters, producing also the accumulation of a large body of data (Giovanonni & Rappé 2000). However, we still lack knowledge about the spatial distribution of dominant prokaryotes at both large and small scales in the ocean (but see Suzuki et al. 2001 and Zubkov et al. 2002) and more importantly, we are largely ignorant of their *in situ* phenotypic characteristics.

Martínez et al (1996) found that marine bacterial isolates showed different metabolic and enzymatic properties, suggesting that phylogenetic diversity may be a critical factor to consider when analyzing spatial and temporal patterns of bacterial activity. Experimental studies have subsequently shown that shifts in marine bacterial assemblage composition can be associated with changes in bacterial growth and activity rates (Pinhassi et al. 1999, Riemann et al. 2000, Kirchman et al. 2004). However, the biogeochemical implications of the phylogenetic diversity of marine bacterial assemblages are poorly understood, since few studies have simultaneously compared *in situ* bacterial assemblage structure with carbon processing, particularly in oceanic open waters (but see Reinthaler et al. 2005, Winter et al. 2005).

Here, we examined the relationship between bacterial assemblage structure and environmental and carbon metabolism parameters throughout an oceanic (bottom depth >1000 m) transition zone between the North Atlantic subtropical Gyre and the NW African upwelling. Upwelling areas are characterized by important inputs of inorganic nutrients, which lead to increased phytoplankton productivity and bacterial heterotrophic production (e.g. Cuevas et al. 2004). In our sampling area, the Cape Blanc upwelling waters also showed higher bacterial abundance and secondary production compared to the oligotrophic stations (Chapter I). In this study we looked at whether changes in bacterial assemblage composition were associated with the observed changes in bacterial carbon dynamics, and whether particular communities could be associated with particular patterns of bacterial carbon processing. The effects of the underlying environmental variables on bacterial assemblage structure and carbon metabolism are also discussed.

Materials and methods

Location and sampling- The study was conducted along two transects from NW African coastal waters to open waters of the North Atlantic subtropical gyre (Fig. 1), during a cruise on board BIO-Hespérides (COCA-II: 20 May-10 June, 2003). For this work water was collected from the surface (5 m) in 10 stations where temperature, salinity and fluorescence were recorded using a CTD system (Mark III-IOC) mounted on a General Oceanic rosette sampler equipped with 24 12-l Niskin bottles.

Basic data. The samples (250 ml) were filtered through Whatman GF/F filters to determine the Chlorophyll a (Chl a) concentration. The filters were homogenized and kept refrigerated in the dark while pigments were extracted in 90% acetone for ca. 1 h. Fluorescence of the

extracts was measured in a Turner Designs fluorometer. Samples (2 l) for POC were filtered through combusted (450°C, 12 hours) 25 mm Whatman GF/F filters. The filters were wrapped in combusted aluminum foil and frozen at –20°C. In the lab, the filters were dried overnight at 65ºC in a desiccator with HCl fumes to remove carbonates, and finally dried overnight in a desiccator with silica gel. Previous to analysis, samples were packed into ultra-clean tin disks. The carbon analyses were performed on a Perkin Elmer-2400 CHN elemental analyzer, according to the JGOFS protocol (UNESCO, 1994). Dissolved inorganic nutrients (nitrate and phosphate) were measured on an autonalyzer using standard colorimetric methods (Hansen & Koroleff 1999).

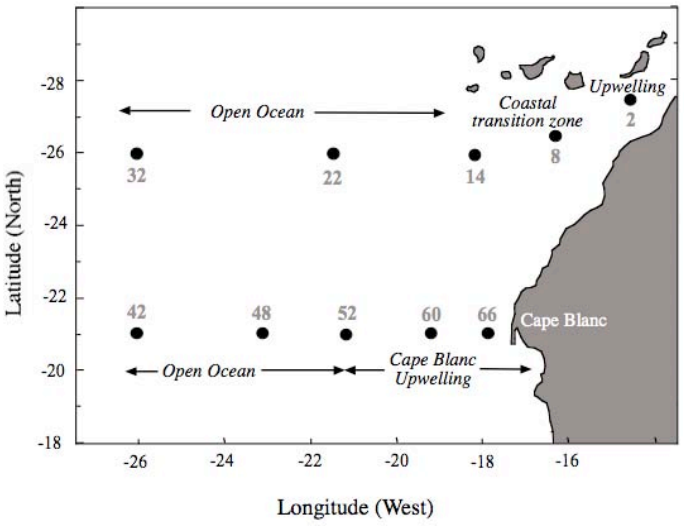

Fig 1. Map of the sampling area indicating the stations' No and location. Different areas are characterized according to hydrographical data (Chapter I).

DGGE- Surface microbial biomass was collected by sequentially filtering around 2 l of seawater through a 3 μm polycarbonate filter (Poretics, 46 mm) and a 0.2 μm polycarbonate filter (Poretics, 46 mm) under gentle pressure. We used these last filters for the analysis. The filters were stored in criovials filled with 1.8 ml of lysis buffer (50 mM Tris-HCl pH 8.3, 40 mM EDTA pH 8.0, 0.75 M sucrose) and kept at -80ºC. Microbial biomass was treated with lysozyme, proteinase K and sodium dodecyl sulphate, and the nucleic acids were extracted with phenol and concentrated in a Centricon-100 (Millipore), as described in Schauer et al. (2000). DGGE and gel analysis were performed essentially as previously described (Schauer et al. 2000). Briefly, 16S rRNA gene fragments (around 550 bp in length) were amplified by PCR, using the universal primer 907rm and the bacterial specific primer 358f, with a GC-clamp. The PCR products were loaded on a 6% polyacrylamide gel with a DNA-denaturant gradient

ranging from 40 to 80%. The gel was run at 100 V for 16 h at 60°C in 1x TAE running buffer. DGGE gel images were analyzed using the Diversity Database software (BIO-RAD).

CARDFISH- Samples were fixed with formaldehyde (3.6% final conc., overnight at 4ºC), filtered and stored at –20ºC until processing by the protocol of Pernthaler et al. (2004). Filters were embedded in agarose (0.1% [wt/vol]) and permeabilized at 37º C with lysozyme (10 mg ml^{-1}; 0.05 M EDTA, 0.1 M Tris-HCl; Fluka) for 60 min., and achromopeptidase (60 U ml^{-1}, 0.01 M NaCl, 0.01 M Tris-HCl, pH 7.6, SIGMA) for 30 min. For the hybridization (35ºC, overnight), 3 µl of horseradish peroxidase (HRP) labeled probe working solution (50 ng μl^{-1}, www.biomers.net) were added to 900 µl of hybridization buffer (HB, 0.9M NaCl, 20 mM Tris-HCl,10% dextran sulfate-wt/vol-, 0.02 % sodium dodecyl sulfate-SDS-, and 1% blocking reagent) containing the following percentages of formamide: 55% for Eub338-III (mixture of probes Eub338, Eub II, and Eub III, Amann et al. 1990, Daims et al. 1999), Ros537 (Eilers et al. 2001), Gam42a and CF319a (Amann et al. 1990), and 20% for Eury806 (Teira et al. 2004), Cren554 (Massana et al. 1997) and Non338 (Wallner et al. 1993). A higher concentration of probe (9 µl in 900µl HB, 45% formamide) was used to detect the cells with SAR11-441R (Morris et al. 2002) and Alf968 (Neef 1997) probes. Probe Gam42a was used with a Bet42a competitor oligonucleotide (Manz et al. 1992). Results of hybridization with the probe Non338 (negative control) never exceeded 1% of DAPI counts, and were not subtracted of CARDFISH counts. Filters were washed for 5 min (37ºC) in prewarmed washing buffer and, subsequently, incubated (15 min, 46ºC) in 1 ml of amplification buffer with 4 µl of tyramide-alexa 488 [1 mg ml^{-1}] containing p-iodophenylboronic acid (20 mg per 1 mg tyramide). Finally, filter sections were mounted in a (4:1) Citifluor-Vecta Shield mixture containing 4'-6'-diamidino-2-phelylindole (DAPI, final conc. 1 µg ml^{-1}) and visualized in a Nikon epi-fluorescence microscope. Between 500 and 1000 DAPI stained cells were counted per sample in a minimum of 10 fields.

Flow Cytometry. Bacterial abundance and the percentage of high nucleic acid cells were measured by flow cytometry (Gasol & del Giorgio 2000). Samples were run in a Becton and Dickinson FACSCalibur cytometer after staining with Syto13 (2.5 µM, Molecular probes), and bacteria were detected by their signature in a plot of side scatter (SSC) vs FL1 (green fluorescence). Regions were established on the SSC versus green fluorescence plot in order to discriminate cells with HNA (high nucleic acid) content from cells with LNA (low nucleic acid) content. Cell abundance was determined for each subgroup. Bacterial biomass was calculated

from abundance assuming a conservative carbon content per cell of 12 fgC $cell^{-1}$ (Fukuda et al. 1998).

Bacterial carbon processing and enrichment experiments.- Details concerning the methodology to determine bacterial carbon flux in the area are presented in Chapter I. Briefly, bacterial heterotrophic production (BHP) was estimated from the incorporation of ^{3}H-leucine (Leu, protein biosynthesis) and thymidine (TdR, DNA biosynthesis). Both tracers were used at 40 nM (final conc.) in incubations of 2-4 h. The samples were processed by the centrifugation method of Smith & Azam (1992). Carbon-to-Leu empirical conversion factors (eCFs) were experimentally determined from dilution cultures at each station and calculated following the cumulative method (Bjørnsen & Kuparinen 1991). Bacterial respiration (BR) measurements were done following the decrease in dissolved oxygen in the bacterial fraction in 24 h incubations by Winkler titrations based on colorimetric end-point detection. We assumed a respiratory quotient of 0.88 (Williams & del Giorgio 2005). Finally, the effects of organic (glucose and acetate, 1 μM each) and/or inorganic (nitrate, ammonium and phosphate, 0.5 μM each) enrichments on BHP were studied in surface samples from all the stations (except for Sta 2). BHP was measured 24-48 h after the nutrients addition and compared to two unamended controls. We accepted a positive response to the enrichment over the control, when BHP increased by a minimum factor of 2 after the nutrients addition.

Statistical analysis. A matrix was constructed for all DGGE lanes taking into account the relative contribution of each band (in percentage) to the total intensity of the lane. Based on this matrix, we obtained a dendrogram by Ward's clustering method (Euclidean distances, Statistica 6.0), and ordinations by nonmetric multidimensional scaling (MDS, Krukal & Wish 1978, Clarke & Green 1988) and principal component analysis (PCA, Chatfield & Collins 1980). MDS is a nonparametric procedure that uses ranks of the similarities between the samples in order to construct a map in which the positions of the samples reflect as close as possible the dissimilarities among them. For this analysis, all DGGE bands were included (Bray-Curtis similarity index, Primer v5). PCA reduces the complexity of multivariate data creating new linear variables that encompass most of the variability in the original data. This analysis was performed taking into account only the more significant bands (those appearing in more than two samples and with a relative intensity > 5%, 18 bands in total), in order to reduce the number of variables used in the analysis (Statistica v 6.0). A PCA carried out with the total number of bands resulted in a nearly identical ordination of the samples. For MDS and PCA analyses, DGGE band percentages were arcsin transformed.

The associations between environmental factors, bacterial assemblage structure (DGGE band pattern), and carbon metabolism parameters were analyzed by the RELATE routine of Primer software (v5). This routine performs correlations of similarity matrices in a procedure similar to a Mantel test (Mantel, 1967), with the difference that the correlation is not the standard product-moment Pearson correlation (of standard Mantel test), but the Spearman non parametric rank correlation (*rho*). The statistical significance of the coefficient *rho* was obtained by a simple permutation test (999 permutations). Correlations were performed between three similarity matrices, which were constructed based on i) DGGE band patterns (arcsin transformed), ii) abiotic data (temperature, salinity, chlorophyll, phosphate and nitrate concentrations, log-transformed), and iii) carbon processing variables (data reviewed in Table 1, BHP measured by Leu uptake using eCFs, BHP measured by TdR uptake rates using a standard CF of 23kg C mol TdR^{-1}, bacterial respiration, and bacteria biomass, log-transformed). Bray-Curtis similarities (for DGGE band patterns) and Euclidean distances (for environmental and carbon metabolism data) were used to construct the similarity matrices (Primer v5).

Results

We visited 10 stations distributed along a Northern (26º N) and a Southern (21º N) transect in the NE Atlantic Ocean (Fig. 1). The Northern transect extended offshore from the boundary of the NW African coastal upwelling, and an upwelling filament affected the station closest to the coast (Stn 2). Stations 8 and 14 were located in the coastal transition zone and stations 22 and 32 exhibited oceanic characteristics. The Southern transect extended from the Cape Blanc coastal upwelling waters to the open ocean, crossing the Cape Vert Frontal Zone (CVFZ). The influence of upwelling waters reached as far as Stn. 52, which was the limit between the upwelling area and oceanic waters. Further details on hydrography of the stations are presented in Chapter I.

DGGE analysis of the samples yielded a total of 40 unique bands with an average (±SD) of 18 ±2 bands per sample and little variation among stations (range 15-22, Fig. 2). Analysis of the DGGE fingerprints (Ward's clustering method) showed a separation between the stations affected by upwelling waters (Stn. 2, 52, 60 and 66) and the offshore stations (Fig. 2).

Table 1. Summary of diagnostic results of the BHP limitation experiments, percentage of HNA (high nucleic acid content) cells, and the data included in the carbon metabolism similarity matrix: BBM (bacterial biomass), BHP1 (Bacterial heterotrophic production measured by Leu uptake and using empirically determined conversion factors), BHP2 (measured by TdR uptake using a theoretical conversion factor of 23 kgC mol TdR^{-1}) and BR (bacterial respiration). These data are presented and discussed in Chapter I. ND non determined

Zone	Stn	BBM ($\mu g\ C\ l^{-1}$)	BHP1 (Prot. synth) ($\mu g\ C\ l^{-1}\ d^{-1}$)	BHP2 (DNA synth.) ($\mu g\ C\ l^{-1}\ d^{-1}$)	BR ($\mu g\ C\ l^{-1}\ d^{-1}$)	HNA cells (%)	BHP Limitation org/inorg nut
NT-UPW	2	18	0.36	2.53	10.9	80	ND
NT	8	16	0.14	0.96	10.6	35	Carbon
NT	14	10	0.08	0.66	9.7	36	Carbon
NT	22	16	0.03	0.87	2.2	31	Phosphorous
NT	32	8	0.63	7.73	11.3	38	Colimitation
ST-UPW	66	51	1.65	1.98	16.4	77	Carbon
ST-UPW	60	68	2.15	3.21	30.8	56	Carbon
ST	52	11	0.26	0.59	12.1	40	Carbon
ST	48	9	0.37	3.48	22.2	31	No limitation
ST	42	5	0.32	7.62	17.7	34	No limitation

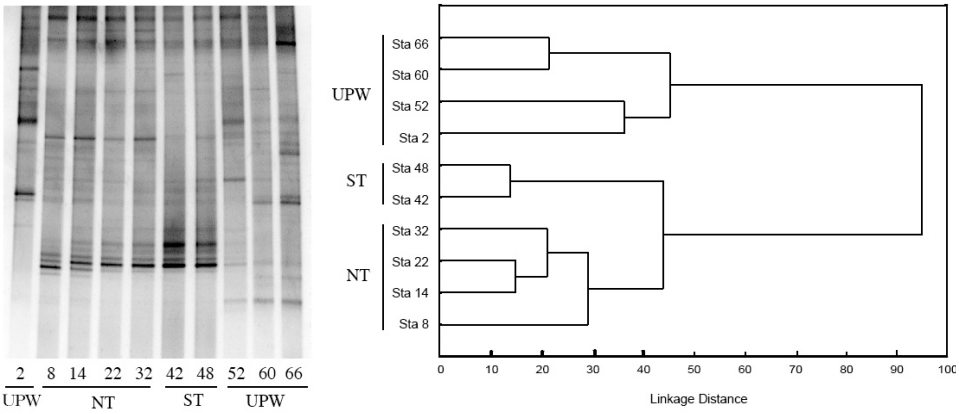

Fig 2. DGGE gel of the surface stations and dendrogram classification (Ward's Method, Euclidean distances according to the band pattern). UPW: upwelling zone, ST: Southern transect offshore, NT: Northern transect offshore.

The dendrogram further showed a separation of the offshore stations into two clusters: (1) Southern transect stations (Sta 42, 48) and (2) Northern transect stations (Sta 8, 14, 22, 32). A dendrogram constructed with the UPGMA method on the DGGE band matrix maintained the same general structure except for Stn. 8, which appeared as an ungrouped branch within the offshore cluster.

The ordination of stations by MDS and principal component analyses based on DGGE band pattern agreed with the clustering results, and showed a separation of the upwelling area from offshore stations, and also the separation of south transect from the north transect offshore stations (Fig. 3). Principal component analysis (PCA) simplified the abundance of bacterial OTUs (DGGE bands) into three new linear variables that encompassed most of the variability in the original data (71% of total variance). The three first principal components of bacterial assemblage structure were correlated to several environmental or bacterial carbon processing variables. The first principal component (PC1, 43% of total variance) was significantly correlated with salinity and chlorophyll (Table 2) as well as several bacterial parameters such as bacterial production, biomass or percentage of high-nucleic acid cells (Table 2). The second principal component (PC2, 14.8% of the variance) was not correlated with any of these environmental or biotic parameters, and the third principal component (PC3, 13.5% of the variance) was only correlated with temperature (Table 2).

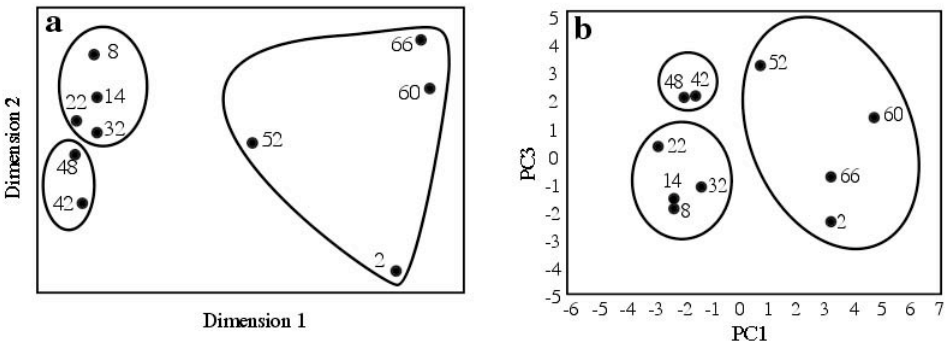

Fig 3. a) Nonmetric multidimensional scaling (MDS) and b) principal component analysis (PCA) of the bacterial assemblage composition data (DGGE band pattern). The stations have been clustered in agreement with the Ward's clustering method results (as shown in Fig 2).

Table 2. Correlation coefficients (Spearman *rho*) between the scores of the stations on the first, second and third principal components (PCs) generated by the bacterial diversity PCA, and environmental (concentrations of particulate organic matter -POC, µM-, chlorophyll a -Chl, µg l^{-1}-, temperature and salinity) and bacterial variables (bacterial production estimated by leucine -BHP Leu-, and thymidine -BHP TdR- uptake, µg C l^{-1} d^{-1}-, bacterial respiration -Bact resp, µg C l^{-1} d^{-1}-, bacterial biomass -Bact biomass µgC l^{-1}-, and percentage of high nucleic acid content-HNA-cells). N varies between 9 and 10. Significant values are in bold *p ≤ 0.05, **p ≤ 0.01. Percentages of variance of bacterial diversity (% var.) explained by each PC are shown.

	PC 1 *(43% var.)*	PC 2 *(15% var.)*	PC 3 *(14% var.)*
Environmental			
Temp.	-0.56	-0.47	**0.64***
Salinity	**-0.73***	0.42	0.03
[POC]	0.63	0.18	0.25
[Chl]	**0.76****	-0.34	0.02
Bacterial			
BHP (Leu)	**0.84***	0.27	0.17
BHP (TdR)	0.28	0.30	0.05
Bact Resp	**0.66***	-0.09	0.58
Bact biomass	**0.64***	-0.37	-0.32
%HNA	**0.82****	-0.18	-0.32

The relationship between bacterial assemblage structure and carbon processing and environmental factors was assessed through correlation (Spearman rank correlation) of the corresponding similarity matrices. We found a strong relationship between environmental data and bacterial assemblage structure through the samples (Spearman *rho* = 0.70, $p < 0.01$), and a weaker but significant relationship between bacterial assemblage structure and carbon processing patterns (Spearman *rho* = 0.36, $p < 0.05$). The relationship between environmental parameters and carbon processing data was not statistically significant ($p > 0.05$).

The results of the experiments of nutrient limitation of bacterial production (Table 1) also showed relative agreement with the grouping of the stations in the bacterial assemblage structure by MDS or PCA (Fig 3). The upwelling stations 52, 60 and 66, which clustered together by the DGGE band pattern, were characterized by carbon limitation (Table 1). In Sta 42 and 48, which also showed a related bacterial assemblage structure, bacteria responded in a similar way to the experimental additions showing no apparent limitation by inorganic or organic resources. We found higher variability in the response to nutrient enrichment within the group of Northern offshore stations, which included stations located in the coastal transition zone and the open ocean. The two stations located in the coastal transition zone (Sta

8 and 14), which were close in the PCA ordination, showed the same response to nutrient addition experiments (i.e. carbon limitation, Table 1).

In situ abundances of bacterial phylogenetic groups were determined by CARD-FISH, which, on average, detected 66% of the DAPI stained cells with the mixture of three HRP-probes covering the domain *Bacteria* (Eub+ cells, Eub 338-II-III, Fig 4). A higher percentage of Eub+ cells was further identified with probes for Alpha- and Gammaproteobacteria and Bacteroidetes (Alf968, Gam42a, CF319a) groups in the upwelling area (average 87% of EUB+ cells) compared to offshore waters (Fig. 4). The amount of cells hybridizing with Archaeal probes (Eury806 and Cren554) in surface waters was below 3% of DAPI-stained cells (details not shown). Alphaproteobacteria was the most abundant group in both transects, with an average of 36% of the DAPI stained cells (55% of Eub+ cells). The SAR11 clade accounted for a majority of the Alphaproteobacteria cells. Bacteroidetes and Gammaproteobacteria constituted, on average, 8 and 7% of Eub+ cells respectively, and like the Roseobacter group, were detected in significantly higher proportions in the upwelling zone (Student t-test, $p < 0.05$, Fig 4).

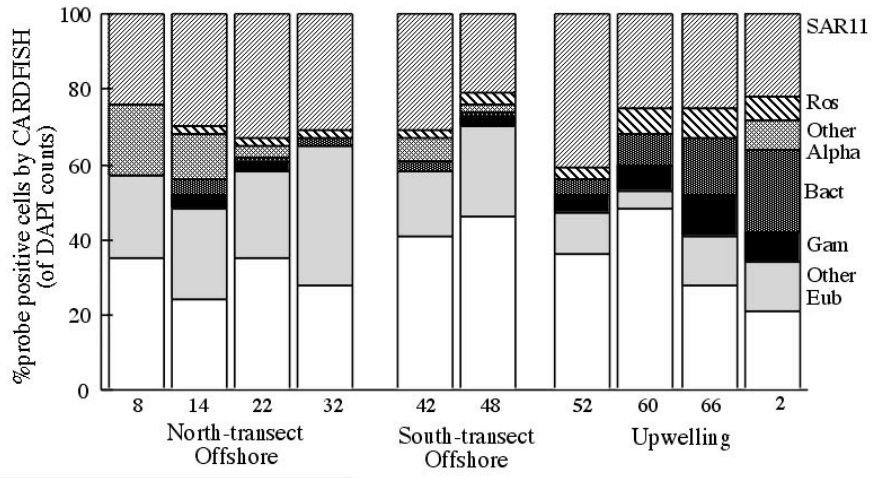

Fig 4- Abundance of bacterial groups detected by HRP-probes (CARD-FISH) scaled to DAPI counts in the stations. Ros: Roseobacter, Alpha: Alphaproteobacteria, Gamma: Gammaproteobacteria, Bact: Bacteroidetes, Eub: Eubacteria.

We analyzed the correlation between the abundance of bacterial groups detected by CARDFISH and environmental or bacterial variables, including the principal components that captured most of the variation of the DGGE banding pattern. The abundance of Roseobacter and Gammaproteobacteria was highly correlated to salinity and concentration of POC and Chl *a*, and Bacteroidetes showed a strong negative correlation with temperature (Table 3). The abundance of these three groups was significantly correlated with the percentage of HNA-cells, as well as with the first PC of the bacterial diversity PCA. Although the abundance of Roseobacter was significantly correlated with bacterial heterotrophic production (Leucine uptake with empirical conversion factors), the abundance of total Alphaproteobacteria was negatively correlated with this parameter and also with bacterial respiration (Table 3). SAR 11 group was not significantly correlated with any of these parameters.

Table 3- Spearman rank correlation coeficients between the proportion of Bacteroidetes (CF319), Gammaproteobacteria (Gam42a), Alphaproteobacteria (Alf968), Roseobacter (Ros537) and SAR11 (SAR11-441R) groups, concentrations of particulate organic matter (POC) and chlorophyll a, bacterial production estimated by leucine (Leu) and thymidine (TdR) uptake, bacterial respiration, bacterial biomass and percentage of high nucleic acid content cells. Abbreviations and units as in Table 2. The percentage of the groups corresponds to abundance scaled to DAPI counts. N varies between 9 and 10. Significant values are in bold *p ≤ 0.05, **p ≤ 0.01.

	%CF319	%Gam42a	%Alf968	%Ros537	%SAR11-441R
Environmental					
Temp.	**-0.75****	-0.60	0.14	-0.50	0.40
Salinity	**-0.65***	**-0.77****	0.27	**-0.76****	0.44
[POC]	0.58	**0.84****	-0.43	**0.91****	-0.38
[CHL]	0.50	**0.77****	-0.32	**0.77****	-0.42
Bacterial					
BHP (Leu)	0.45	0.36	**-0.79****	**0.70***	-0.37
BHP (TdR)	-0.17	-0.28	-0.58	0.06	-0.30
Bact Resp	0.11	0.26	**-0.64***	0.58	-0.26
Bact biomass	**0.64***	**0.67***	-0.25	0.61	-0.25
%HNA	**0.877****	**0.849****	-0.16	**0.67***	-0.14
PC 1	**0.69***	**0.66***	-0.51	**0.81****	-0.18
PC2	-0.04	-0.01	-0.11	-0.01	0.18
PC3	-0.26	0.01	-0.18	0.18	0.45

Discussion

Bacterial assemblage composition appears to be uniform over large oceanic areas (Acinas et al. 1997), even when significant differences in environmental or bacterial activity parameters exist (Riemann et al. 1999, 2002). As an example, Arrieta et al. (2004) found that the phylogenetic composition of the bacterioplankton community did not change after an iron fertilization experiment in the Southern Ocean, despite significant changes in bacterial production, abundance and ectoenzymatic activities. This suggests that bacterial community structure can remain stable under different trophic conditions, and probably perform redundantly in carbon processing.

On the other hand, substantial changes in bacterial community structure have also been observed spatially in the sea, particularly in upwelling and frontal systems (Kerkhof et al. 1999, Suzuki et al. 2001, Pinhassi et al. 2003). To know whether changes at the bacterial diversity level can affect the in situ carbon metabolism is crucial to understand whether bacterioplankton can be regarded as an uniform unit, or a more detailed phylogenetic picture is needed in carbon flux models. Such linkage, though, has very seldom been explored in oceanic waters, and almost exclusively comparing changes in bacterial richness with several carbon processing parameters (Reinthaler et al. 2005, Winter et al. 2005).

Here, we explored the linkage using a multivariate approach, in which we analyzed changes in bacterial assemblage composition by DGGE and simultaneously, a range of bacterial carbon processing variables. PCA was used in order to reduce the complexity of bacterial community structure (DGGE banding pattern) into a few linear variables, which were subsequently correlated to environmental and bacterial variables, similarly to previous studies in different systems (e.g. Fry et al. 2006). The ordination of the stations with the PCA analysis of DGGE banding pattern was highly coherent with results from a non-parametric approach based solely on similarity distances of stations (MDS) and Ward's clustering method, making our results robust.

Spatial variability in bacterial assemblage structure in the NE Atlantic Ocean.

In previous studies DGGE has provided insights into changes in microbial diversity along temporal and spatial scales (Murray et al. 1998, Schauer et al. 2000, 2003) but, as all the PCR-based methods, is potentially prone to problems and biases (Wintzingerode et al. 1997). The

use of a PCR-independent method (i.e. CARDFISH) allowed the identification and quantification of the dominant bacterial groups by the use of specific probes. Both complementary approaches have been very seldom compared (Castle & Kirchman 2004) and, in our study, showed good agreement in the differences of bacterial assemblage structure throughout the area, especially between the upwelling and the offshore stations. As an example, the high similarity shown by CARDFISH between Stn. 66 (closest to the upwelling area in the Southern transect) and Stn. 2 (upwelling filament reaching the Northern transect), in spite of being located almost 800 km away, was particularly relevant as to the role of oceanographic regimes in shaping bacterial community structure.

There are still few studies in open ocean waters that use fluorescence in situ hybridization (FISH) to provide a detailed description of the composition of bacterioplankton including probes for broad and more specific phylogenetic groups. Glöckner et al. (1999) provided first insights into differences in assemblage compositions of different marine regions with group-specific oligonucleotide probes. Eilers et al (2001) showed for the first time seasonal differences in the contribution of Alpha-, Gammaproteobacteria, Bacteroidetes and other groups to bacterial assemblage in the North Sea. More recently, Fuchs et al. (2005) used the adapted CARDFISH protocol (Pernthaler et al. 2002) to study the composition of bacterioplankton in different areas of the Arabian Sea. These authors found minor differences in the proportion of major phylogenetic groups, but higher differences in the proportion of more specific groups such as SAR86, SAR11 and SAR116. In our study we also used the improved CARDFISH methodology, which probably contributed to the high detection of groups, such as SAR11, in oligotrophic oceanic samples.

On average, we found that SAR11 made up 28% of the DAPI counts, which are values comparable to the few previous studies that have detected this group by means of fluorescence in situ hybridization with the use of multiple probes (Morris et al. 2002, Malmstrom et al. 2004). Our findings corroborate that SAR11 is one of the most abundant bacterial groups in the oceans and suggest that this clade was not favored, in terms of contribution to assemblage composition, by upwelling conditions (in agreement with the results of Fuchs et al. 2005).

In contrast, we found maximal contributions to assemblage structure of Roseobacter, Gammaproteobacteria and Bacteroidetes in the upwelling region. The Roseobacter group has been detected in high proportions in association with natural phytoplankton blooms (González et al. 2000, Suzuki et al. 2001). Furthermore, members of this group have been suggested to be active colonizers of particles under algal bloom conditions (Riemann et al. 2000). Similarly, the

Gammaproteobacteria group is also characterized by having members with high growth rates that could be favored by nutrient enrichment pulses (Eilers et al. 2000, Fuchs et al. 2000, Pinhassi & Berman 2003, Yokokawa et al. 2004).

The Bacteroidetes cluster has also been found in upwelling events (Suzuki et al 2001), and their members have a high ability to degrade high-molecular weight compounds (Cottrell & Kirchman 2000, Kirchman 2002). This carbon source was probably abundant in the upwelling area, where we also encountered high POC concentrations (Chapter I). Indeed, members of the Bacteroidetes group have been reported to be quantitatively important components of both, the free-living and the particulate fraction of bacterial assemblages, during phytoplankton blooms (Abell & Bowman, 2005, Riemann et al. 2000).

Linkage between environmental factors, bacterial assemblage structure, and carbon processing.-

The linkage between bacterial assemblage structure and carbon metabolism is a difficult issue to elucidate under in situ conditions, since environmental factors are probably affecting both parameters. In this study we found that environmental factors significantly affected the composition of the bacterial assemblages (Spearman *rho* 0.66, $p<0.01$), but did not significantly affect carbon processing. The first principal component of bacterial assemblage structure, which included most of the variation of the DGGE banding pattern, was significantly correlated with environmental variables characteristic of the upwelling, such as salinity and Chl a. These results are in agreement with reported changes in bacterial diversity in upwelling regimes (Suzuki et al. 2001).

In our samples, the composition of the bacterial assemblage was also significantly correlated with the bacterial processing of carbon, although the strength of this relationship was not very strong by this correlation approach (Spearman *rho* 0.36, $p<0.05$). Similarly, the first principal component of the DGGE banding pattern was significantly correlated with bacterial production (Leu uptake) and biomass, suggesting that the community developing in the upwelling was performing differently in terms of carbon use.

The coupling between bacterial assemblage composition and carbon metabolism is in contrast with some other studies in marine open waters that have reported no such relationship, like the experimental study by Arrieta et al. (2004). However, a recent study by Fuhrman et al. (2006) has shown that the temporal patterns in distribution and abundance of bacterial taxa (analyzed by automated ribosomal intergenic space analysis, the ARISA-

fingerprint technique) off the California coast were significantly influenced and could be predicted by a range of abiotic and biotic factors including bacterial production by TdR uptake. These results suggest low levels of redundancy in functional bacterial communities.

Pinhassi et al. (2003) have suggested that not only quantitative but also qualitative differences in variables that affect bacterial growth, are required to understand the variability in bacterioplankton assemblage composition. In their study across the Skagerrak front, these authors hypothesized that N versus P limitation could affect the variability in bacterial assemblage structure. In contrast, Van Mooy et al. (2004), using the T-RFLP methodology, found that the growth response to different organic amendments was generally unrelated to bacterial assemblage structure in the North Pacific. In our case, nutrient limitation seemed to be generally related to bacterial assemblage structure, although with some exceptions (sta 22 and 32 in the north transect).

Relationship between the in situ abundance of bacterial groups and environmental or biotic parameters.

SAR11 was the dominant group of Alphaproteobacteria, and their abundance was not significantly correlated with any carbon processing variables, suggesting that this group was not importantly affecting the carbon metabolism in this system. Although this group has shown to be highly active in the Sargasso Sea in some studies by means of microautoradiography combined with FISH (Malmstrom et al. 2004), their activity seems to be significantly lower in more coastal waters, such as the North Sea or the NW Mediterranean (Alonso & Pernthaler 2006a, Chapter V).

The percent contribution of *Roseobacter* was highly correlated with POC and Chl *a*, suggesting that this group can be a good competitor under high levels of resources, efficiently consuming the carbon produced by the phytoplankton. This is in agreement with the common association of this lineage with phytoplankton cultures (Schäfer et al. 2002) or natural blooms (Suzuki et al. 2001). This group was also highly correlated with bacterial production, in agreement with their high single-cell activity in terms of substrate uptake (Alonso & Pernthaler 2006b, Chapter V).

The three groups -Roseobacter, Gammaproteobacteria and Bacteroidetes- were positively correlated to the %HNA cells (Table 5). The %HNA cells has been shown to be a relatively good correlate of bacterial activity in coastal waters (Lebaron et al. 2001, 2002), and shifts in bacterial metabolism have been associated with changes in the percentages of active cells (del

Giorgio & Bouvier 2002). Although the positive correlation is not a direct observation, it suggests that these groups could be composed of high nucleic acid ("active") bacteria. This would agree with the results of Zubkov et al. (2001), who flow-cytometrically sorted the HNA bacterial community of an offshore sample and found high proportions of Bacteroidetes and Roseobacter within this group. On the contrary, SAR11 always fell in the low-nucleic-acid-group in a study conducted in the Arabian Sea by Fuchs et al. (2005).

In summary, bacterial assemblage structure was highly influenced by environmental factors such as temperature, salinity and chlorophyll, showing a distinct community in the upwelling, and different spatial regions within offshore waters. Bacterial carbon metabolism was weakly but significantly correlated with bacterial assemblage structure, as shown by Mantel procedure and by the significant correlations between the first principal component of bacterial assemblage structure data and several carbon processing parameters. The bacterial groups Roseobacter and Gammaproteobacteria showed positive correlations with bacterial heterotrophic production and biomass, respectively. SAR11 group, which was approximately constant throughout the area did not correlate with any of these parameters, suggesting their lower participation in carbon metabolism. The correlative approaches, such as the one we use, can provide first insights on the linkage between bacterial assemblage structure, biotic and abiotic parameters. Even if correlation does not imply causality, it is of great interest to know whether changes on bacterial assemblages are associated with changes in carbon metabolism for carbon modeling and predictive purposes. Further studies with the use of single-cell techniques will undoubtedly help us to identify the groups with the most important roles in the flux of carbon in the ocean and accelerate the potential incorporation of bacterial assemblage structure into carbon cycle models.

Acknowledgements

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Chapter III

Seasonality in bacterial diversity in NW Mediterranean coastal waters: assessment through clone libraries, fingerprinting, and fluorescence in situ hybridization

L. Alonso-Sáez, V. Balagué, E.L. Sà. O. Sánchez, J.M. González, J. Pinhassi, R. Massana, J. Pernthaler, C. Pedrós-Alió & J. M. Gasol

FEMS Microb. Ecol. (submitted)

Abstract

We combined 16S rRNA gene clone libraries, denaturing gradient gel electrophoresis (DGGE), and catalyzed reporter deposition-fluorescence in situ hybridization (CARD-FISH), to investigate the seasonality of the bacterial assemblage composition in NW Mediterranean coastal waters. DGGE analysis indicated that bacterial diversity changed gradually throughout the year, although with a clear separation of the summer period. Alphaproteobacteria were the dominant group (annual average, 29% of the DAPI counts by CARD-FISH, and 70% of the bacterial clones), and SAR11 showed very high proportions during spring and summer (>20% of DAPI counts). However, the sequencing of the most predominant DGGE bands missed the SAR11 cluster, possibly due to the large microdiversity found within this group. Roseobacter were less abundant than SAR11, and reached higher proportions in winter and spring (up to 7% of DAPI counts). Bacteroidetes constituted the second most important group and were quantitatively uniform throughout the year (average 11% of the DAPI counts). Gammaproteobacteria showed a peak during summer (8% of DAPI counts), when most of them belonged to the NOR5 cluster, as shown by CARD-FISH. Clone libraries and CARD-FISH showed reasonable agreement in the quantitative proportions of Bacteroidetes and Gammaproteobacteria, but also a bias towards overrepresentation of Alphaproteobacteria in clone libraries.

Introduction

First insights into marine bacterial diversity were obtained by the identification of easily cultured species from seawater, which were assumed to be dominant (Zobell 1946). The finding that cultured bacteria make up less than 1% of the in situ assemblages (reviewed in Staley & Konopka 1985), and the introduction of rRNA-based molecular techniques in microbial ecology (Pace et al. 1986), promoted the study of the species that are actually dominant in the environment. Early applications of such methodologies, by cloning and sequencing 16 rRNA genes extracted from open water samples, revealed that most in situ marine bacteria, such as the SAR11 cluster, were unknown (Giovanonni et al. 1990, Schmidt et al. 1991). Since then, many clone libraries have been constructed with samples from different oceans, resulting in the hypothesis that a relatively few major phylogenetic groups are widespread and predominate in marine bacterioplankton (Giovanonni & Rappé 2000, Hagström et al. 2002, Rappé & Giovanonni 2003). However, given that clone libraries are relatively expensive and time-consuming, most of these studies are based on single sampling

points, what seriously limits our understanding of temporal-spatial variations in bacterial diversity.

The application of fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE) or terminal restriction fragment length polymorphism (T-RFLP), has facilitated the study of changes in the bacterial assemblage composition at different spatial and temporal scales (e.g. Schauer et al. 2000, 2003, Ghiglione et al. 2005). These methods allow a reasonably straightforward comparison of the phylogenetic composition of a large number of samples, and some of them, such as DGGE, further allow the identification of some phylotypes by subsequent sequencing. Like other fingerprint techniques, however, DGGE is not able to assess the diversity of the assemblage to the same level of detail that clone libraries allow.

Both fingerprinting and clone libraries methods are subjected to different sources of bias. The use of different primers significantly changes the bacterial assemblage composition obtained by these techniques (Cardinale et al. 2004), and PCR differential amplification of sequences can importantly affect the quantitative interpretation of results (Wintzigerode et al. 1997). The appropriate quantification of distinct bacterial groups can be achieved by fluorescence in situ hybridization (FISH) with specific probes. However, this technique is limited by the number, specificity and coverage of the available probes, and only allows accounting for the previously known lineages. Due to the counting effort, it is a time-consuming procedure specially if a large number of probes are used, and thus, this technique usually cannot be applied in practice to detect all the phylotypes retrieved in clone libraries. It is a tool more useful for the analysis of populations, rather than for describing the diversity of the whole community (Pernthaler & Amann 2005).

Different molecular approaches have been applied in the relatively few studies that have assessed seasonal bacterial diversity in marine waters (Schauer et al. 2003, Ghiglione et al. 2005, Brown et al. 2005). Pinhassi & Hagström (2000) used whole-genome DNA hybridization in the Baltic Sea and found a clear differentiation between the bacterial assemblages during spring, dominated by Bacteroidetes, and summer, dominated by Alphaproteobacteria. Eilers et al. (2001) studied the seasonality of marine bacterial groups by FISH in the North Sea, and found that Bacteroidetes dominated during spring and early summer, while a group of Gammaproteobacteria (i.e. NOR5) was abundant during summer. Mary et al. (2006) also found dominance of Bacteroidetes in spring and early summer in the English Channel by catalyzed reporter deposition (CARD)-FISH, and reported dominance of Alphaproteobacteria from late

summer to winter. In the ocean surface (Bermuda Time Series Station, BATS), the groups SAR11, SAR86 and SAR116 exhibited the strongest increases during summer periods, as shown by T-RFLP and bulk nucleic acid hybridization (Morris et al. 2005). Even if some comparisons between the various molecular approaches have been carried out (Cottrell & Kirchman 2000, Castle & Kirchman, 2004), it is still unknown to what extent the different techniques can be quantitatively compared.

Schauer et al. (2003) provided a first approach to the seasonality of bacterial assemblages of Blanes Bay, a well-studied coastal station in the NW Mediterranean, using the DGGE technique. In the present study we constructed 5 clone libraries and obtained monthly DGGE and CARDFISH data throughout one year, with two main objectives: (a) to obtain a detailed picture of the seasonal changes in bacterial diversity in Mediterranean coastal waters, and (b) to compare the results obtained by the three different methods, in order to describe the strong and weak points of each approach, and how they affect the overall image of bacterioplankton diversity and assemblage structure generated by each technique. To our knowledge, this is the first study that compares results of the three different approaches simultaneously on the same set of marine samples.

Materials and methods

Location and sampling. We carried out a monthly study in Blanes Bay (The Blanes Bay Microbial Observatory, NW Mediterranean) from 4 March 2003 to 22 March 2004 (14 samples). Surface waters were monthly sampled at about 1 km offshore (41°40'N, 2º 48'E), filtered through a 200 μm net and transported to the lab under dim light (within 1.5 hours) in 25 l polycarbonate carboys. For convenience, the sampling on 4 March 2003 will be referred to as "February 2003", to avoid confusion with the sampling on 25 March 2003 (i.e. March 2003). Samples were filtered (for DGGE and clone libraries) or fixed (for CARDFISH) immediately upon arrival to the lab.

Basic data. Surface water temperature was measured in situ with a mercury thermometer. For determination of Chlorophyll *a* concentration, 150 ml of seawater were filtered on GF/F filters (Whatman) and subsequently extracted in acetone (90% v/v) in the dark at 4ºC for 24h. Fluorescence was measured with a Turner Designs fluorometer. Hourly rain values (mm) were obtained from the automatic meteorological station located at Malgrat de Mar (6 km away

from Blanes Bay), run by the SMC (Servei Meteorològic de Catalunya) and integrated for the 7 days before each sampling date.

Abundance of prokaryotes and photosynthetic picoplankton. *Synechococcus*, *Prochlorococcus* and photosynthetic picoeukaryotes were enumerated by flow cytometry and distinguished by their different size and pigment properties in unstained samples following common procedures (i.e. Marie et al. 1997). Heterotrophic prokaryotes were also counted by flow cytometry (Gasol & del Giorgio 2000) after staining with Syto13.

DNA extraction. Surface microbial biomass was collected by sequentially filtering around 8 l of seawater through a 3 μm pore size Polycarbonate filter (Poretics) and a 0.2 μm Sterivex filter (Durapore, Millipore), using a peristaltic pump. The Sterivex units were filled with 1.8 ml of lysis buffer (50 mM Tris-HCl pH 8.3, 40 mM EDTA pH 8.0, 0.75 M sucrose) and kept at -80ºC. Microbial biomass was treated with lysozyme, proteinase K and sodium dodecyl sulphate, and the nucleic acids were extracted with phenol and concentrated in a Centricon-100 (Millipore), as described in Massana et al. (1997).

DGGE, band sequencing and phylogenetic analysis. DGGE and band sequencing were performed as previously described (Schauer et al. 2003). Briefly, 16S rRNA gene fragments were amplified by PCR using the universal primers 907rM and 358f with a GC-clamp. PCR products were loaded on a 6% polyacrylamide gel with a DNA-denaturant gradient ranging from 40 to 80%. The gel was run at 100 V for 16 h at 60°C in 1x TAE running buffer using DGGE-2000 system (CBS Scientific company). These conditions had been previously optimized for the Blanes samples. DGGE gel images were analyzed using the Chemidoc software (BIO-RAD). A matrix was constructed for all lanes taking into account the relative contribution of each band (in percentage) to the total intensity of the lane. Based on this matrix, we obtained a dendrogram by UPGMA clustering method (Euclidean distances, Statistica 6.0). DGGE bands were excised and reamplified with the original primer set. The position of the bands was confirmed in another DGGE gel. Bands were purified and subsequently sequenced using the primer 358f without the clamp, with the Big Dye Terminator Cycle Sequencing Kit v3.1 (PE Biosystems) and an ABI 3100 (Applied Biosystems) automated sequencer. The sequences obtained were compared with public database DNA sequences by using BLAST (Altschul et al. 1997) to determine their phylogenetic affiliation. Seven bands (i.e. OTUs, Operational Taxonomic Units) were excised at their corresponding position in several lanes in order to analyze the similarity within each band or OTU. Excluding

one case (out of 20), the average similarity of sequences within the same band position was 99.1%.

Clone libraries, RFLP analysis and sequencing. Cloning and RFLP analysis were performed as previously described (Ferrera et al. 2004). 16S rRNA genes were amplified by PCR with the universal primers 27f and 1492r. Products from 3 individual PCR reactions were pooled and cleaned with the QIAGEN PCR purification kit and cloned using the TOPO-TA cloning kit (Invitrogen). PCR amplifications were digested with the restriction enzyme *Hae*III (Invitrogen), and the RFLP patterns of the clones were compared. Clones showing the same RFLP pattern (DNA fragments of equal size) were grouped together and considered to belong to the same OTU. We analyzed the similarity between clones from the same OTU (a total of 58 clones within 17 different OTUs) and the average similarity was 98.8%. One clone for each OTU was partially sequenced with the internal primer 358f. Chimeric sequences were identified by using the CHECK_CHIMERA (Maidak et al. 2001) and by BLAST search with different sequence regions. Sequences were aligned using ClustalW 1.82 (Thompson et al. 1994) and very variable regions of the alignment were automatically removed with Gblocks (Castresana 2000). Maximum likelihood analysis was carried out with PAUP 4.0b10 (Swofford 2002), using the model of evolution and the parameters estimated by ModelTest 3.7 (Posada & Crandall 1998). Gene sequences were deposited in Genbank under accession numbers DQ778132-DQ778298.

CARDFISH. Samples were fixed with paraformaldehyde (2% final conc., overnight at 4ºC) for the determination of in situ abundance of different bacterial populations by CARD-FISH (Pernthaler et al. 2004). Filters were permeabilized with lysozyme (37ºC, 1h) and hybridization was performed at 35°C for a minimum of 2 hours. Horseradish peroxidase (HRP) labeled probes (50 ng μL^{-1}) were added to the hybridization buffer (HB, 1:300) containing the following percentages of formamide: 20% for probe Eury806 (Teira et al. 2004) and Cren554 (Massana et al. 1997), 50% for NOR5-730 (Eilers et al. 2000), 60% for Alt1413 (Eilers et al. 2000) and 55% for Eub338-III (Amann et al. 1990; Daims et al. 1999), Ros537 (Eilers et al. 2001), SAR86/1245 (Zubkov et al. 2001), Gam42a and CF319a (Amann et al. 1990). We used higher concentration of probes in the HB (1:100, 45% formamide) and overnight hybridization to detect the cells with probes SAR11-441R (Morris et al. 2002) and Alf968 (Neef 1997). The Eub antisense probe Non338 (Wallner et al. 1993) was used as negative control. For the amplification, we used tyramide labeled with Alexa 488. Counter-staining of CARDFISH preparations was done with 4'-6'-diamidino-2-phenylindole (DAPI, final conc. 1 $\mu g\ ml^{-1}$).

DAPI and FISH-stained cells were counted by automated image analysis (Pernthaler et al. 2003).

Diversity estimates. The relative distribution of OTUs in each library was used to calculate coverage values (Good 1953) and the non-parametric S_{Chao1} estimator (Chao 1984) using the software tool provided by Kemp & Aller (2004). Good's coverage is a non-parametric estimator of the proportion of phylotypes in a library of infinite size that would be represented in a smaller library. As defined by Good (Good 1953), coverage is calculated as: $C= 1-n_1/N$ where n_1 is the number of phylotypes appearing only once in a library and N is the library size. The S_{Chao1} non parametric estimator yields an estimate of the probable total number of phylotypes present in the source assemblage (Chao et al. 1993, Lee & Chao 1994). When it reaches a plateau, the library could be considered "large enough" to provide an unbiased estimate of OTU richness (Kemp & Aller 2004).

Results

The summer period (from June to September) was characterized by high temperatures (23-25ºC) and low concentrations of Chl *a* (around 0.2 μg l^{-1} Chl *a*, Fig. 1A). Water transparency was also higher in summer (June-September), and nutrient concentrations were substantially lower from May to October (details not shown). Higher concentrations of Chl *a* were found in winter (from December to the end of March), generally over 1 μg l^{-1} Chl *a*. Prokaryotic abundance followed somehow the pattern of Chl *a*, with two peaks at the end of March (2003) and December (Fig. 1B). There was a replacement of picophytoplankton populations of picoeukaryotes (peak during the winter), *Synechococcus* (peaks in spring and summer), and *Prochlorococcus* (higher abundances from September to January, Fig. 1C).

Seasonal pattern of bacterial assemblage structure as revealed by denaturing gradient gel electrophoresis (DGGE)- The analysis of the DGGE gel yielded a total of 73 different band positions or OTUs (Fig. 2). The number of bands per sample was significantly lower (t-Test, $p<0.0001$) in the spring-summer period (end or March to September, ranging between 21 and 32) than in the autumn-winter period (October to beginning of March, range between 35 and 41). In general, less than 25% of the bands (4 to 10 bands) accounted for more than 50% of the total intensity per lane. A total of 30 band positions were excised in order to determine

their phylogenetic affiliation, and informative sequences were obtained from 23 bands (Table 1). These bands accounted for 35 to 80% of the total band intensity in each sample. Most bands showed high similarity (>97%) with sequences from uncultured clones by BLAST search in public databases (Table 1).

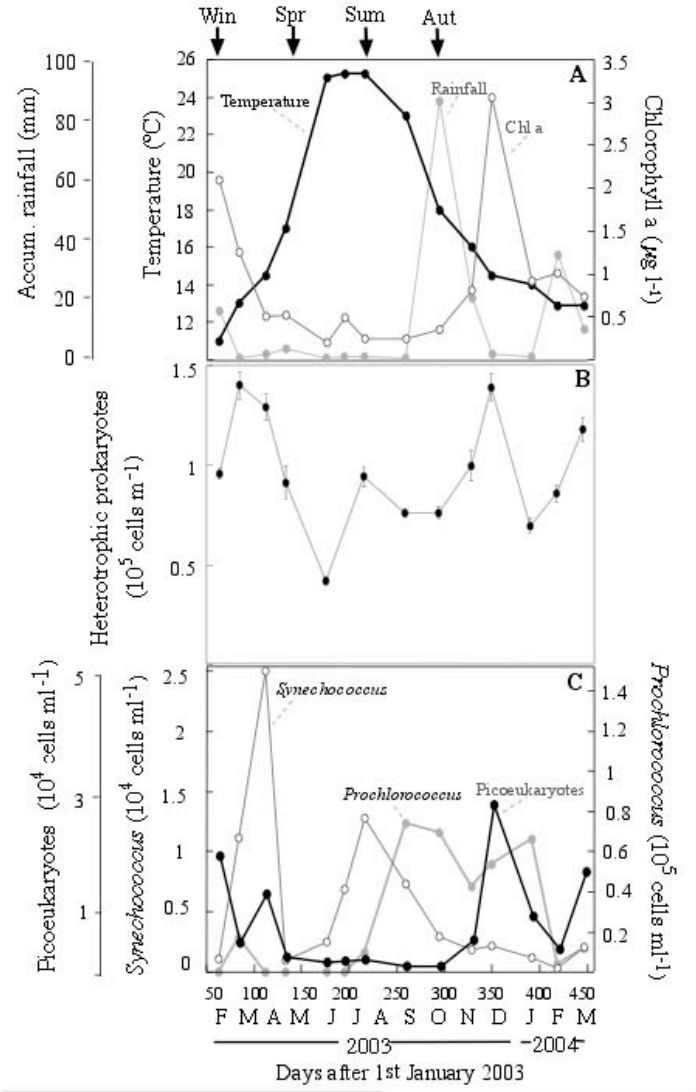

Fig 1- A) Seasonal changes in chlorophyll *a* concentration, temperature, and accumulated rain (during the week before each sampling), B) Abundance of prokaryotes, and (C) abundance of photosynthetic picoeukaryotes, *Synechococcus* and *Prochlorococcus* in Blanes Bay. Arrows indicate the samples from which seasonal clone libraries were carried out.

Only three bands of bacterial origin (7% of the bacterial phylotypes) persisted throughout the year. These bands were affiliated with two Roseobacter phylotypes (bands 35 and 43, Table 1) and the SAR116 group (band 33, Table 1), although this last band was always present at low intensities (lower than 3% of total intensity per lane). A plastid of *Micromonas* (band 27, Table

1) was also detected throughout the year, but with very low intensities during the summer. On the other hand, 9 bands (21% of the OTUs) were exclusively detected at only one sampling date.

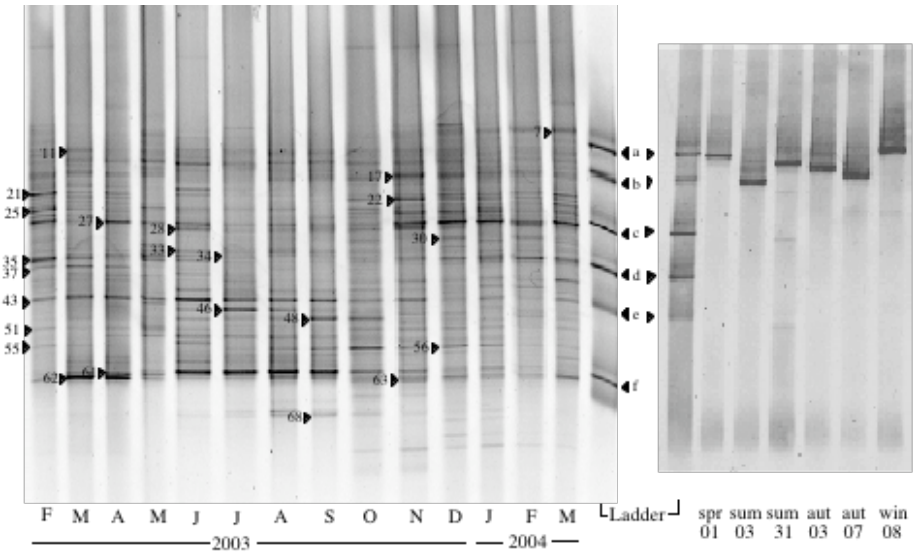

Fig 2- DGGE gel of bacterial 16S rRNA gene fragments from the seasonal samples, indicating the band positions which were succesfully sequenced (presented in Table 1). On the right, DGGE gel of clones affiliated with the SAR11 cluster retrieved from the spring (clone BL03-SPR01), summer (BL03-SUM03 and SUM31), autumn (BL03-AUT03 and BL03-AUT07) and winter (BL03-WIN08) clone libraries. A ladder including different environmental clones from Blanes Bay (a:BL03-AUT19-Bacteroidetes, b:BL03-SPR52-SAR11, c:BL03-SPR25-Plastid of *Dinophysis norvegica*, d: BL03-SPR09-Roseobacter NAC11-7, e: BL03-JUL18-Gammaproteobacteria, and f:BL03-JUL23-*Synechococcus*) was included in both DGGE gels in order to compare the band positions.

The DGGE results showed rather gradual changes of the bacterial assemblage throughout the year, following a clear seasonality (Fig. 3). The dendrogram separated the samples taken in the summer period (June to September) from the samples taken in other seasons. Within the non-summer cluster, spring samples (end of March to May) were separated from winter and autumn samples, which also formed different clusters.

Three bands were affiliated with *Synechococcus* (Table 1), and the relative intensity of these bands was significantly correlated with the relative abundance of this population (over total prokaryote abundance) determined by flow cytometry (arcsine transformed, Pearson's r= 0.64, n= 14, p= 0.01). Similarly, the relative abundance of band 56, corresponding to *Prochlorococcus marinus,* was highly correlated with the relative abundance of *Prochlorococcus* by flow cytometry

(arcsine transformed, r=0.74, n=14, p=0.0025). The intensity of bands of plastidial origin was not included in total band intensity for these and the following calculations.

Table 1- Phylogenetic affiliation of sequences obtained from DGGE bands, closest uncultured and cultured matches and the presence and average relative intensity of the band in different samples. Number of bases used to calculate the sequence similarity is shown in parentheses in the third column.

Band	Closest match (environmental or culture)	Seq. sim.(%) (no. of bases)	Taxonomic group	GenBank Acc. number	Closest cult. match (% similarity)	Presence (nº samp)	Avg Intensity(%)
BL03-7	Clone OUT_A	100 (374)	Bacteroidetes	AF207850	*Tenacibaculum mesophilum* (98)	6	5.2
BL03-11	S1-005-F-B-Nr3	95.9 (439)	Bacteroidetes	AJ508420	*Owenweeksia hongkongensis (87)*	9	1.8
BL03-17	SCF-4969	98.1 (263)	Bacteroidetes	AJ630719	*Bizionia paragorgiae* (93)	9	4.0
BL03-21	clone OM5	100 (496)	Prasinophyte chloroplast	U70715	*Ostreococcus* sp. RCC393 (95)	13	2.4
BL03-22	SPOTSAPR01_5m159	99.2 (266)	Bacteroidetes	DQ009089	*Owenweeksia hongkongensis* (87)	11	2.3
BL03-25	*T. amphioxeia*	99.8 (491)	Cryptophyte chloroplast	AY453067	*Teleaulax amphioxeia* chlorop (99.8)	9	3.1
BL03-27	*Micromonas* RCC434	98.8 (430)	Prasinophyte chloroplast	AY702163	*Micromonas* sp (98.8)	14	7.3
BL03-28	CONP48	98.8 (342)	Bacteroidetes	AY828419	*Gelidibacter algens* (92)	7	3.1
BL03-30	clone SBI04_177	97.5 (324)	Bacteroidetes	DQ186969	*Chryseobacterium* (95)	7	2.2
BL03-33	NAC11-16	99.5 (402)	Alphaprot./ SAR116	AF245641	*Ahrensia kielensis* (90)	14	1.7
BL03-34	*M. methylotropha*	97.3 (299)	Alphaprot./*Roseobacter*	U62894	*Marinosulfonomonas methylotropha* (97.3)	10	4.3
BL03-35	NAC11-7	99.8 (499)	Alphaprot./*Roseobacter*	AF245635	*Ophiopholis aculeata* symbiont (99)	14	6.0
BL03-37	NAC11-6	99.3 (437)	Alphaprot./*Roseobacter*	AF245634	*Roseobacter* sp. 3008 (98)	6	2.4
BL03-43	EF100-65C12	100 (499)	Alphaprot./*Roseobacter*	AY627371	*Roseobacter* sp. LA7 (98)	14	6.3
BL03-46	DGGE band DI-12	99.5 (434)	Alphaprot./*Roseobacter*	AY919600	*Roseobacter* sp. (97)	10	2.7
BL03-48	*Erythrob.* 12IX/A01/170	100 (430)	Alphaprot./*Erythrobacter*	AY612770	*Erythrobacter citreus* (97)	10	3.4
BL03-51	clone 185	97.6 (411)	Alphaprot.	DQ187755	*Rhodothalassium salexigens* (91)	12	2.3
BL03-55	SPOTSAPR01_5m244	99.1 (424)	Alphaprot./Rhodobacteraceae	DQ009316	*Antarctobacter* sp. (91)	3	1.5
BL03-56	*P. marinus* MED4	99.2 (381)	Cyanobacteria/*Prochloroc.*	BX572090	*Prochlorococcus marinus* (99)	9	3.2
BL03-61	*Synechococcus* CC9605	100 (471)	Cyanobacteria/*Synechococcus*	CP000110	*Synechococcus* sp (100)	4	10.2
BL03-62	*Synechococcus* Almo3	99.8 (457)	Cyanobacteria/*Synechococcus*	AY172800	*Synechococcus* sp (99.8)	12	9.8
BL03-63	SPOTSAUG01_5m57	100 (256)	Bacteroidetes	DQ009288	*Aquaspirillum peregrinum* (91)	2	6.3
BL03-68	*Synechococcus* CC9605	99.8 (409)	Cyanobacteria/*Synechococcus*	CP000110	*Synechococcus* sp. (99.8)	10	2.5

Bands affiliated with Alphaproteobacteria represented a rather constant percentage of total band intensity (average of 32%). Five of these bands were affiliated with the Roseobacter cluster (21% of total band intensity, Table 1), showing seasonal substitution of OTUs. Bands 35 and 37 (highly similar to NAC11-7 and NAC11-6 clones respectively) were found with higher intensities in winter and spring, while band 46 was mainly found during the summer. Band 48, affiliated with genus *Erythrobacter* in the Alphaproteobacteria, was mainly found during the summer-autumn period. Seven bands were affiliated with Bacteroidetes (Table 1), with higher contributions to total band intensity in the autumn-winter (average of 19%) than in the spring-summer period (only 3% on average). Remarkably, none of the sequenced bands were affiliated with the SAR11 cluster or with Gammaproteobacteria. In order to observe the band positions that correspond to SAR11 phylotypes, we amplified and run several SAR11 clones from our clone libraries in a DGGE gel (Fig. 2). These clones migrated within a quite narrow region of the gel, where several faint bands were visible in the environmental samples. Several attempts to obtain clean sequences from these bands of the gel were unsuccessful.

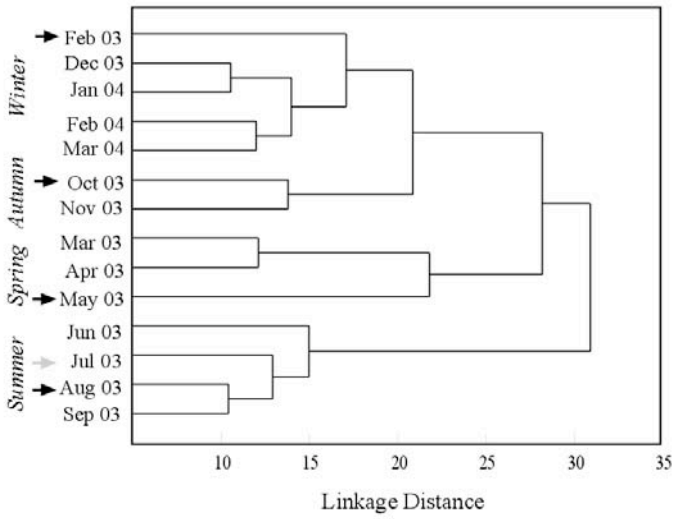

Fig 3- Dendrogram of the DGGE banding pattern constructed from the intensity matrix, grouping the Blanes Bay samples. Arrows indicate the samples from which seasonal clone libraries were carried out.

Quantitative analysis of microbial assemblage composition by CARDFISH- On average (±SD), 73 ± 10% of DAPI-stained cells were detected with the universal set of probes for *Bacteria* (Eub+ cells). Most of Eub+ cells were identified with probes for broad phylogenetic

groups (Bacteroidetes, Alpha- and Gammaproteobacteria, average 75%), with a lower proportion of identified Eub+ cells in autumn-winter (63%) than in the spring-summer period (87%). We found that *Euryarchaea* reached higher proportions in winter (up to 6% of DAPI cells) and remained below detection in summer (Table 2). Several counts of *Crenarchaea* (probe Cren 554) in different months indicated that this group was below 3% of DAPI counts throughout the year (data not shown).

Table 2- Percentage of total DAPI counts (±SD of replicate filters) detected with the group-specific HRP-probes Eury906 (*Euryarchaea*), Eub338-II-III (*Eubacteria*),), CF319a (Bacteroidetes), Gam42a (Gammaproteobacteria), Alf968 (Alphaproteobacteria), Ros537 (Roseobacter), SAR11-441R (SAR11 clade), Alt1413 (Alteromonas), NOR5-730 (NOR5 cluster), and SAR 86/1245 (SAR86 cluster).

	Fraction (%) of total DAPI counts detected with HRP-probe									
Date	**Eury906**	**Eub338**	**CF319a**	**Alf968**	**Gam42a**	**SAR11**	**ROS537**	**NOR5-730**	**Alt1413**	**SAR86**
4 Mar 03	4 ± 3	63 ± 0	12 ± 2	22 ± 2	2 ± 2	15 ± 7	4 ± 0	2 ± 2	<1	3 ± 3
25 Mar 03	<1	63 ± 4	7 ± 3	42 ± 4	4 ± 2	28 ± 2	2 ± 3	3 ± 3	2 ± 3	<1
22 Apr 03	3 ± 1	69 ± 4	8 ± 4	30 ± 4	4 ± 4	23 ± 2	3 ± 2	2 ± 2	1 ± 2	<1
12 May 03	3 ± 1	75 ± 6	16 ± 1	37 ± 2	5 ± 0	33 ± 1	7 ± 0	1 ± 1	1 ± 0	<1
25 Jun 03	<1	67 ± 1	11 ± 5	39 ± 8	7 ± 0	37 ± 6	1 ± 1	1 ± 3	<1	<1
14 Jul 03	1 ± 0	91 ± 0	8 ± 3	33 ± 3	50 ± 1	16 ± 5	4 ± 1	6 ± 3	30 ± 1	<1
4 Aug 03	1 ± 1	74 ± 2	12 ± 5	38 ± 4	8 ± 1	20 ± 2	1 ± 1	5 ± 4	<1	<1
16 Sep 03	<1	59 ± 5	12 ± 6	35 ± 3	3 ± 4	33 ± 1	1 ± 1	2 ± 1	<1	<1
21 Oct 03	1 ± 1	63 ± 1	6 ± 1	19 ± 2	1 ± 2	6 ± 0	<1	<1	<1	<1
25 Nov 03	3 ± 3	78 ± 5	11 ± 3	34 ± 3	4 ± 0	18 ± 2	3 ± 2	1 ± 0	<1	<1
16 Dec 03	5 ± 3	85 ± 3	12 ± 2	27 ± 3	3 ± 1	15 ± 1	5 ± 1	1 ± 0	1 ± 0	<1
26 Jan 04	6 ± 4	73 ± 7	14 ± 0	24 ± 2	2 ± 1	22 ± 1	3 ± 2	<1	<1	<1
23 Feb 04	3 ± 2	84 ± 1	14 ± 3	21 ± 1	4 ± 1	15 ± 3	7 ± 1	<1	<1	<1
22 March 04	5 ± 3	84 ± 0	13 ± 3	24 ± 2	4 ± 2	29 ± 6	5 ± 2	1 ± 0	<1	<1

Alphaproteobacteria were the dominant bacterial group (average ± SD: 30 ± 7% of DAPI counts), with the exception of the sample taken in July 2003. At this time, the bacterial assemblage structure showed a drastic change with an unusual burst of Gammaproteobacteria (50% of DAPI counts), and more specifically Alteromonadaceae (probe Alt1413, 30% of DAPI counts, Table 2). This unusual event was not considered as a seasonal feature, and for this reason data from the July sampling are presented as outliers in Fig 4. Within the Alphaproteobacteria, the SAR11 cluster showed very high proportions in the spring-summer period (27 ± 8 % of DAPI counts), and lower proportions during the autumn-winter period (18 ± 8 % of DAPI counts; Fig. 4B). Roseobacter were generally found in significantly lower proportions than SAR11, and showed an opposite seasonal trend to this group. After a peak in May, Roseobacter were almost absent during the summer (1% of DAPI counts) but showed higher contributions to the bacterial assemblage in the autumn-winter period (up to 7%, Fig. 4B).

Bacteroidetes were the second most abundant broad phylogenetic group, with a rather constant contribution to the bacterial assemblage throughout the year (average ±SD: 11± 3% of DAPI counts, Fig. 4A). Gammaproteobacteria were not abundant (average ±SD: 4± 2% of DAPI counts, with the exception of the sample from July), but increased during the summer period with a peak in August (8% of DAPI counts, Fig. 4C). Most identified Gammaproteobacteria hybridized with the NOR5-730 probe at this time, with very similar dynamics of both groups through the year except in spring (Fig. 4C). Other tested groups within the Gammaproteobacteria (Alteromonas and SAR86) were almost undetectable for most of the year (Table 2), again with the exception of the July sample.

Identification and seasonality of taxonomic groups of bacteria by clone libraries. We analyzed from 91 to 107 clones in each of four clone libraries constructed with samples from winter (3 March 2003), spring (14 May 2003), summer (4 August 2003) and autumn (21 October 2003). A smaller number of clones were analyzed in a clone library constructed with the July sample (48 clones), with the aim of confirming the unusual bacterial assemblage composition obtained by CARD-FISH for this month. Similarly to CARD-FISH, Gammaproteobacteria dominated the bacterioplankton assemblage (57% of the clones) in July, with a phylotype belonging to Alteromonadaceae (*Glaciecola*) representing 38% of the clones (details not shown).

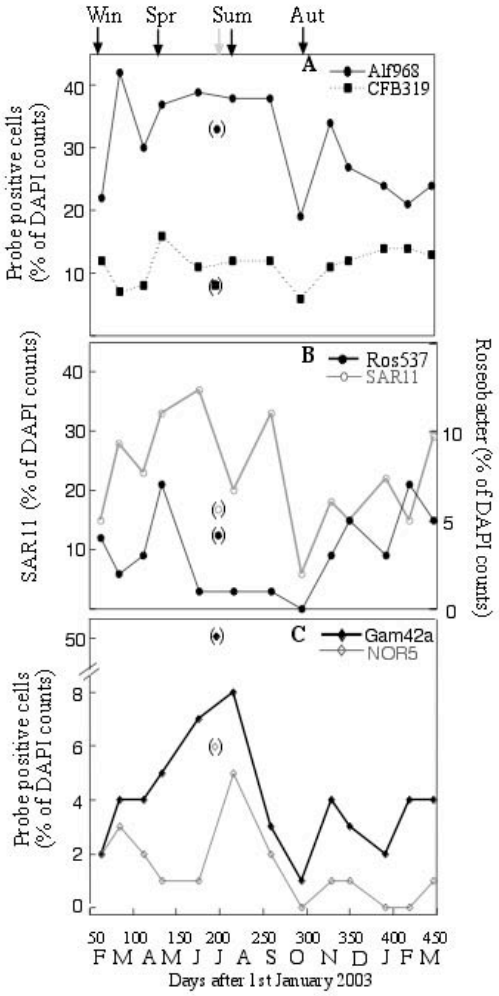

Fig 4- Proportions of bacterial groups detected by CARD-FISH with HRP-probes specific for: (A) Alphaproteobacteria (Alf968) and Bacteroidetes (CF319), (B) Gammaproteobacteria (Gam42) and Nor5 cluster (NOR5-730), and (C) Roseobacter (Ros537) and SAR11 (SAR11-441R). Samples taken in July, when a drastic change in the bacterial assemblage structure was found, are presented as outliers. The arrows indicate the samples from which seasonal clone libraries were carried out.

We obtained informative sequences from 78 to 93 clones in each of the four seasonal clone libraries (corresponding to 28 to 52 OTUs per library after screening with RFLP). The number of clones analyzed seemed to be large enough to appropriately describe the diversity of the bacterial assemblage in spring and autumn, as revealed by the asymptotic behavior of the S_{Chao} index (Fig. 5). By contrast, a linear behavior of S_{Chao} versus library subsample size was found for the winter and summer clone libraries (Fig. 5). Good's coverage values ranged from 40% in winter to 80% in spring, with an average of 62%.

Most of the clones showed >97% similarity to sequences of uncultured marine bacteria deposited in GenBank. However, in the autumn clone library, we found a high percentage of

clones (35%) with similarities below 97% to published sequences, which could be considered as a cutoff for species level (Stackebrandt & Göbel, 1994). Also remarkably, 21% of the clones (mostly affiliated with Verrucomicrobia or Alphaproteobacteria) did not match published sequences with similarity larger or equal to 93%, which could be considered as a cutoff for the genus level (Devereux et al. 1990, Dobson et al. 1993).

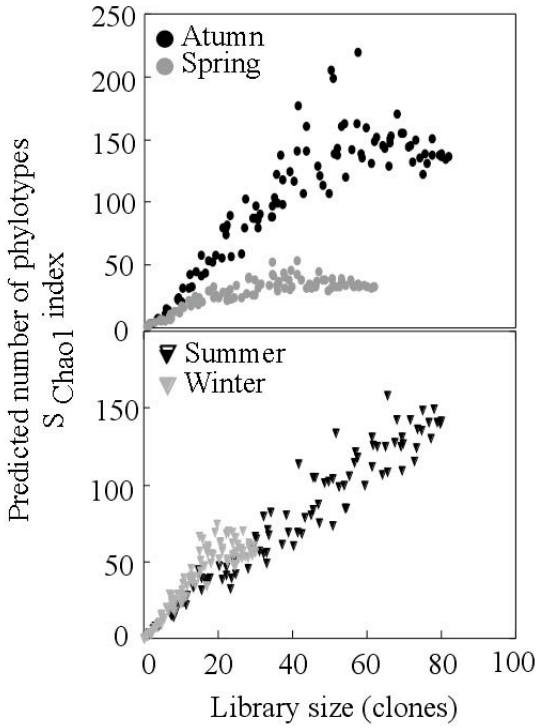

Fig 5- Predicted number of phylotypes based on S_{Chao1} index versus size of subsamples of the four clone seasonal libraries from Blanes Bay. Each point is the mean of 10 replicate subsamples of the library. When the estimated phylotype richness reaches an asymptote, the library can be considered large enough to yield a stable estimate of phylotype richness (Kemp & Allen 2004).

We recovered a high proportion of plastids in winter (61% of the clones, mostly from Cryptophytes and Prasinophytes), and spring (19% of the clones, mostly from Prymnesiophytes and Prasinophytes). For the comparison between different clone libraries, we only considered the bacterial (not plastidial) origin clones, as shown in Fig. 6. In this figure, we show the results of the clone libraries together with the counting results obtained by CARDFISH (to be discussed later).

Pronounced changes in bacterial assemblage structure were detected among different seasons (Fig. 6, "LIB" columns). A remarkably low diversity was found in the spring clone library, in which 65% of the clones were affiliated with the SAR11 cluster, and 26% to the

Roseobacter group. Other groups, such as Bacteroidetes and Gammaproteobacteria, represented less than 4% of the clones each (Fig. 6).

The SAR11 cluster also dominated in the other clone libraries (around 40% of the clones, Fig. 6). Some Alphaproteobacteria clones appearing in summer and autumn (14 and 3% of the clones, respectively, included in "other Alphaproteobacteria" in Fig. 6) were closely related to a group of clones found in the Aegean Sea, which clustered with SAR11 (AEGEAN_233, AEGEAN_169 and AEGEAN_112, Fig 7A). Roseobacter clones were less abundant, showing higher proportions in winter (13% of clones) compared to summer and autumn (4 and 1% of clones, respectively). Bacteroidetes and SAR86 groups were rather constant (around 10% and 7% of the clones, respectively), while other groups, such as Verrucomicrobia and Actinobacteria, only appeared in substantial proportions in the Autumn clone library (Fig. 6).

Clones affiliated with the SAR11 cluster were distributed into two separate clusters (sequence similarity between clusters of approximately 91-94%) without a marked seasonality, indicating that a diverse set of SAR11 clones were present throughout the year (Fig. 7A). Most of the clones clustered with *Pelagibacter ubique*, showing a sequence similarity of around 98% to this recently isolated bacterium and the original SAR11 clone. A group of seven clones (which did not include any winter sequences) clustered with ZD0410 and MB11D08 phylotypes, which have been found in the North Sea and the Pacific Ocean, respectively.

Clones affiliated with Roseobacter were distributed in several different positions in the Roseobacter phylogenetic tree (Fig. 7B). Some spring clones clustered with cultivated strains of Roseobacter such as *Octadecabacter antarcticus* (SPR10), and *Nereida ignava* (SPR23, SPR21 and SPR19), which was isolated from Mediterranean Sea waters. The other clones were included in clusters mostly represented by clone sequences (i.e. CHAB-I-5, NAC11-7).

Clones affiliated with the SAR11 cluster were distributed into two separate clusters (sequence similarity between clusters of approximately 91-94%) without a marked seasonality, indicating that a diverse set of SAR11 clones were present throughout the year (Fig. 7A). Most of the clones clustered with *Pelagibacter ubique*, with a sequence similarity of around 98% to this recently isolated bacterium and the original SAR11 clone. A group of seven clones (which did not include any winter sequences) clustered with ZD0410 and MB11D08 phylotypes, which have been found in the North Sea and the Pacific Ocean, respectively.

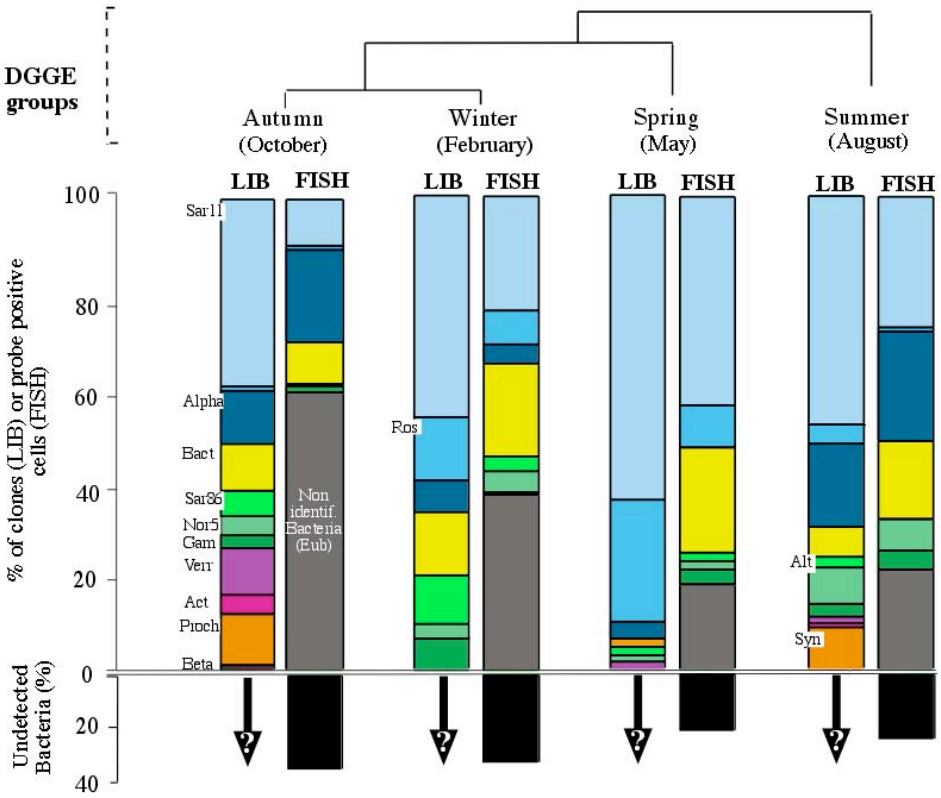

Fig 6- Proportions of clones or Eub+ cells affiliated with different phylogenetic groups (Sar11, Roseobacter-Ros-, Other Alphaproteobacteria-Alpha-, Bacteroidetes-Bact-, Sar86, Nor5, Alteromonas-Alt-, Other gammaproteobacteria-Gam-, Betaproteobacteria-Beta-, Verucomicrobia-Verr-, Actinobacteria-Act-, *Prochlorococcus*-Proch-, and *Synechococcus*-Syn-) found by cloning and sequencing in the four seasonal clone libraries (LIB columns), or by CARD-FISH (FISH columns), respectively. The samples from winter (February 2003), spring (May 2003), summer (August 2003) and autumn (October 2003) were chosen as representative of the groups found in the DGGE dendogram. The proportion of undetectable *Bacteria* in "FISH" columns refers to the percentage of DAPI cells (excluding *Archaea*) that did not hybridize with the set of probes EubI-III. The arrows with question marks indicate that an unknown proportion of phylogenetic groups cannot be retrieved by the set of primers used in the clone libraries.

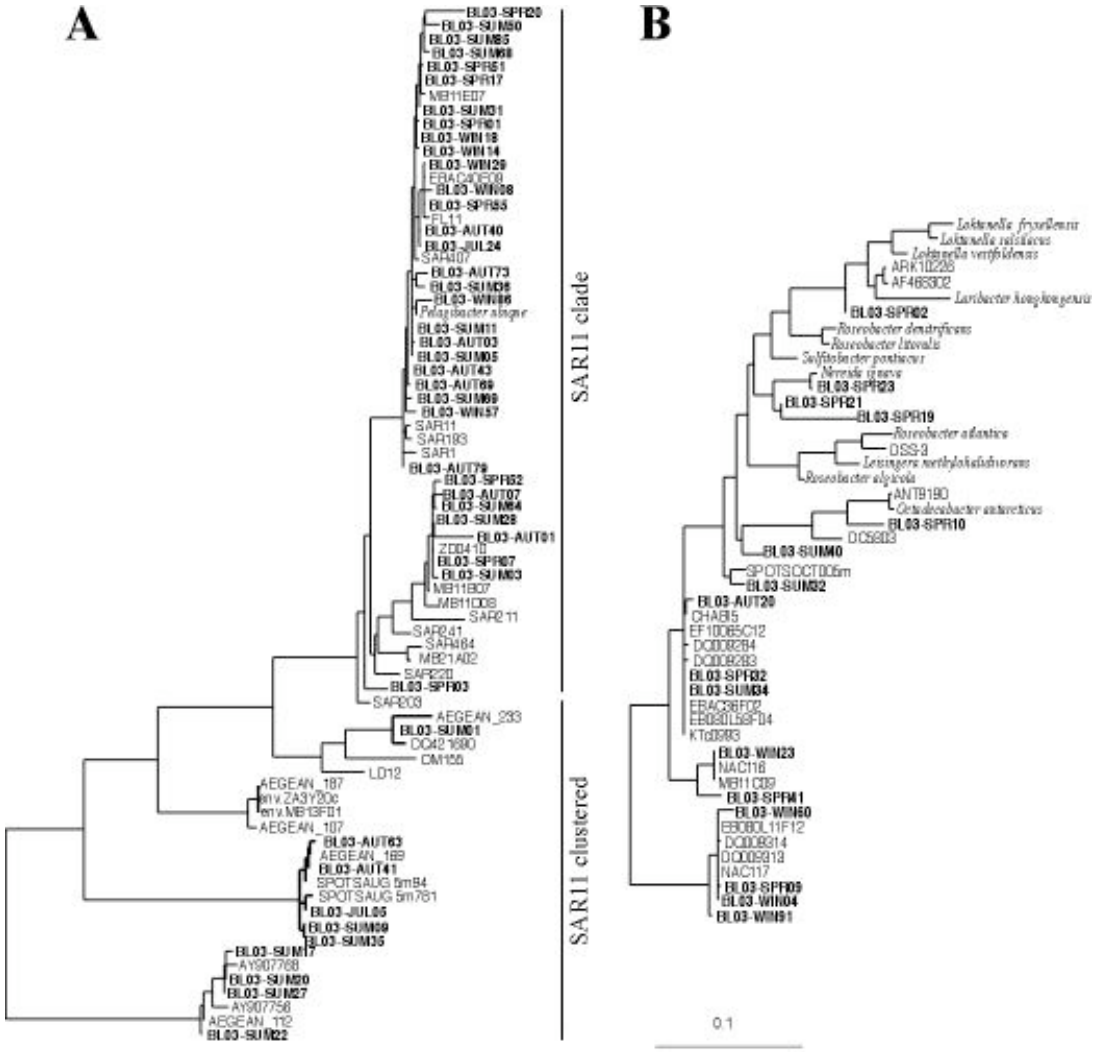

Fig 7- Maximum likelihood phylogenetic trees of environmental clones affiliated with (A) SAR11 and (B) Roseobacter in the four seasonal clone libraries: Clones from the winter (WIN), spring (SPR), summer (SUM) and autumn (AUT) clone libraries are shown in boldface. The SAR11 phylogenetic tree includes several clones isolated in the summer and autumn clone libraries related to the SAR11 cluster, and with high similarity to a group of clones from the Aegean Sea (AEGEAN clones, Moeseneder *et al.* 2005). The trees were rooted with the sequence of *Sinorhizobium melitoti* (not shown). Scale bar denotes 0.1 substitution per nucleotide position.

Quantitative comparison between the three molecular approaches (DGGE, CARD-FISH and clone libraries). The analysis of DGGE bands showed a remarkably different picture of the seasonality of bacterial assemblage structure compared to CARDFISH and clone libraries. Although most bands were affiliated with Alphaproteobacteria, their quantitatively most important group (i.e. the SAR11 cluster) was not detected by DGGE. Roseobacter and Bacteroidetes were detected in higher proportions compared to clone libraries or CARDFISH (Tables 1 and 2, Fig. 6). Likewise, the Gammaproteobacteria group was not detected in DGGE bands, even during July, when the proportion of Gammaproteobacteria increased to around 50% of clones and DAPI counts. However, we found that changes in the relative intensity of some species agreed with their proportions obtained from flow cytometric counts (*Synechococcus* and *Prochlorococcus*) and from clone libraries (such as the appearance of *Erythrobacter*-band 48-in Autumn-Fig. 2-, as well as in the clone library constructed in Autumn, clones BL03-AUT04 and BL03-AUT12).

We compared the proportion of clones (bacterial origin) of different phylogenetic groups to the proportion of cells with the same phylogenetic affiliations analyzed by CARDFISH with specific probes (Fig. 8). This figure shows the ratio between both percentages, including only those samples in which the group was present in significant percentages (≥3%) in either clone libraries or CARDFISH. Data points above or below the line (in which the ratio equals 1), indicate samples in which the groups were overrepresented in clone libraries or CARDFISH, respectively. Most of the cases where groups were detected by FISH but not in clone libraries (ratio equal to 0), belonged to the July clone library (open circles), which was constructed with a significant lower number of clones. Remarkably, in this clone library the percentages of Alphaproteobacteria, SAR11, Gammaproteobacteria and *Alteromonas* were equal by both methods.

Alphaproteobacteria, and specifically within this group the SAR11 and Roseobacter clusters, were generally overrepresented in clone libraries as compared to CARDFISH counts (Fig. 8). Bacteroidetes, on the other hand, were generally underrepresented in clone libraries. The proportions of Gammaproteobacteria were more proportionate by both methods (Fig. 8), but SAR86 was always overrepresented in clone libraries. The NOR5 group showed variable results, being under- or overrepresented in clone libraries in different samplings.

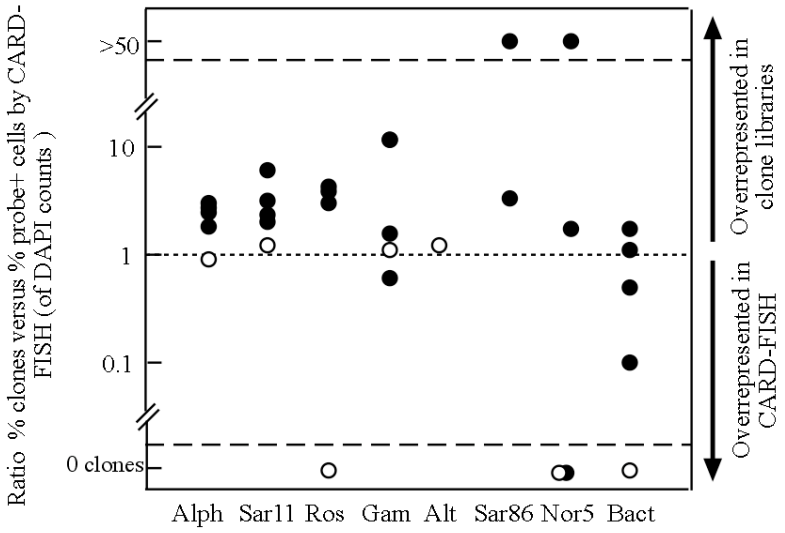

Fig 8- Ratio of the percentage of clones (scaled to clones of bacterial origin) of the different phylogenetic groups to the percentage of bacteria (scaled to total DAPI counts) detected by CARD-FISH with specific HRP-probes for the same groups. If percentages of bacteria scaled to Eub probe (instead of DAPI) were used, the general picture would be very similar. Open circles refer to the clone library constructed in July, and closed circles to the other clone libraries. Alph: Alphaproteobacteria, Ros: Roseobacter, Gam: Gammaproteobacteria, Alt: Alteromonas, Bact: Bacteroidetes.

Discussion

Seasonal changes in bacterial diversity could be relevant to understand the year-round variability in important bacterially-mediated processes in the ocean, such as carbon metabolism. However, there are still relatively few studies that have assessed this topic in marine waters. Although different approaches have been used, including whole genome hybridization (Pinhassi & Hagström2000) and FISH (Eilers et al. 2001, Mary et al. 2006), most studies have relied on PCR-based techniques (Ghiglione et al. 2005, Morris et al. 2005, Schauer et al. 2003), which may not provide a reliable quantification of the abundance of different bacterial groups. In our study, the objective was to obtain a detailed picture of the seasonality of bacterial assemblages in a coastal oligotrophic site, combining and comparing different molecular approaches (DGGE, CARD-FISH, and clone libraries).

Seasonal changes in bacterial assemblage structure in Blanes Bay- We carried out a monthly sampling, which had been previously shown to be an adequate time scale to detect seasonal changes in bacterial assemblage composition in our sampling area (Schauer et al. 2003). These authors hypothesized that the gradual change in bacterial assemblage composition was due to the complex control by bottom-up or environmental factors (such as temperature) on bacterial assemblage composition, since top-down factors seem to vary at much shorter time scales. During the July sampling, Gammaproteobacteria, and particularly the phylotype *Glaciecola* (Alteromonadaceae*),* increased their proportions drastically, reaching 50% of the bacterial counts (by CARD-FISH). This burst of growth is striking, since the abundances of these organisms are typically <1% over the year in Blanes, as well as in other marine environments (Eilers et al. 2000). However, high proportions of this group have been described in clone libraries from particle-associated bacteria from the Mediterranean Sea (Acinas et al. 1999, García-Martínez et al. 2002). These results suggest that episodic events can promote the blooming of specific populations (Pernthaler & Amann 2005), superimposed on the smooth seasonal changes generally captured by the monthly sampling

Leaving apart the July event, the change in the bacterial assemblage was as gradual as commonly observed in other studies, including the one in our same system 5 years before (Schauer et al. 2003). It is noticeable that the three main broad phylogenetic groups (Bacteroidetes*,* Alpha- and Gammaproteobacteria) showed a decrease in their proportions during the sampling in October, concomitant with the increase in the proportion of unusual groups such as Verrucomicrobia, Actinobacteria and Betaproteobacteria. Verrucomicrobia and Betaproteobacteria are commonly found in freshwater environments (Zwart et al. 2002). Thus, the appearance of these unusual phylotypes could be related to the increase in the riverine discharge and coastal runoff to the Bay after the stormy events that took place at that time of the year (Fig. 1). It is also remarkable that, in October, a high number of clones showed lower similarity than the cutoff for genus level (<93%) with published sequences. This suggests that a substantial amount of additional diversity can be found in water samples in which drastic environmental changes occur, such as during stormy periods.

Alphaproteobacteria, and more specifically the SAR11 cluster, dominated year-round in agreement with previous studies that suggest that SAR11 could be the most abundant bacterial group in the ocean (Giovannoni & Rappé 2000, Morris et al. 2002). The proportion of SAR11 clones was higher at our sampling site than in other coastal systems (12% of the clones in Oregon coast and Cappe Hatteras, Rappé et al. 1997, 2000), but was similar to open waters

from the Mediterranean (35% of clones, Acinas et al. 1999) and other marine regions (Giovanonni & Rappé 2000). This could be related to the open ocean influence in Blanes Bay by the intrusions of offshore waters through a nearby submarine canyon (Masó et al. 1989), which seems to affect the bacterial assemblage composition, making it more similar to that of open-sea waters (i.e. Schauer et al. 2000).

The marked seasonality in the proportions of the SAR11 group, with increasing values in spring and maximal values during the summer, is also in agreement with results by Morris et al. (2002, 2005) in the Atlantic Ocean. Lower proportions of SAR11 cells have been found during the summer in the English Channel (Mary et al. 2006). However, these authors apparently did not adapt the protocol for overnight hybridization, and we found that this can be crucial for the successful hybridization of SAR11 cells (details not shown). The high capacity of these bacteria to grow in nutrient limited waters (Rappé et al. 2002) could be related to the importance of SAR11 in Blanes Bay during summer, the season in which the phosphorus limitation of bacterial activity is maximal (Pinhassi et al., in press).

Another group of Alphaproteobacteria clones, which clustered with the SAR11 group, appeared in summer and autumn in Blanes Bay (Fig. 7A). These clones showed high similarities with some phylotypes retrieved from the Aegean Sea (AEGEAN233, AEGEAN169 and AEGEAN 112, Moeseneder et al., 2005), and some clones recently isolated from the San Pedro Channel (Brown et al. 2005) and Arabian Sea (Fuchs et al. 2005). Some of the phylotypes (clone AEGEAN 233, Moeseneder et al. 2005) appeared in a RNA-based clone library, suggesting that members of this group can be metabolically active.

Remarkably, a seasonal pattern opposite to that of SAR11, with higher proportion in winter compared to summer, was found for the other relevant group of Alphaproteobacteria, Roseobacter. This suggests that this group is favored by nutrient rich conditions, in agreement with the common association of Roseobacter with phytoplankton blooms (González et al. 2000, Suzuki et al. 2001). Most of the clones during the Chl *a* rich season (winter) were highly similar (>97%) to the NAC11-7 phylotype, which is primarily represented by clone sequences, some of them associated with algae and algal blooms. This phylotype was also found in higher proportions by DGGE during the winter in Blanes Bay in our study (band 35, Fig.2), as well as in the study conducted by Schauer et al. (2003) five years before. Another peak of abundance of Roseobacter was found in spring, coincident with a high diversity of clones within this cluster. Higher abundances of Roseobacter in spring, associated to higher nutrient availability, have been reported from offshore the Californian Coast (Brown et al. 2005). Finally, another

group of Roseobacter clones (BL03-SPR32, BL03-SUM34, BL03-AUT20) were present in all seasons except winter, and were similar to CHAB-I-5, which is a coastal group found mainly in surface coastal waters (Buchan et al. 2005).

Bacteroidetes were remarkably constant over the year, in contrast with other studies where the abundance of this group increased with higher levels of chlorophyll (Pinhassi & Hagström2000). Regarding Gammaproteobacteria, their higher abundance detected by CARD-FISH during the summer, is in agreement with the results presented by Mary et al. (2006) in the English Channel. Most of the clones in this group were affiliated with the SAR86, NOR5 and Alteromonas lineages in Blanes Bay, although only NOR5 could be detected in substantial proportions by CARDFISH year-round. Specifically, three clones affiliated with SAR86 (BL03-AUT16, BL03-SUM93 and BL03-JUL28), showed 97-99% similarity to a clone retrieved in the Aegean Sea (AEGEAN_234; Moeseneder et al. 2005). This clone was found in much higher proportions in an RNA- compared to a DNA-based clone library, indicative of its high metabolic activity. However, it is remarkable that we usually could not hybridize SAR86 cells by CARDFISH even if the group was present in the clone libraries, a problem that could be due to low ribosomal content.

The NOR5 cluster has been retrieved in several clone libraries in the Mediterranean Sea (Schäfer et al. 2001), Atlantic Ocean (Rappé et al. 1997) and North Sea (Eilers et al. 2000). A strain of this group was isolated from open waters in the German Bight, using low nutrient concentration seawater medium (Eilers et al. 2001). Similar dynamics of the NOR5 group was found in Blanes and in the German Bight, with higher proportions in the summer months (peaks of 5 and 8% of DAPI counts in Blanes and the German Bight, respectively) and lower proportions in autumn and winter. The contribution of this group to total Gammaproteobacteria was also similar (around 60% of Gammaproteobacteria) in both studies. Interestingly, Eilers et al., (2001) found that the peaks of NOR5 coincided with biomass peaks of a diatom (*Lauderia* sp). In our study, the dynamics of this group (measured by CARDFISH) was rather similar to that of *Synechococcus*, suggesting a possible association of this lineage with some phytoplankton populations.

In summary, Alphaproteobacteria dominated the assemblage composition throughout the year in this oligotrophic coastal site. During the nutrient limited season (summer) we observed a peak of SAR11, in agreement with their capability to grow at low levels of nutrients. During the spring and winter, we found higher abundances of the Roseobacter group, possibly due to their closer associations to nutrient rich environments. Bacteroidetes were the second most

important group in Blanes Bay, with year-round uniform contributions to the bacterial assemblage. Gammaproteobacteria showed low abundances, and the NOR5 group was the predominant Gammaproteobacteria during the summer.

Shortcomings and biases of clone libraries, DGGE and CARD-FISH methods in environmental studies. The comparison between clone libraries, DGGE and FISH results is not straightforward because of the different levels of phylogenetic resolution of each technique. The amplification by PCR and the specificity of primers and probes are important sources of bias when comparing the quantitative results by the three methods. There is a general agreement among researchers about the limitations of each technique (Amann et al. 1995, Wintzigerode et al. 1997), but there are still very few studies that have compared the results of different techniques in marine waters (Cottrell & Kirchman 2000, Díez et al. 2001), and none has compared them through a complete annual cycle.

The assemblage structure depicted by DGGE was rather different from that obtained by the other two methods, especially with respect to the SAR11 cluster. This group was not retrieved in any of our sequenced DGGE bands, unlike in other studies (Selje & Simon 2003 Balagué et al., unpublished results), although the set of primers used (358f and 907rM) covered reasonably well the SAR11 cluster as well as the other broad bacterial groups. Thus, our results clearly caution against the common assumption that the most prominent DGGE bands represent all the important bacteria present in the system (Selje & Simon 2003).

In a comparative study between DGGE and FISH, Castle & Kirchman (2004) frequently identified the same phylogenetic group as being dominant by both techniques but, as in our results, in some samples DGGE missed the most abundant phylogenetic group detected by FISH (in their case Betaproteobacteria). Besides primer specificity, Castle & Kirchman (2004) argued that high richness within groups could lead to an underestimation compared to FISH, since different sequences would appear as different faint bands. Those bands could be difficult to excise from the DGGE gels for sequencing. Our results support this hypothesis, given the high microdiversity found in SAR11 clones in this and in previous studies (Fig. 7, García-Martínez & Rodríguez-Valera 2000, Brown & Furhman 2005), the close location of SAR11 bands in the gel (Fig. 2) and the unsuccessful sequencing of the bands at those positions.

The dominance of Alphaproteobacteria in general, and of SAR11 in particular, in the clone libraries, is in agreement with the predominance of this group ever since the first clone library was constructed from marine waters (Giovanonni 1990). Even if this group has been shown to

be numerically dominant (Morris et al. 2002), clone libraries still seem to overestimate their proportion (up to 6-fold) as compared to CARDFISH counts with the probe SAR11-441R (Fig. 8). The presence of multiple RNA operons seems not to be a reason for such a bias, since a recent report on the genomic content of a member of this group (*Pelagibacter ubique,* Rappé et al. 2002), has shown that it only contains one copy of the 16S rRNA gene (Giovanonni et al. 2005).

The overestimation of SAR11 in clone libraries could be explained by PCR bias favoring their amplification, or alternatively, due to possible mismatches of the specific probes, which would lead to the underestimation of this group by FISH. We analyzed the specificity of the probe used in this study (SAR11-441R) using the ARB-software (Ludwig et al. 1998), and found that this probe matched most (91%) of our clones. This points to PCR bias as the most probable explanation for the overestimation of SAR11 in clone libraries

A remarkable result in this respect was that the Alf968 probe had mismatches with all our SAR11clones and the group of Alphaproteobacteria clones related to those retrieved by Moeseneder et al. (2001). The mismatch with the SAR11 clones is located in the last nucleotide for most of the clones (87% of clones). Therefore, the hybridization is possibly not critically affected by this mismatch (otherwise the percentage of Alphaproteobacteria would drastically decrease), but it is probably not optimal. The mismatch with the group of clones related to those retrieved by Moeseneder et al. (2005), was located in the center of the probe, and this was probably critical for the detection of these cells by CARDFISH. This suggests that Alf968 can severely underestimate the real proportions of groups of Alphaproteobacteria, at least in some cases.

Regarding the Bacteroidetes, Cottrell et al. (2000) found a clear underestimation of this group in clone libraries, something that is partially supported by our results (Fig. 8). Our underestimation was not as high as in their study, but it has to be considered that the proportion of Bacteroidetes determined by FISH was significantly higher in their original samples (20-30% of DAPI counts) than in Blanes Bay. Our results also agree with Cottrell et al. (2000) regarding the Gammaproteobacteria group, which was found either on the 1:1 line or overrepresented in clone libraries (Fig. 8).

Another group that was overrepresented in clone libraries was SAR86, in agreement with previous studies (Eilers et al. 2000). This group was abundant in numerous clone libraries in both coastal (up to 29% of bacterial clones, Rappé et al. 1997) and open waters (Giovanonni &

Rappé 2000), but its optimal detection by in situ hybridization required the development of the CARDFISH methodology (Pernthaler et al. 2002). Although we used this optimized protocol in our study, we could only detect this group in one out of the 14 months sampling (Table 1). Even if SAR86 has been detected in important proportions by in situ hybridization in relatively eutrophic marine areas such as the North Sea (Eilers et al. 2000, Pernthaler et al. 2002) or the English Channel (Mary et al. 2006), it remains unknown whether this widely distributed group of uncultivated Gammaproteobacteria is quantitatively abundant in more oligotrophic oceanic waters.

In summary, the combination of the three approaches was very useful for assessing changes in bacterial diversity, but not a single technique alone can be trusted to cover all aspects of a good description of the bacterial phylogenetic diversity or population dynamics. Fig. 6 presents a snapshot of the kind of information and results that can be obtained by each technique. DGGE allowed an assessment of the changes in the composition of the bacterial assemblage through the seasons with sufficient time resolution. This would have been expensive, impractical and time-consuming using clone libraries or FISH. Failure in the sequencing of faint bands (Schauer et al. 2003) and phenomena such as high microdiversity prevent using DGGE to describe bacterial diversity. Such problems, however, do not affect its power as a fingerprinting technique, and thus DGGE allowed reasonable grouping of the samples according to their similarities.

Clone libraries provided the highest phylogenetic resolution, and a detailed picture of the species within each phylogenetic group. However, the PCR bias can produce over- or underestimations of specific groups, such as Alphaproteobacteria and Bacteroidetes, respectively, compared to the direct quantification obtained by CARDFISH. The specificity of the PCR primers also determines the number of groups that can be recovered at all, and the proportion of groups that are not targeted by the primers used is always unknown. CARDFISH allowed a direct quantification of the most important groups of bacterioplankton (combining probes from lower to higher levels of resolution), although the picture of the bacterial assemblage composition was substantially limited by the number and phylogenetic resolution of the probes. As usually happens in studies with natural marine samples, substantial proportions of the cells remained unidentified with the broad phylogenetic probes used, or undetectable by the method (with the EubI-III probes). Importantly, a great part of the disagreement between PCR-based and direct methods such as FISH can be due to mismatches in the commonly used probes (such as that for Alf968). This indicates that more effort should

be devoted to new probe design and the specificity of old probes should always be checked with the growing public databases.

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Chapter IV

Seasonality of bacterial carbon use in NW Mediterranean coastal waters and controlling factors

L. Alonso-Sáez, E. Vázquez-Domínguez, J. Pinhassi, C. Cardelús, M.M. Sala, I. Lekunberri, M. Vila-Costa, V. Balagué, F. Unrein, R. Massana, R. Simó & J.M. Gasol

Manuscript

Abstract

We carried out a seasonal study on the carbon flux through bacteria at a NW Mediterranean coastal site (Blanes Bay), including monthly measurements of heterotrophic production (BHP), respiration (BR), growth efficiency (BGE) and ectoenzyme activities. We specifically assessed whether temperature, DOM lability, nutrient concentrations or bacterial assemblage composition affected the *in situ* rates of bacterial carbon processing and its partitioning into BR and BHP. Leucine uptake rates (as an estimate of BHP) showed high interannual variability. On average, lower values were found in winter (around 50 pmol Leu^{-1} h^{-1}) as compared to summer (around 150 pmol Leu^{-1} h^{-1}), but BHP was only weakly correlated to temperature ($r= 0.30$, $p< 0.03$, $n= 53$). Over a full-year cycle (in 2003), BHP peaked concomitantly with winter Chl *a* maxima, or in periods of high ectoenzyme activities in spring and summer. This suggests that both low molecular weight (LMW-)DOM released by phytoplankton during the algal bloom, or high molecular weight (HMW-)DOM in periods of low Chl *a*, can enhance BHP. BR ranged between 7 and 48 μg C l^{-1} d^{-1} and was not correlated to BHP, but it showed a significant correlation to DOC concentration. The total bacterial carbon demand (sum of BHP and BR) was only met by dissolved organic carbon produced by phytoplankton during the winter period. BGE ranged from 3 to 42%, and increased during the phytoplankton blooms in winter (during the Chl *a* peaks), and in spring. Changes in bacterial carbon use were not significantly related to changes in bacterial assemblage structure as depicted by denaturing gradient gel electrophoresis (DGGE) fingerprinting. Our results, therefore, did not reveal any significant coupling of bacterioplankton metabolism and diversity in this coastal ecosystem.

Introduction

Marine bacterioplankton play a central position in the oceanic carbon cycle with two main ecological roles: 1) the incorporation of dissolved organic matter (DOM) into biomass (i.e. bacterial heterotrophic production, BHP) and 2) the remineralization of organic carbon (i.e. bacterial respiration, BR). A large number of studies have focused on determining the BHP and bacterial biomass in the ocean (Ducklow & Carlson 1992), resulting in a substantially larger dataset compared to BR measurements. However, most of bacterial carbon demand (BCD, generally over 80%) is accounted for by BR, and thus, this parameter currently

constitutes the biggest gap in our understanding of carbon cycling by bacterioplankton (Janhke & Craven 1995).

BR measurements are needed in order to estimate not only BCD, but also bacterial growth efficiency (BGE), i.e. the proportion of carbon consumed by bacteria that can be transferred to higher trophic levels (BGE=BHP/(BHP+BR)). A wide range of BGEs has been found in marine waters (1 to >60%, del Giorgio & Cole 1998), and there is a great interest in constraining these values across spatial and temporal scales in the ocean for the purposes of large scale modelling, especially in oligotrophic areas, which cover most of the ocean. Because of methodological problems, high uncertainties are associated with many current estimates of BGE (Briand et al. 2004, Chapter I), but also importantly, the *in situ* controlling factors of BGE and BCD are still unclear, despite the great biogeochemical importance of these parameters.

Temperature, nutrient concentrations, DOM lability and bacterial taxonomic composition have been regarded among the main factors that can influence BGE and DOM consumption by bacteria. Significant correlations between temperature and BHP (White et al. 1991) or BGE (Rivkin & Legendre 2001) have been found on large-scale comparative analyses. However, in field studies, the relative importance of temperature as a modulator of these parameters is less clear (del Giorgio & Cole 1998). The few previous seasonal studies carried out in marine waters that included BGE estimates suggest that this parameter is mainly determined by DOC lability (Lemée et al. 2002, Reinthaler & Herndl 2005). Importantly, no attention has been paid to bacterial assemblage structure, which may substantially change throughout the year (Schauer et al. 2003, Ghiglione et al. 2005, Chapter III). Such changes could have relevance in terms of carbon use since, as has been observed in previous studies, distinct phylogenetic groups of bacterial can carry out different patterns of DOM consumption (Cottrell & Kirchman 2000, Covert & Moran 2001).

In this study we aimed to characterize the flux of carbon through bacterioplankton in a coastal oligotrophic site using an exhaustive approach which included the measurement of parameters that are seldom assessed in seasonal studies such as BR and BGEs, empirical carbon-to-leucine conversion factors (to accurately constrain BHP), ectoenzyme activities (as indicators of the bacterial consumption of high molecular weight (HMW-)DOM), or dissolved primary production by phytoplankters, which is a primary source of carbon for bacteria. We integrated the information obtained from these measurements with two main objectives: 1) to depict a comprehensive view of seasonal changes in the flux of carbon through

bacterioplankton over the year, and 2) to analyze the underlying controlling factors on BGE and BCD, including not only environmental parameters (temperature, Chl a...), but also the effect of changes in bacterial assemblage structure, which have been reported in Chapter III.

Materials and methods

Sampling and basic parameters We carried out a monthly study in Blanes Bay (The Blanes Bay Microbial Observatory, NW Mediterranean) during 1998, and from March 2001 to March 2006. During the three first years (1998, 2001 and 2002) temperature, chlorophyll *a* and inorganic nutrients were measured, and bacterial abundance was also determined during 1998. From 4 March 2003 to 22 March 2004 a full study was conducted including a wide range of bacterial parameters (see below), some of which were measured until March 2006 (Fig. 1). Surface waters were sampled at about 1 km offshore (41°40'N, 2º 48'E), filtered through a 200 μm mesh net and transported to the lab under dim light (within 1.5 hours) in 25 l polycarbonate carboys. Surface water temperature was measured in situ with a mercury thermometer. For determination of Chlorophyll *a* concentration, 150 ml of seawater was filtered on GF/F filters (Whatman) and subsequently extracted in acetone (90% v/v) in the dark at 4ºC for 24h. Fluorescence was measured with a Turner Designs fluorometer.

Chemical analyses. For analyses of dissolved nutrient and organic carbon concentration, seawater samples were filtered through 0.2 μm pore size polycarbonate filters (47 mm diameter, Supor-200; Gelman sciences) using a polycarbonate filtration device (Millipore). Dissolved inorganic nutrient concentrations were determined spectrophotometrically with an Alliance Evolution II autoanalyzer following standard procedures (Grasshof et al. 1983), except for phosphate concentration, which was determined manually, using a 10-cm cuvette to increase the detection limit (Pinhassi et al., in press). For analysis of DOC concentration, samples (20 ml) were acidified with 16 mM HCl (final conc.) in acid-clean polypropylene tubes, and stored at 4ºC until analysis. DOC was measured with a high temperature carbon analyzer (Shimadzu TOC 5000) at the intercalibrated facilities at Umeå Marine Research Station (UMF), Sweden. All utensils in contact with the samples (filters, filter holder, tubes) were acid rinsed with 1M HCl and extensively washed with MilliQ water prior use.

Bacterial abundance and biomass.- Samples (1.6 ml) were preserved with 1% paraformaldehyde + 0.05% glutaraldehyde (final conc.). Bacterial abundance was analyzed by flow cytometry (FACSCalibur cytometer, Becton and Dickinson) after staining with Syto13

(Molecular probes). Bacteria were detected by their signature in a plot of side scatter (SSC) versus FL1 (green fluorescence) as explained in Gasol & del Giorgio (2000). Picocyanobacteria were discriminated in a plot of FL1 versus FL3 (red fluorescence). Flow cytometric counts were calibrated with DAPI counts. Bacterial cell size (biovolume) was estimated using the relationship between average bacterial size (obtained by image analysis of DAPI preparations following common procedures) and average fluorescence of the SYTO-13 stained sample relative to beads as showed by Gasol & del Giorgio (2000). Bacterial biomass was calculated by using the carbon-to-volume relationship derived by Norland (1993) from the data of Simon & Azam (1989): pg C $cell^{-1}$ = 0.12 pg $(\mu m^3\ cell^{-1})^{0.7}$.

Bacterial heterotrophic production- BHP was estimated using the [^{3}H]-leucine incorporation method (Kirchman et al. 1985). For each sample, triplicate or quatriplicate aliquots (1.2 ml) and one or two TCA killed controls were incubated with 40 nmol l^{-1} leucine (Leu) for about 2 hours at in situ temperature. The incorporation was stopped with the addition of 120 µl of cold TCA 50% to the vials and samples were kept frozen at –20ºC until processing, which was carried out by the centrifugation method of Smith & Azam (1992). Finally, samples were counted on a Beckman scintillation counter, 24 hours after addition of 1 ml of scintillation cocktail. During 2003, empirical carbon-to-leucine conversion factors (eCFs) were determined monthly in replicate dilution cultures, which were also used to determine one estimate of BGE (long-term incubations). Details on the preparation of the seawater dilution cultures are described below (BGE determination section). Factors were computed with the cumulative (Bjørnsen & Kuparinen 1991) and the integrative (Fuhrman & Azam 1980, Riemann et al. 1987) methods.

Ectoenzyme activity. For the determination of the activities of ectoenzymes (beta-glucosidase, aminopeptidase, xylase and chitinase) we used fluorogenic substrates (Hoppe 1983) and followed a modification of the methodology described in Sala et al. (2001). In brief, each ectoenzyme activity was assayed by observing the release of fluorescent methylumbelliferone (MUF) from the following MUF-linked analogues: 4-MUF-beta-glucoside for betaglucosidase, L-leucine-7-amido-4-methyl-coumarin for aminopeptidase, 4-MUF-beta-xyloside for xylase, and 4-MUF-chitine for chitinase. Substrates were added at saturating concentrations (100 µM final concentration) to 0.9 ml replicate subsamples and fluorescence was measured immediately after addition, and after an incubation of 1-3 h in the dark at room temperature. Fluorescence was read on a Shimadzu spectrofluorometer RF-540 at 365 nm excitation and 446 nm emission wavelengths. Increase of fluorescence units during the

incubation time was converted into activity by preparing a standard curve with the end product of the reaction, 4-methyllumbelliferone. Since aminopeptidase may have a phytoplankton origin, samples for aminopeptidase activity determination were previously filtered through 1 μm (Millipore) size pore filters.

Bacterial respiration. BR was measured by following changes in dissolved oxygen during dark incubations of filtered water (0.8 μm, mixed esters of cellulose) to include only the bacterial fraction. After filtration, we recovered on average (±SE) 48 ± 4 % of total bacteria. Values of BR were compared to measurements performed with unfiltered water, and on average (±SE), bacterial respiration accounted for 71 ±12% of total community respiration. In October a strong increase in the respiration rate of the filtered fraction compared to the unfiltered water was found (almost three fold). Therefore, for this month, the measurement obtained from the unfiltered water, and not the filtered fraction, was used as an estimate of bacterial respiration. Boro-silicate glass bottles bottles were carefully filled, and 4-8 replicate bottles were immediately fixed with Winkler reagents to determine the initial oxygen concentration. Eight to ten replicate bottles were incubated in the darkness at in situ temperature and fixed with Winkler reagents after 24 hours. Dissolved oxygen measurements were made with an automatic titrator based on potentiometric endpoint detection (Outdot et al. 1988). The rate of respiration was determined by regressing O_2 against time for the 0-24 hours interval, assuming a respiratory quotient (RQ) of 0.88 (Williams & del Giorgio 2005).

Bacterial growth efficiency (BGE). BGE was estimated as the ratio of bacterial production to total carbon demand by two independent approaches. In the first approach (BGE short-term), BR was estimated as described in the previous section, and bacterial net production was estimated as the average between BHP at time zero of the incubation experiment (with empirical CFs) and the change in bacterial biomass estimated from the increase in cell biomass during the 24 h incubation. In the second approach (BGE long-term) we followed the increase in POC (estimated from bacterial biomass) and decrease in the concentration of DOC in seawater cultures (Carlson & Ducklow 1996). For each dilution culture, approximately 1900 ml of sample was filtered through a 0.2 μm pore size Sterivex filter capsule (Millipore) using a peristaltic pump. The inoculum (100 ml) was prepared by gravity filtration (0.8 μm pore-size polycarbonate filter, Nucleopore) and added to obtain a 20-fold dilution of bacterial abundance. Seawater cultures were incubated at *in situ* temperatures in the dark for 2-4 days. Samples for bacterial abundance and BHP (Leu uptake) determination were taken every 8 to

24 hours. Samples for DOC were taken in replicates at the beginning and the end of the incubation, in order to determine DOC consumption (DOC initial - DOC final).

Primary production. For the measurement of particulate primary production (pPP), fourteen 70 ml-bottles (Corning) and one dark control (bottle wrapped with aluminum foil) were filled with seawater and inoculated with (5 to 15 μCi) $NaH^{14}CO_3$. The incubation was carried out in a water bath at *in situ* temperature for 2 hours in a gradient of light irradiance (ca 10-1000 μmol photons m^{-2} h^{-1}). Circulating water connected to a water bath maintained the temperature. Light was measured with a small size spherical light meter (Illuminova AB, Sweden). After the incubation, the samples were filtered at low vacuum pressure through cellulose ester filters (Millipore 0.22 μm), and the filters were subsequently exposed overnight to concentrated HCl fumes. Scintillation cocktail (4.5 ml Optiphase Hisafe 2) was then added to each filter, and the radioactivity was measured in an Beckton-Dickinson LS6000 scintillation counter. In situ pPP was determined from the P-E curve and the *in situ* irradiance obtained with a Li-Cor sensor (Li-193S).

Parallel to these incubations, the percentage of extra-cellular release (PER) was estimated in a different set-up. Sixteen bottles (Corning) were filled up with sample and half of them were covered with aluminium foil. They were inoculated the same amount of ^{14}C and incubated for 2.5 hours at a fixed light intensity (ca. 500 μmol photons m-2 h-1). At varying times throughout the incubation, 2 clear and 2 dark bottles were taken, the samples filtered through glass fiber GF/F filters, and the radiactivity measured on both filters (labeled POC, pPP) and filtrates (labeled DOC, dPP). The filters were treated as described before and the filtrates were acidified with 1 ml of HCl 50%. Scintillation cocktail was then added to both filters and filtrates. Average percentages of labeled DOC over total labeled carbon (DOC + POC) over the time-course evolution were used to calculate the PER, manually checking and removing data when DOC production had reached an asymptote or started to decrease due to bacterial consumption. Rates of dissolved primary production (dPP) were estimated from in situ pPP and PER estimates. Total primary production (tPP) was calculated as the sum of pPP and dPP. Rates of PP were converted from hourly to daily values using the irradiance at the Blanes Latitude and the Straskraba & Gnauck (1985) model. We assume that the in situ irradiance at 10:00 am (sampling time) was the average irradiance of the day (light period).

DGGE. Details of the analysis of seasonal samples by DGGE are given in Chapter III. In brief, genes encoding for rDNA were amplified with primers 358f and 907rM, and DGGE

analysis was performed as previously described (Schauer et al. 2003). Each band was considered one OTU, and relative band intensities were used to obtain an intensity matrix.

Statistical treatment. Nonmetric multidimensional scaling (MDS) was used in order to analyze the similarities between the samples based on bacterial assemblage structure (DGGE OTUs intensities), environmental factors (temperature, chlorophyll, DOC concentration and nitrate), and carbon processing including the following parameters: BHP (leucine uptake using empirical conversion factors), BR and bacterial biomass. The associations between environmental factors, bacterial assemblage structure (DGGE band pattern), and carbon metabolism parameters were analysed by the RELATE routine of Primer software (v5). This routine performs correlations of similarity matrices in a procedure similar to a Mantel test (Mantel 1967), with the difference that the correlation is not the standard product-moment Pearson correlation (of standard Mantel test), but the Spearman non parametric rank correlation (*rho*). The statistical significance of the coefficient *rho* was obtained by a simple permutation test (999 permutations). Correlations were performed between three similarity matrices, which were constructed based on DGGE band pattern (arcsin transformed), environmental and carbon processing variables (log-transformed). Bray-Curtis similarities (for DGGE band pattern) and Euclidean distances (for environmental and carbon metabolism data) between the stations were used to construct the similarity matrices (Primer v5).

Results

Environmental factors- Average five-year (March 2001-2006) series of monthly measurements of several environmental parameters such as temperature, inorganic nutrients and chlorophyll *a* (Chl *a)* concentration are shown in Fig 1. These averages also include the monthly measurements of temperature and chlorophyll determined during 1998. The period from June to September was characterized by high water temperature (over 22ºC, Fig. 1b), and low concentrations of inorganic nutrients and Chl *a* (around 0.4 μg Chl *a* l^{-1}, Fig 1b). Based on these characteristics, we will refer to this period as representative of summer conditions. On the contrary, the period from December to March showed water temperatures around 13ºC, and higher concentrations of nutrients and Chl *a* (over 1 μg Chl *a* l^{-1}, Fig 1b), and we will refer to it as the winter period. The concentration of nitrate (Fig 1b) and silicate (details not shown) usually peaked in March. Phosphate concentration, which was only analyzed during 2003, showed low values over the entire year (<0.06 μM, Pinhassi et al. in press) except for October

(0.2 μM, coincident with a stormy period) and December (0.1 μM, coincident with a Chl *a* peak). The peak of Chl *a* always occurred during the winter, but at variable months depending on the year (Fig. 1).

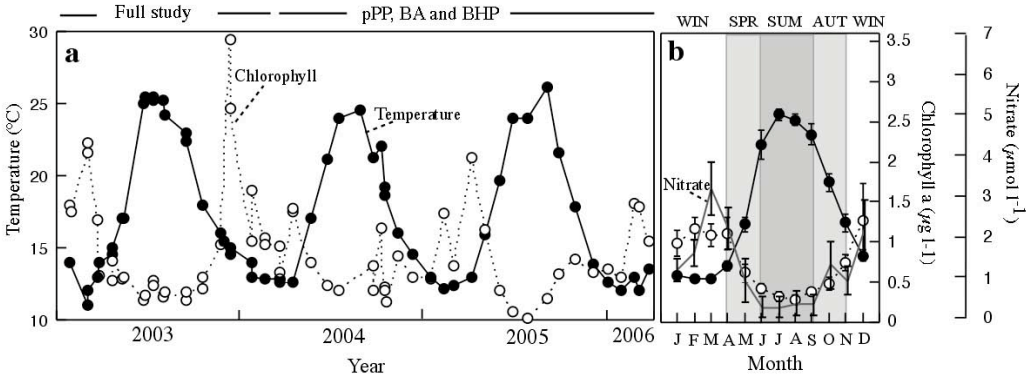

Fig 1- . a) Monthly measurements of temperature (solid circles) and chlorophyll a concentration (open circles) in Blanes Bay. Measurements of bacterial abundance (BA), bacterial heterotrophic production (BHP) and particulate primary production (pPP) were conducted throughout the entire period, while in 2003 a more exhaustive study was performed including measuremets of bacterial respiration, growth efficiency, dissolved primary production, empirical carbon-to-leucine conversion factors and ectoenzyme activities, b) Monthly-averaged measurements of temperature (solid circles), chlorophyll *a* (open circles) and nitrate concentrations (grey line) during the period 1998 and March 2001-March 2006, including a distinction of winter, spring, summer and autumn periods based on the values of these parameters (see text).

Seasonal variations in particulate primary production, bacterial production and abundance over three annual cycles- Phytoplankton particulate primary production (pPP), bacterial heterotrophic production (BHP) and bacterial abundance were studied over three seasonal cycles (March 2003-March 2006, Fig. 1a). Phytoplankton pPP showed a marked seasonality, with higher average values during the winter (over 1.5 mg C m^{-3} h^{-1}) compared to the summer (Fig 2a). We found a repeatable peak of pPP in February (2.6 mg C m^{-3} h^{-1}) and variable peaks in December and March (Fig. 2a). From spring to autumn, the average pPP was around 1 mg C m^{-3} h^{-1}, with the exception of an unusually high value obtained during July 2003 (2.5 mg C m^{-3} h^{-1}, Fig. 2a).

Leucine uptake rates (as an estimate of BHP) showed a different pattern from that of primary production, and average values tend to be higher in spring and summer periods (Fig.

2b). However, there was a substantial interannual variation in Leu uptake rates during summer, with values ranging around 14-fold. The high variability in BHP measurements was confirmed even at a much shorter time-scale in this site. Measurements carried out on consecutive dates for several months during 2003 varied, on average, by a factor of 3 (details not shown).

Bacterial abundance (BA) measurements showed a much narrower range, and after a decline in June (average of 5.3 10^5 bact ml^{-1}), BA tended to increase during the summer period reaching maxima in August (1.3 10^6 bact ml^{-1}, Fig. 2c). BA average values were quite stable (around 0.8 10^6 bact ml^{-1}) the rest of the year (Fig. 2c). Weak but significant correlations were found between BHP and temperature ($r= 0.30$ $p< 0.03$ $n= 53$, January 2003-March 2006), and BA and Chl *a* ($r= 0.30$, $p< 0.02$, $n= 63$, over four years).

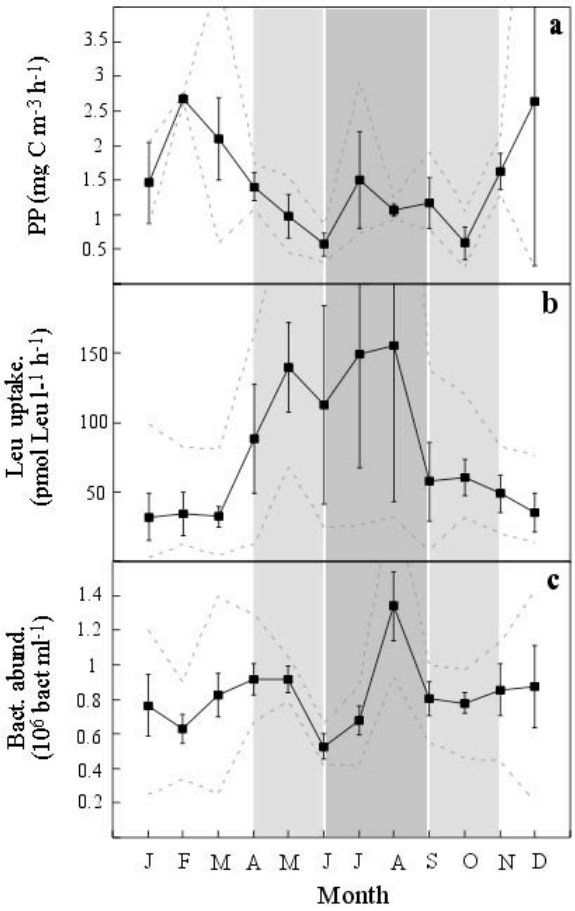

Fig 2- Monthly-averaged (± SE) measurements of a) particulate primary production (pPP), b) leucine uptake rates and, c) bacterial abundance (BA, panel c) from ca. 5 determinations per month through the period March 2003-March 2006 in Blanes Bay. In the case of pPP, measurements were carried out only until December 2005. The dotted lines above and below the lines indicate the monthly maximum and minimum values obtained for each measurement over the period studied.

Annual cycle of bacterial production, respiration and growth efficiency- Seasonal determinations of BHP and BA were complemented by measurements of bacterial respiration (BR) and growth efficiency (BGE) during a full annual cycle (March 2003-March 2004, Fig. 3). Empirical Leu-to-carbon conversion factors (eCFs) were determined monthly during almost all this period (until January 2004), in order to obtain reliable determinations of BHP (Table 1). Empirical CFs (range 0.97-3.6 kgC mol Leu^{-1}) generally showed higher values during the winter period and May, compared to the summer-autumn period, and were positively correlated with Chl *a* concentration (Pearson's $r= 0.69$, $n= 13$, $p< 0.01$).

Table 1- Empirical carbon-to-leucine conversion factors (± SD) calculated by the integrative (Riemann et al. 1987) and cumulative (Bjørnsen and Kuparinen 1991) methods. SD refers to two replicate incubations.

Date	Emp. CF (integrative) (Kg C mol Leu^{-1})	Emp. CF (cummulative) (Kg C mol Leu^{-1})
28-Jan-03	3.40 ± 0.47	3.52 ± 0.50
4-Mar-03	1.66 ± 0.05	1.65 ± 0.08
25-Mar-03	1.04 ± 0.06	0.98 ± 0.02
22-Apr-03	1.07 ± 0.06	1.32 ± 0.43
13-May-03	2.46 ± 0.14	2.25 ± 0.04
25-Jun-03	1.36 ± 0.20	1.25 ± 0.18
14-Jul-03	1.29 ± 0.12	1.31 ± 0.26
4-Aug-03	1.37 ± 0.23	1.35 ± 0.11
16-Sep-03	1.38 ± 0.02	1.33 ± 0.06
21-Oct-03	0.97 ± 0.31	0.98 ± 0.25
25-Nov-03	1.36 ± 0.04	1.28 ± 0.11
16-Dec-03	3.62 ± 0.40	3.62 ± 0.53
26-Jan-03	1.67 ± 0.13	1.65 ± 0.15

During the two winter periods (January to March 2003, and December 2003 to March 2004), BHP followed the pattern of Chl *a*, with peaks in February and December 2003 (Fig. 3a). However, maximal values of BHP were found in May, when Chl *a* concentration was low (Fig 3a), and a secondary peak was found in August. BHP was low from the end of the summer through autumn. An unusually high BHP value was found in July 2003, which was never repeated in other years, and was concomitant with a drastic shift of bacterial assemblage structure, as well as important changes in other parameters (Gasol et al. in prep). Therefore,

the results of July 2003 have not been considered as a seasonal feature and are presented as outliers in Fig. 3.

The dynamics of BR was not correlated to those of BHP. Remarkably, minimum values of BR were found at the time of the two BHP peaks (May and December 2003, Fig 3b), leading to high BGEs in these months (Fig. 3c). High values of BR were found in March 2003 and January 2004, during the decline of the bloom. Intermediate values of BR (around 25 μgC l^{-1} d^{-1}) were found during the summer. BR measurements (unlike BHP) tended to follow the pattern of DOC concentration (Fig. 3) and both parameters were positively correlated (Pearson's r= 0.74, n= 12, p< 0.01).

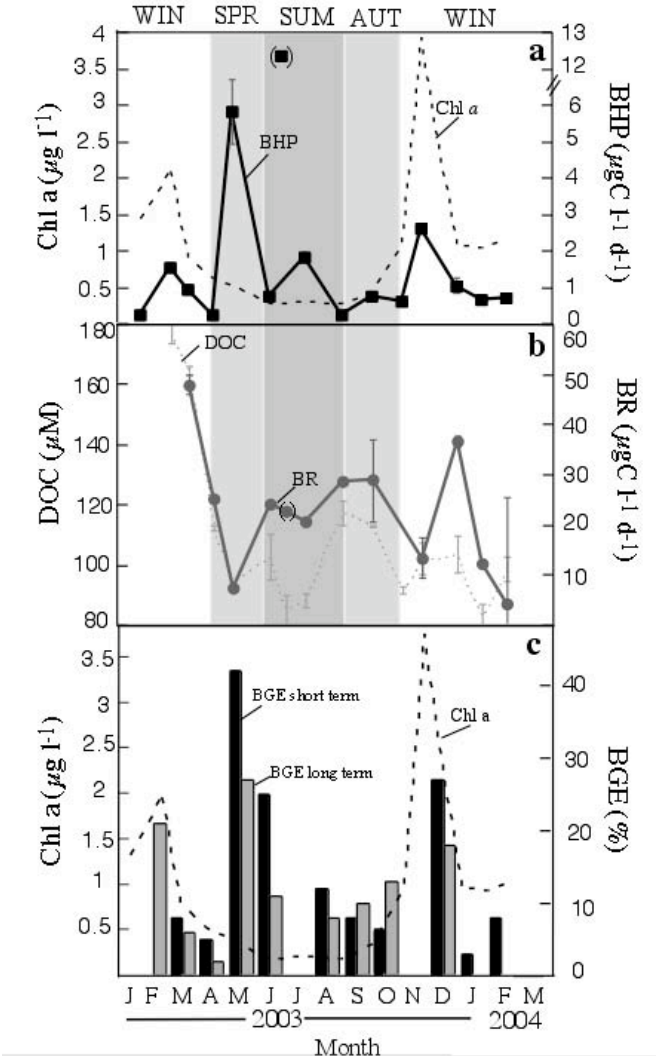

Fig 3- Determinations (± SE) of a) bacterial heterotrophic production (BHP), b) bacterial respiration (BR) and c) growth efficiency (BGE) over the period January 2003-February 2004 in Blanes Bay. Dotted lines indicate the concentrations of Chlorophyll *a* (panels a, c), or dissolved organic carbon (DOC, panel c) in the waters.

BGE was determined monthly by two independent approaches. First, we used short incubations (generally for 24 h) with filtered water, where we measured the decrease in oxygen concentration (BGE short-term estimate, Fig. 3c). Secondly, we carried out long-term incubations of seawater dilution cultures, during which we followed the increase in POC and decrease in DOC (BGE long-term estimate, Fig. 3c) over 3-5 days. Although the range of BGE obtained with the two methods was different (3-42% and 2-27% for the short-term and long-term estimates respectively), both approaches were correlated (Pearson correlation coefficient r= 0.84, p< 0.01, n= 8) and showed a similar trend throughout the year, with maximum values in May and December (Fig. 3c). BGE decreased after the Chl *a* peaks, and from May to October (Fig. 3c).

Ectoenzyme activities- The activities of some bacterial ectoenzymes (Betaglucosidase, aminopeptidase, xylase and chitinase) were measured from January 2003 to March 2004. We found low concentrations of the enzymes during winter in 2003, although the activity of betaglucosidase and aminopeptidase increased during the winter 2004 (Fig. 4). A common peak of activity of all the enzymes studied was found in May, and a second peak was found in August for all the enzymes except for the aminopeptidase, which peaked in September and December. All enzyme activities were significantly correlated with bulk bacterial production (Leu uptake, r> 0.5, n= 15 p< 0.04), but xylase and chitinase showed the highest correlation coefficients (r= 0.88 and 0.82, respectively, p< 0.0001).

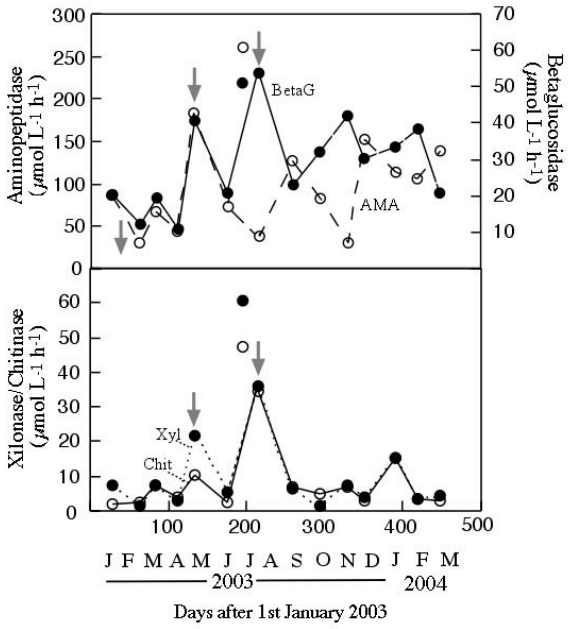

Fig 4- Activity of bacterial ectoenzymes a) betaglucosidase (betaG) and aminopeptidase (AMA, in the fraction <1μm), and b) xylase (Xyl) and chitinase (Chit) through January 2003-March 2004 in Blanes Bay. Arrows indicate peaks in ectoenzyme activities

DOC concentration and release by phytoplankton- The in situ concentration of DOC ranged between 83 and 176 μM with maximal values during the winter 2003 (Fig. 3b). These high values were not repeated during the winter of 2004 (Fig 3b). During the spring and summer periods, peaks of BHP (May, August) were coincident with substantial decreases in the concentration of DOC. The DOC release by phytoplankton was measured over an annual cycle (March 2003-March 2004). On average, the percentage of photosynthetically extracellular release (PER) constituted 45% of total PP (range 22-73%), and the lowest values were found in winter (24 and 22% in December and January respectively, details not shown).

Values of dissolved (dPP) and total PP (tPP) were compared to total bacterial carbon demand (BCD, Fig. 5). BCD met autochthonous dPP during some months of the winter period (Fig 5a). When total (dissolved plus particulate) PP was taken into account, BCD could also be balanced by autochthonous PP during spring and August (Fig. 5b).

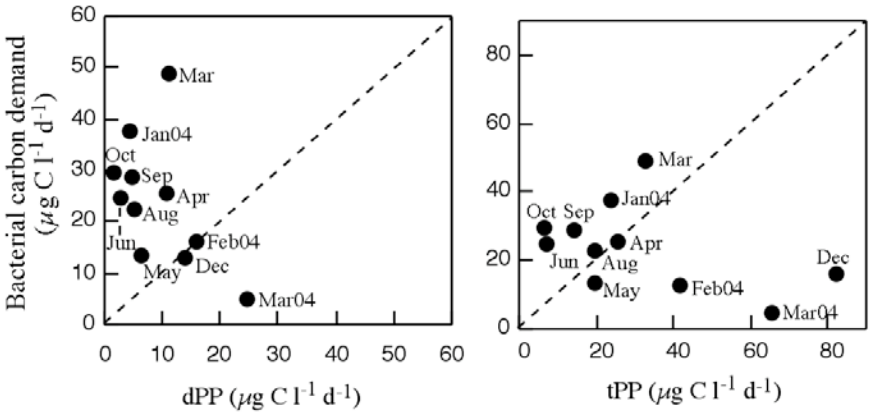

Fig 5- Dissolved (dPP) or total primary production (tPP) compared to bacterial carbon demand from March 2003 to March 2004. The dotted line indicates the 1:1 line.

Relationship between bacterial assemblage structure and carbon metabolism- Seasonal changes in bacterial assemblage structure were assessed during 2003 by means of denaturing gradient gel electrophoresis (DGGE, Chapter III). Nonmetric multidimensional scaling (MDS) and Ward's clustering method were used in order to explore the similarities between the samples based on bacterial assemblage structure, carbon metabolism and environmental factors (Fig. 6). We found a reasonably similar grouping of seasonal samples based on

environmental and bacterial assemblage structure parameters, suggesting that summer versus winter and spring conditions were differentiated, as it is clearly shown in the bacterial diversity MDS. However, in the environmental MDS, the November sample clustered together with spring samples, and the March sample clustered with winter samples. Opposite to environmental and diversity parameters, carbon metabolism parameters did not produce a seasonal grouping of the samples, clustering together samples from different seasons.

The relationship between these multivariate parameters was assessed through correlation (Spearman rank correlation) of the corresponding similarity matrices. We found a marginally not significant relationship between environmental data and bacterial assemblage structure through the samples (Spearman rho= 0.20 p= 0.062). Bacterial carbon metabolism was not related to environmental factors (Spearman rho= -0.06 p= 0.6) nor to bacterial diversity (Spearman rho= -0.09 p= 0.7).

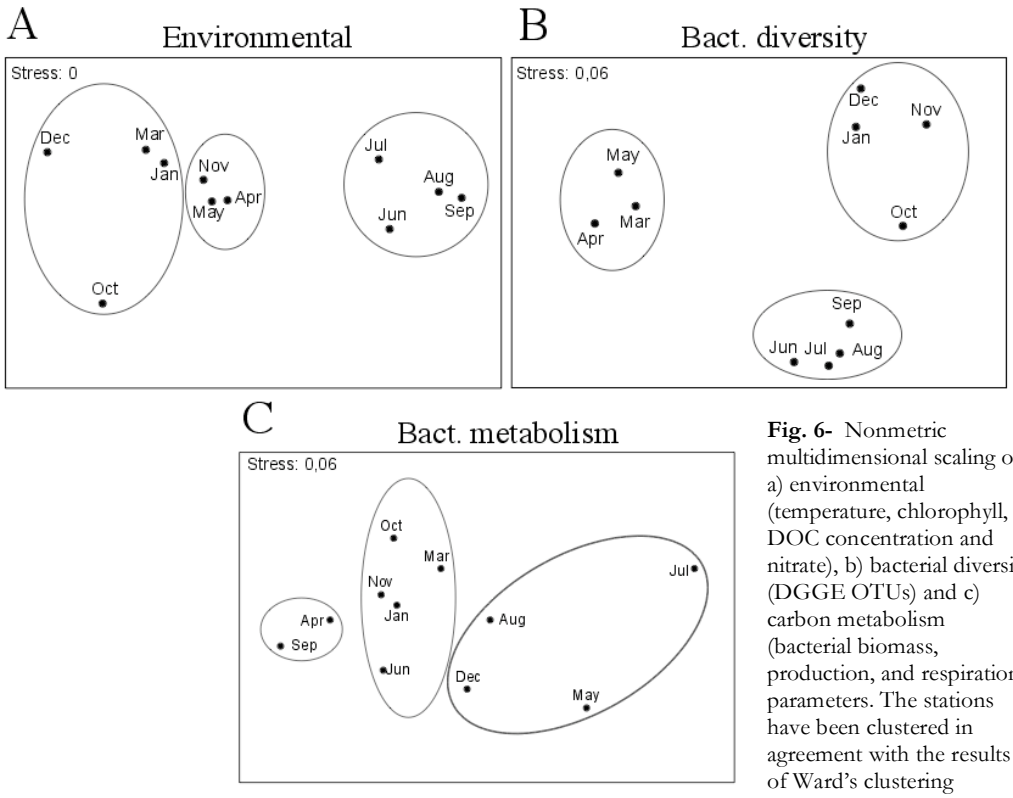

Fig. 6- Nonmetric multidimensional scaling of a) environmental (temperature, chlorophyll, DOC concentration and nitrate), b) bacterial diversity (DGGE OTUs) and c) carbon metabolism (bacterial biomass, production, and respiration) parameters. The stations have been clustered in agreement with the results of Ward's clustering method.

Discussion

Seasonal changes in physico-chemical variables and plankton organisms had been described in Blanes Bay thanks to some long-term studies (Duarte et al. 1999), which included measurements of microplankton respiration and net production (Satta et al. 1996, Lucea et al. 2004). However, the characterization of the bacterial carbon flux, including measurements of BR and BGE and seasonal estimates of BHP, had not been reported before. The year-round measurements of BHP and BR in Blanes Bay were in the range of previous studies in coastal oligotrophic sites (Ducklow & Carlson 1992, Robinson & Williams 2006). In fact, the annual BR average (26 μg C l^{-1} d^{-1}) was closer to the mean respiration rate calculated by Robinson & Williams (2006) for open-ocean areas (35 μg C l^{-1} d^{-1} assuming a RQ of 0.88), than that of coastal areas (78 μg C l^{-1} d^{-1}, assuming a RQ of 0.88). Similarly, several environmental parameters in this site are more similar to those found in open ocean waters, such as the low Chl *a* and nutrient concentrations (Krom et al. 1993). This makes this coastal area a highly suitable site to study microbial processes that could be representative of what occurs in more oceanic conditions. Besides, the strong phosphorous limitation of BHP throughout the year in this site (Pinhassi et al. in press), which is characteristic of the Mediterranean (Sala et al. 2002, Van Wambeke et al. 2002, Zohary & Robarts, 1998), allowed the examination of how this type of limitation can affect the pattern of carbon use by bacterioplankton. Drastic differences in the bacterial carbon assimilation and growth efficiency were found in nutrient-replete conditions (such as the winter bloom), which were characterized by higher BGEs (over 20%), versus nutrient limited assemblages, such as those of the end of the summer, when BGE was as low as 5%.

Seasonality of bacterial carbon consumption in Blanes Bay- During a full-year cycle (2003), BHP roughly followed chlorophyll concentration during the winter, with peaks of production associated with maxima of Chl *a* (Fig. 3a). This suggests that bacteria were initially growing on the dissolved compounds exudated by phytoplankton, probably monomers (carbohydrates) easily transported and degraded. However, peaks of BHP were also found during the spring and summer periods, when phosphorous limitation was maximal (Pinhassi et al. in press) and Chl *a* was low (Fig 3). These peaks were concomitant with high ectoenzymes activities (Fig. 4) suggesting that they could be associated with the degradation of high molecular weight (HMW)-DOM. The utilization of this fraction of DOM was probably carried out after the exhaustion of simple easily-degraded monomers, since the degradation of

polymers requires the expenditure of extra energy. Higher energetic costs due to HMW-DOM degradation could be related to the lower BGEs found during summer.

Chróst (1992) showed similar results: the specific betaglucosidase activity was low during the spring phytoplankton bloom (although in a different system, a eutrophic lake), and reached the highest values at the late stage of the phytoplankton bloom collapse. This increase in enzyme activity was concomitant to an increase in bacterial biomass and production, similar to that found in Blanes Bay during May. Chróst (1992) postulated that excretion of photosynthetic products during active growth of phytoplankton repressed the bacterial synthesis of the enzyme, which was later induced by the high amount of polysaccharides released by the autolysis of senescent algae.

The induction of the use of polymers in the nutrient-limited season (summer) could also be due to the increase in the release of HMW-DOM by phosphorous-limited phytoplankton, or alternatively, to the increase in cell lysis during this season. In this line, several studies have shown that phosphorous depletion may cause an increase in total polysaccharide exudation by phytoplankton (Obernosterer & Herndl, 1995, Urbani et al. 2005). Increased levels of phytoplankton cell lysis during the summer have also been reported in Mediterranean waters (Agustí et al. 1998) based on high concentrations of esterases found in the waters.

The high interannual variability of Leu uptake (as a BHP estimate) in summer (Fig. 2b), despite the consistently low nutrient and chlorophyll concentrations during this period, supports the hypothesis that BHP does not follow a clear seasonal pattern, and is probably highly affected by the rate of DOM supply. On average (over three years), Leu uptake was lower in winter compared to spring and summer (Fig. 2b), in agreement with previous seasonal studies in marine (Sherry et al. 2002) and estuarine systems (Staroscik & Smith 2004, Shiah & Ducklow, 1994). However, low summer Leu uptake measurements, like those we found in 2003, have also been found in NW Mediterranean open waters (Lemée et al. 2002).

Empirical carbon-to-leucine conversion factors (eCFs) are crucial to constrain bacterial production measurements (Ducklow & Carlson 1992, Chapter I). However, the temporal variability of CFs is usually neglected in seasonal studies of BHP and, to our knowledge, only Murrell (2003) have reported seasonal eCFs in marine waters. These authors did not found a relationship between the variability of eCFs and environmental parameters. Conversely, we found a positive relationship between eCFs and Chl *a*, and interestingly, the peaks of eCFs coincided with BGE peaks (May and December). Even if we could not find a significant

positive relationship between both parameters, a highly significant correlation between BGE and eCFs has been found in oceanic North Atlantic (Chapter I) suggesting that both metabolic processes are related, at least in open ocean waters. In our study, eCFs were close to 1.55 kg C mol Leu^{-1} throughout the year, which is the theoretical value calculated by Simon & Azam (1992) if no intracellular leucine is present in the waters. This agrees with the oligotrophic state of Blanes Bay year-round, and the probably low concentrations of amino acids in the waters. Higher conversion factors were found during winter (table 1), probably because of isotope dilution with extracellular leucine released by primary producers in this season.

With regard to respiration measurements, previous studies in Blanes Bay have shown a repeatable seasonality in total community respiration (CR), with higher values during the summer (Satta et al. 1996, Lucea et al. 2005). This pattern did not show up in our seasonal cycle, where BR also peaked after the collapse of phytoplanton blooms in winter (Fig.3b). BR was not correlated with BHP in our annual study, contrary to the positive relationship found between both parameters by del Giorgio & Cole (1988), in a large-scale comparison which included samples from a wide range of aquatic systems. This lack of relationship suggests that both processes were not coupled, and would be controlled by different factors. In this sense, it is interesting that BR, and not BHP, was positively correlated to the in situ DOC concentration ($r= 0.74$, $n= 12$, $p< 0.006$), similarly to other reports (González et al. 2003, but see Lemée et al. 2002). BR is usually regarded as a more integrative and constant measurement compared to BHP, which can rapidly respond to environmental changes. The BHP measurement probably reflected the incorporation of the most labile fraction of DOC, while BR integrated the global use of DOC.

Seasonality of bacterial growth efficiency- To measure BGE, we applied the two most often used approaches: short and long-term incubations (del Giorgio & Cole 1998). In a review of BGEs in aquatic ecosystems, del Giorgio & Cole (1998) did not find a significant effect of the type of method used on the relationship between BHP and BR, or that between BHP and BGE. However, to our knowledge this is the first published study that has directly compared both types of BGE estimates simultaneously on the same set of samples. Results from these independent approaches show a relatively good agreement (Pearson correlation coefficient $r= 0.84$, $p< 0.01$, $n= 8$, excluding the unusual sample from July) although the values tended to move away from the 1:1 line at high BGEs (over 20%) with higher estimates in the short-term experiments (Fig. 7). This suggests that, at least in some cases, the rates of bacterial carbon processing in seawater cultures can be extrapolated to in situ conditions with relatively good

precision, despite the drawbacks of the long incubation approach (e.g., changes in bacterial assemblage composition, Massana et al. 2001).

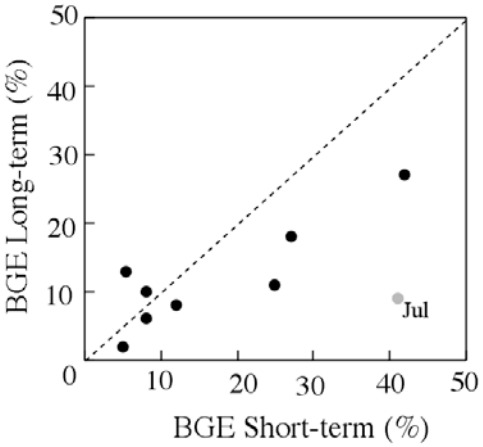

Fig 7- Bacterial growth efficiency measured with short-term assays, versus long-term assays (i.e. seawater cultures).

The range of BGE found (3-42%) is in close agreement with previous estimates in an oceanic station (DYFAMED) of the NW Mediterranean (Lemée et al. 2002), and other oligotrophic sites (Carlson & Ducklow 1996, Sherry et al. 1999). The few previous studies that have addressed the seasonality of BGE in marine waters found different patterns. Higher BGEs were found in spring and summer in the North Sea (Reinthaler & Herndl 2005), and during the spring bloom and fall in the NW Mediterranean (Lemée et al. 2002). Sherry et al. (1999) also found high variability in BGE measurements along a transect in the subarctic NE Pacific, with maximum values of BGE in the spring near the coast, and in the summer offshore. Our results, showing higher BGE during the winter bloom and spring, concur to suggest that temperature should not be a critical factor regulating BGE, as discussed below.

Bacterial carbon demand and DOM production: autochthonous versus allochthonous carbon supply- Positive correlations between bacterial and phytoplankton biomass or production have been observed in mesocosms and large-scale comparative studies (White et al. 1991, Cole et al. 1988, Gasol & Duarte 2000). However, it is often not evident in field studies, such as the one presented here, what has been attributed to either consumption of allochthonous DOM, inefficient DOM consumption due to inorganic nutrient limitation (Thingstad et al. 1997), or a time lag between DOM production by phytoplankton and consumption by bacteria (Sherr & Sherr 1996). It could also be possible that our sampling

frequency did not have enough resolution to find a positive relationship between primary and bacterial production, but other more frequent-sampling studies have also failed to capture such relationship (weekly sampling, Starostik & Smith 2004).

It has been postulated that the fact that dissolved primary production (dPP) is frequently neglected in carbon production measurements, can obscure the relationship between phytoplankton and bacterioplankton production, since it constitutes one of the most important sources of labile organic molecules for heterotrophic bacterial growth (Cole et al. 1982, Norrman et al. 1995). The measurement of phytoplankton photosynthetic extracellular release (PER) allowed us to analyze whether bacterial carbon demand could be met by dPP throughout the seasonal cycle. PER showed high variability, with generally lower values in the chlorophyll-rich season, in agreement with previous results (Morán et al. 2002, Teira et al. 2001). It has been postulated that PER can be high under low nutrient concentrations because of carbon fixation exceeding the build-up of new cell material.

The average PER value (45%) measured is in the highest range of previous reports for the Mediterranean (2-44%, Hagström et al. 1988, Fernandez et al. 1994, Morán & Estrada 2001), and other marine regions (Nagata 2000). Despite these generally high values, we found that bacterial carbon demand could only be met by autochthonous dPP in the winter period (with the exception of January), or winter, spring and august, if pPP was also considered (Fig. 5). These results support previous reports of the net heterotrophy of this site (Satta et al. 1996, Lucea et al. 2005). Other studies have found similar results in different marine systems. For example, Sherry et al (1999) found that pPP could meet BCD in winter and spring, but not during the summer in slope waters of the NE Pacific without considering the dissolved fraction of PP. This fraction can reach high levels in oligotrophic areas (Karl et al. 1998). The same could be applied to the study of Lemée et al. (2002), in which BCD systematically exceeded pPP over an annual cycle, but dPP was not measured. In a much more eutrophic marine area, the North Sea, Reinthaler & Herndl (2005) found that BCD was only met by pPP in April, July and September, also without considering dPP.

In order to illustrate the differences in the ratio BCD/PP throughout the year, we constructed a carbon budget (Fig. 8) for two contrasting seasonal situations: the winter bloom period, characterized by high PP and high input of nutrients, and the summer period when inorganic nutrient (phosphorous) limitation was maximal (September, Pinhassi et al. in press). As shown in the budget, while dissolved production could meet carbon demand in nutrient rich conditions (winter bloom), an important source of allochthonous or not contemporary

carbon is needed in order to cover BCD during the summer. The Bay of Blanes sporadically receives inputs of nutrients and terrestrial carbon from the Tordera River during stormy periods (Satta et al. 1996), which can be equivalent to about 50% of PP (Lucea et al. 2005). These authors found that the excess net benthic community production could also balance the carbon deficit in the pelagic compartment over a seasonal cycle. Thus, benthic community production together with allochthonous DOC from river inputs (and coastal runoff) could meet the unbalance between BCD and PP in Blanes Bay.

Constraining BGE in different regions of the ocean is of great interest, since BHP is easier and more frequently determined than BR (Robinson & Williams 2006), and total BCD could be extrapolated from in situ BHP measurements if reliable estimates of BGE were available. However, it is important to note that BGE estimates are highly sensitive to BHP measurements, especially in low productivity systems. BHP estimates, unlike BR, vary greatly over the incubations, and frequently are quite different to the values measured in situ. In different studies, distinct BHP determinations have been used for the computation of BGE without a clear consensus among researchers. The initial BHP of the incubation and the increase in bacterial biomass are the most widely used estimates of BHP (Chapter I) and, in this study, we used the average from both estimates to minimize errors and biases.

The uncertainty in BHP measurements can greatly affect the BGE estimates, which, if are later used for extrapolation from *in situ* measurements of BHP, can conduce to great under- or overestimation of BR and BCD. In this sense, it is remarkable that if we applied the BGE estimated for September (8%) to extrapolate BR based on the in situ BHP measurement (0.2 μg C l^{-1} d^{-1}), we would calculate a BR of 3 μg C l^{-1} d^{-1} instead of that directly measured (13 μg C l^{-1} d^{-1}). Therefore, by this extrapolation, dPP would meet BCD in September, contrary to our suggestion based on in situ measurements. Clearly, more research effort should be focused on the calculation and interpretation of BGE measurements in order to constrain these values, especially in non-productive regions.

Effect of environmental factors on bacterial carbon flux and growth efficiency. Temperature and nutrient availability are among the most important environmental factors that modulate BHP (Pomeroy & Wiebe 2001). While different nutrients have been shown to limit BHP in the ocean, such as phosphate (Sala et al. 2002, Cotner et al. 1997), nitrogen or iron (Kirchman et al. 2000), the effects of temperature are more contradictory. A positive relationship between temperature and BHP has been found in several studies (White et al.

1991), but other authors suggest that it is not a critical factor (del Giorgio & Cole, 1998). Our results are in this latter line, since we found a weak but significant relationship between BHP and temperature. With regard to inorganic nutrients, Pinhassi et al (in press) found phosphorous limitation all throughout the year in Blanes Bay (yet much stronger in spring and summer conditions), with the exception of October, coincident with a stormy period, when bacterial production was carbon limited.

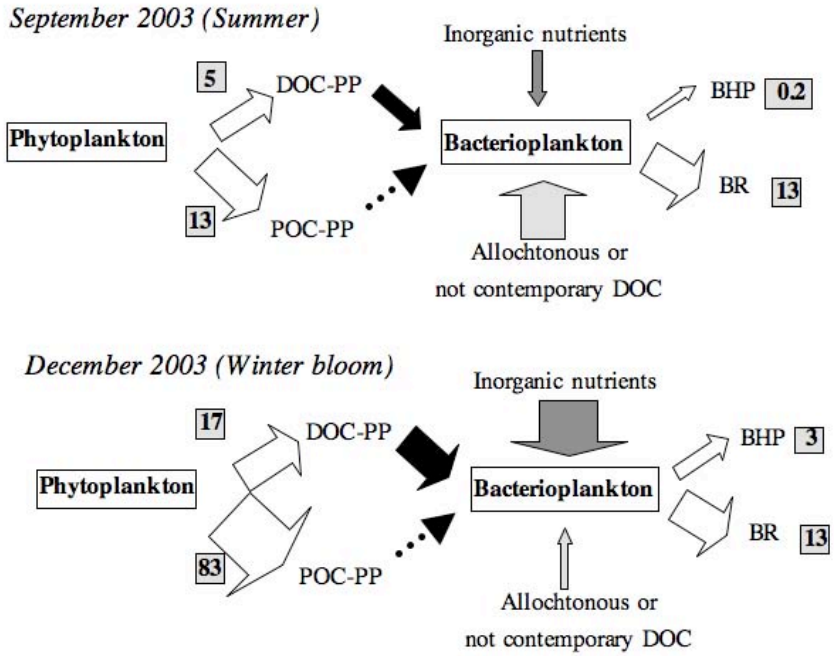

Fig 9- Carbon budget corresponding to summer conditions (upper panel) and the winter bloom (lower panel). Numbers beside the arrows indicate carbon flow measurements (μg C l^{-1} d^{-1}). The vertical grey and white arrows are hypothetical.

The factors affecting BR and BGE have been less studied. Phosphorous limitation on BR has been reported in some studies (Pomeroy et al. 1995, Obernosterer et al. 2003), although a lack of such effect was also found in the subtropical North Atlantic (Chapter I). Rivkin & Legendre (2001) proposed a temperature dependence of BGE, but in their study they ignored the covariation between temperature and nutrient supply. They also included data from a wide range of temperatures. Such relationship has not been shown in studies covering narrower ranges of temperature, such as our seasonal study and others (del Giorgio & Cole 1998, Lemée

et al. 2002, Reinthaler et al. 2005), although possibly, ranges of 10ºC such as that shown in this study, should have been sufficient to observe a trend if temperature exerted a strong control on BGE.

Inorganic nutrient availability has been proposed as a controlling factor enhancing BGE in experimental (Goldman et al. 1987) and field studies (Kroer 1993, Smith & Prairie 2004, Chapter I). In contrast, Lemée et al. (2002) found a negative relationship between BGE and the concentrations of nitrate and phosphate. DOC lability has also been suggested as a main factor that affects BGE in seasonal studies (Reinthaler & Herndl 2005, Lemée et al. 2002). Indeed, nutrient limitation can also affect DOC lability, and therefore BGE. Obernosterer & Herndl (1995) demonstrated that heterotrophic bacteria could not utilize extracellular products released from P-limited phytoplankton efficiently because their metabolism is affected by the same P limitation. Puddu et al. (2003) found higher BGE in bacteria growing in P-balanced conditions (56%) compared to P-limited conditions. Thus, nutrient limitation during the summer period in Blanes Bay could lead to the phytoplankton release of extracellular products that are more difficult to use by bacteria, leading to higher energy costs and lower BGEs.

The association of BGE with PP seems to be a common feature in seasonal studies. Lemée et al. (2002) found a positive relationship between BGE and Chl a, and Reinthaler & Herndl (2005) found higher BGE in months with higher PP. These authors concluded that BGE could be directly linked to the bioavailability of DOC and indirectly to PP. We also found BGE peaks during Chl *a* maxima. However, a significant peak in BGE was also found in May, unrelated to Chl *a* concentration, which suggests that factors other than PP can also affect BGE.

Effects of bacterial assemblage structure on carbon metabolism- We could not find a direct relationship between changes in bacterial assemblage structure and carbon metabolism with the use of several multivariate methods. The integration of different metabolic parameters produced a grouping of samples quite different from that based on bacterial assemblage structure (Fig 6). This would suggest that there is a large degree of redundancy in the bacterial community, and different combinations of organisms can produce similar patterns of carbon metabolism. Despite the relevance of knowing whether bacterial assemblages perform redundantly in carbon processing, or that specific bacterial populations conduct different patterns of carbon metabolism, such topic has been very seldom assessed in situ in marine waters. Very recently, Fuhrman et al. (2006) have found highly repeatable temporal patterns in

the bacterial assemblage structure (by the automatic ribosomal intergenic spacer analysis-ARISA-, fingerprint method) off the California coast, and more importantly, such patterns in distribution were significantly influenced and predictable from a range of abiotic and biotic factors. Such results suggest that marine bacteria exhibit low levels of functional redundancy, in contrast to our results. However, their fingerprint technique (ARISA) provides a much higher resolution than DGGE (e.g. many more OTUS could be analyzed). Furthermore, most of the factors analyzed by Fuhrman et al. (2006) were environmental variables, but bacterial abundance and production had lower predictive power. In our data set, the environmental factors were also more related with bacterial assemblage structure than to carbon metabolism data.

The lack of relationship between bacterial diversity and carbon metabolism could also be due to the fact that changes in the proportions of different bacterial groups may mask changes of the really active populations, which cannot be discriminated by the DGGE method. Several studies have shown that the single-cell activities of different groups can vary widely (Cottrell & Kirchman 2000), but little is known yet about temporal changes in the single-cell activity of given groups of species. Results from microautoradiography combined with fluorescence in situ hybridization (MARFISH) in Blanes Bay have shown substantial differences in the activity of distinct populations, being Roseobacter one of the most important components of the active bacterial community (Chapter V). Thus, it is possible that ecosystem functioning (with respect to C-processing) is mainly carried out by populations that represent a low percentage of the total bacterial community and that, therefore, do not drive the variations in community structure. Interestingly, Roseobacter abundance peaked in May and December, when higher BHP and BGE were found. These active populations could be the preferred target of bacterial predators (either because of their cell size, or because of their high activity, del Giorgio et al. 1996) and, thus, never end up dominating the community in terms of biomass.

In a parallel study to this one, we have encountered that a specific group of bacteria (*Glaciecola*) channelled most C when it became a very dominant part of the bacterial community in Blanes Bay (Gasol et al., in prep). However, this population was subject to a strong predation pressure and disappeared fast from the site. This suggests that, occasionally, active populations of bacteria can escape predation control, and then their abundance reflects their dominance in carbon processing. Definitely, more research will be needed in order to determine which species are driving most of the carbon flux in oceanic waters and this will

require a combination of bulk process measurements with single-cell measurements of bacterial activity.

In summary, bacterial assemblages in Blanes Bay showed different patterns of carbon metabolism throughout the year. Bacterial heterotrophic production showed higher variability than bacterial respiration, and peaked concomitantly with chlorophyll maxima in winter or with peaks of ectoenzyme activities in spring and summer. This suggests that both LMW- and HMW-DOM can be important sources of carbon for marine bacteria. Bacterial growth efficiencies were also related to chlorophyll peaks, and were higher in the winter or spring periods (>20%), compared to summer (<10%). Such changes in carbon metabolism were apparently not related to changes in bacterial assemblage structure, suggesting a high functional redundancy in the bacterial assemblage at the phylogenetic resolution level analyzed (DGGE band pattern). However, the dynamics of some groups, such as Roseobacter, were much better related to peaks of BHP or BGE, suggesting that some bacterial groups can be driving most of the carbon metabolism.

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Chapter V

Seasonal variation in the contribution of different bacterial groups to the uptake of LMW-DOM compounds in NW Mediterranean coastal waters

L. Alonso-Sáez & J.M. Gasol

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Abstract

We analyzed the contribution of different heterotrophic bacterial groups to the uptake of several low-molecular weight (LMW) compounds during a seasonal cycle in the NW Mediterranean coast (Blanes Bay Microbial Observatory). Bacterial assemblage structure has been shown to change substantially year-round for this site. However, whether changes in the activity of the different bacterial groups also occurred at the seasonal scale was unknown. Microautoradiography combined with catalyzed reporter deposition fluorescence in situ hybridization (MARCARDFISH) was used to analyze the patterns of glucose, amino acids and ATP uptake by different bacterial groups. Gammaproteobacteria and Bacteroidetes were not very active in the uptake of glucose year-round (<10% of cells were active) as compared to Alphaproteobacteria (generally >20% of cells were active). Dissolved free amino acids were considerably taken up by Alphaproteobacteria and Gammaproteobacteria, but not by Bacteroidetes. Relatively high percentages of cells of the three broad phylogenetic groups actively took up ATP, which could be related to the high phosphorous limitation of bacterial production during most of the year in Blanes Bay. SAR11 contributed moderately to the uptake of the monomers, with less than 30% of the cells being active. On the contrary, Roseobacter was highly overrepresented in the uptake of all the substrates year-round, with more than 50% cells being active in all the samples and for all substrates. Seasonal changes in the activity of some bacterial groups were also found, with generally lower uptake of the monomers in the summer period. Our results suggest that seasonal changes in the uptake activities of some bacterial groups are substantial, and different uptake patterns cannot be explained solely based on the changes in bacterial community composition, at least with the use of broad phylogenetic level probes.

Introduction

The heterotrophic activity of marine bacteria and their role in carbon cycling have been extensively studied in the ocean as bulk, global, measurements. Consequently, current carbon models implicitly assume that bacterioplankton constitute a single element in the system and that all heterotrophic bacteria perform equally in carbon processing (Fasham et al. 1999). This view has been challenged by the application of single-cell techniques (i.e. CTC, BrdU, autoradiography, cell-sorting), which have showed that activity is not homogeneously distributed throughout the bacterial assemblage, and that a wide range of metabolic states

coexists in each bacterial assemblage (Smith & del Giorgio 2003). These findings have pointed to the need of using methods with single-cell resolution for the study of the ecological role that different bacterial populations play in the ocean,

Some key biogeochemical processes that occur in the sea, such as anaerobic methane oxidation or anaerobic ammonium oxidation, have been shown to be mediated by very specific microbial populations (Boetius et al. 2000, Schmidt et al. 2005). However, very little is known about the partition of carbon processing among the different phylogenetic groups of bacteria that coexist in the upper oxygenated ocean (Cottrell & Kirchman, 2000). Considerable understanding of the phylogenetic diversity of marine bacteria has been gained by rRNA-based techniques. To understand the role that each one of these populations plays in the oceanic carbon cycle is the logical next objective (Giovanonni & Rappé, 2000).

One approach to that objective is obtaining oceanic relevant microbes in culture. Efforts with new culturing approaches have reached successful results, such as the isolation of a member from the ubiquitous SAR11 group (Rappé et al. 2002). The sequence of its genome has further allowed insights into the ecology of this group (Giovanonni et al. 2005). However, still most cultured phylotypes are not representative of the true oligotrophic marine bacteria, which are frequently limited in situ by carbon or inorganic nutrients such as N, P and Fe. Approaches such as metagenomics (e.g. DeLong 2005) allow to circumvent the culturing approach. By this approach, metabolic characteristics and potential ecological functions can be linked to specific in situ phylotypes. However, it is a extremely expensive and time-consuming procedure and, in a way, more suited to explore "potential" microorganism functions, than the actually expressed ones. The combination of single-cell and molecular techniques, such as microautoradiography combined with FISH (MARFISH) has revealed as a powerful alternative to study the metabolic strategies of natural communities.

MARFISH has been used to show different uptake patterns of simple LMW-DOM compounds in several studies (Overney & Fuhrman 1999, Cottrell & Kirchman 2000, Vila et al. 2004). Usually, however, only the activity of broad phylogenetic groups has been assessed (but see Malmstrom et al. 2004, Alonso & Pernthaler 2006a, 2006b). This can be difficult to interpret since each of these broad groups contains several subgroups, which can greatly differ in space and time (Giovanonni & Rappé 2000). Furthermore, although some MARFISH studies have focused on changes in the bacterial use of organic compounds along spatial gradients (Cottrell & Kirchman 2003, Elifantz et al. 2005, Malmstrom et al. 2004), very little attention has been paid to temporal changes, usually limited to the comparison of two different

periods (Cottrell & Kirchman 2000, Vila et al. 2004). Thus, it is difficult to know whether patterns of uptake of specific compounds by bacterial groups can be generalized.

We could expect that the activity of different bacterial groups could change with time, driven by variations in the availability of organic compounds or the need for specific nutrients in resource-limited situations. Indeed, a well-known response of organisms to nutrient depletion is an increase in the capacity for uptake of the limiting nutrient (e.g. Lean & Pick 1981). Giovanonni & Rappé (2000) hypothesized that the dominance of ecologically successful heterotrophs in the surface ocean may be the result of a competitive advantage in procuring limiting inorganic nutrients.

In this study we studied the uptake of three kinds of organic compounds (glucose, dissolved free amino acids and ATP) in order to reflect the supply of carbon (glucose), or carbon with extra nitrogen (amino acids) and phosphorous (ATP). We carried out this study across a seasonal range of trophic conditions from nutrient depleted (in summer) to nutrient replete (winter) periods. Bulk bacterial production has been shown to be severely limited by phosphorous throughout most of the year in Blanes Bay (Pinhassi et al. in press), and is probably affecting the single-cell activity of specific groups. Substantial year-round changes in bacterial assemblage structure have been described in this site (Schauer et al. 2003, Chapter III). However, the identity of the active bacterial groups and whether the activity of specific bacterial groups also changes seasonally is unknown. Beside probes for broad phylogenetic groups (such as Alphaproteobacteria, Gammaproteobacteria and Bacteroidetes), we used more specific probes for the groups Roseobacter and SAR11, since the dynamics of both alphaproteobacterial lineages differed year-round (Chapter III). We specifically wanted to test whether the uptake activity of these bacterial groups was a permanent feature throughout the year, despite differences in environmental conditions, or whether seasonal patterns occurred. Ultimately, we wanted to know which members of the bacterioplankton community are important in terms of carbon and energy flux in this coastal site. To our knowledge, this is the first study to show seasonal dynamics on the single-cell activity of bacterial populations.

Materials and methods

Basic data. Samples were taken from Blanes Bay (The Blanes Bay Microbial Observatory, NW Mediterranean) at several dates along a seasonal cycle (March 2003-February 2004, Fig. 1). Surface water temperature was measured in situ with a mercury thermometer. For determination of Chlorophyll *a* concentration, 150 ml of seawater was filtered on GF/F filters (Whatman) and subsequently extracted in acetone (90% v/v) in the dark at 4ºC for 24h. Fluorescence was measured with a Turner Designs fluorometer. For analyses of dissolved nutrient concentration, seawater samples were filtered through 0.2 μm pore size 47 mm diameter polycarbonate filter (Supor-200; Gelman sciences) using a polycarbonate filtration device (Millipore). Dissolved inorganic nutrient concentrations were determined spectrophotometrically with an Alliance Evolution II autoanalyzer following standard procedures (Grasshorff et al. 1983). Phosphate concentrations were determined manually, using a 10-cm cuvette to increase the detection limit (Pinhassi et al., in press).

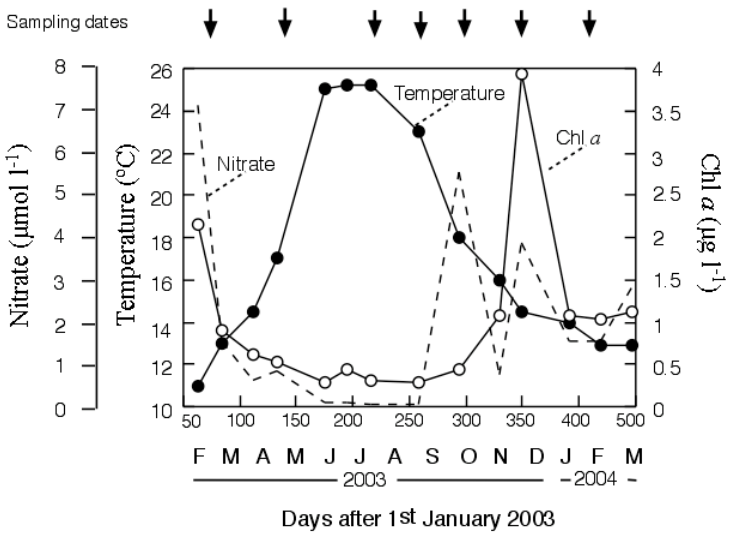

Fig 1- Temperature, Chl a and nitrate concentrations throughout the seasonal study in Blanes Bay. The arrows indicate the dates at which samples were taken for MAR-CARDFISH analyses.

Bulk uptake of amino acids, glucose and ATP. Bulk uptake of the substrates (glucose, amino acids and ATP) was monthly determined by the measurement of the radioactivity incorporated in subsamples withdrawn from the incubations used for MARCARDFISH analysis. As explained below, the three substrates were added at 0.5 nM concentration, the incubations lasted 4 hours and were done in an incubator adjusted to the in situ temperature.

For each sample (20 ml), four aliquots (1.2 ml) were taken in Eppendorf tubes, and 120 µl of cold 50% TCA was added to stop the incorporation. For every sample and compound, aliquots (5 ml) killed with formaline before the isotope addition were used as controls. Samples were kept frozen at –20ºC until processing, which was carried out by the centrifugation method of Smith & Azam (1992). Finally, 1 ml of scintillation cocktail (Optimal HiSafe) was added to each Eppendorf tube, and samples were counted on a Beckman scintillation counter after 24 hours.

Catalyzed Reporter Deposition (CARD)-Fluorescence In Situ Hybridization. CARDFISH was carried out following the protocol described by Pernthaler et al. (2004). Several horseradish peroxidase (HRP)-probes were used to characterize the composition of the microbial community in the original water samples: Eub 338-II-III (target most Eubacteria [Daims et al. 1999, Amann et al. 1990]), Alf968 (targets most Alphaproteobacteria [Neef, 1997]), Gam42a (targets most Gammaproteobacteria [Manz et al. 1992]), CF319 (targets many groups belonging to the Bacteroidetes group [Manz et al. 1996]), Ros537 (targets members of the Roseobacter-Sulfitobacter-Silicibacter group [Eilers et al. 2001]) and SAR11-441R (targets the SAR11 cluster [Morris et al. 2002]). The Eub antisense probe Non338 (Wallner et al. 1993) was used as a negative control. All probes were purchased from biomers.net (Ulm, Germany). Hybridizations were carried out at 35ºC overnight and specific hybridization conditions were established by addition of formamide to the hybridization buffers (20% formamide for NON338 probe, 45% formamide for ALF968 and SAR11-441R probes, and 55% for the other probes). Counterstaining of CARD-FISH preparations was done with 4,6-diamidino-2-phenylindole (DAPI, 1 µg ml^{-1}). Between 500 and 1000 DAPI-positive-cells were counted manually in a minimum of 10 fields.

MARCARDFISH. MARCARDFISH was performed in seven occasions during the seasonal cycle. We followed the protocol described by Alonso & Pernthaler (2005). Briefly, samples (20 ml) were incubated for 4 h in an incubator adjusted to the in situ temperature with the following tritiated substrates (0.5 nM final. conc.): [^{3}H] glucose (Amersham TRK85), [^{3}H]-amino acids mixture (Amersham TRK440), and [^{3}H]-ATP (Amersham TRK747). Single samples (for each compound and treatment) were killed with formaldehyde before the addition of the tritiated compounds and were used as controls. After the incubation, samples were fixed overnight with formaldehyde (1.8%) at 4ºC, and gently filtered on 0.2-µm polycarbonate filters (Millipore, GTTP, 25 mm diameter). The filters were then hybridized following the CARD-

FISH protocol, and subsequently glued onto glass slides with an epoxy adhesive (UHU plus; UHU GmbH, Bühl, Germany). For microautoradiography, the slides were embedded in 46ºC tempered photographic emulsion (KODAK NTB-2) containing 0.1% agarose (gel strength 1%, >1 kg cm^{-2}) in a dark room. The slides were placed on an ice-cold metal bar for about 5 min to allow the emulsion to solidify and subsequently placed inside black boxes at 4 ºC until development. The optimal exposure time was determined for each experiment and compound, which resulted between 13 and 19 days depending on the compound and time of the season. For development, we submerged the exposed slides for 3 min in the developer (KODAK D19), 30 s of rinsing with distilled water, and 3 min in fixer (KODAK Tmax) followed by 5 min of washing with tap water. The slides were then dried in a dessicator overnight, stained with DAPI (1 μg ml^{-1}), and counted in an Olympus BX61 epifluorescence microscope.

Results

Seven samples were chosen in order to cover a full seasonal cycle throughout March 2003-February 2004 (Fig. 1), being representative of the different bacterial assemblages that develop in Blanes Bay year-round, as assessed by monthly DGGE and CARD-FISH analyses (Chapter III). Three samples were taken during winter, when the temperature was relatively low (12-13ºC), and the concentrations of nutrients were quite high (Fig. 1). One of the winter samplings coincided with a Chl *a* peak (December), and the other two were taken in post-*bloom* situations (March 2003 and February 2004). The summer period was characterized by high temperatures (over 22ºC), and low Chl *a,* and concentration of nutrients (nitrate below 0.1 μM, Fig. 1). Phosphorus followed the nitrate pattern (results not shown) but showed a lower concentration range (0.12-0.24 μM). In this period, MARCARDFISH analyses were carried out in August and September. Two additional samples were analyzed in spring (May) and autumn (October), the last coincident with a stormy period, which produced a peak in inorganic nutrient concentrations (Fig. 1), and the only measured instance of C instead of P limitation of bacterial production (Pinhassi et al. in press).

Bulk incorporation rates- The bulk rates of uptake of glucose, amino acids and ATP (added at trace concentrations, 0.5 nM) ranged between <1 to 12 pM^{-1} h^{-1}, throughout the year (Fig. 2). In general, higher uptake rates were found in winter and spring, concomitant with higher concentrations of Chl *a*, while lower uptake rates were detected for the three compounds during the summer (Fig. 2). Generally, the highest incorporation rates throughout the year

were found for ATP (range 1-12 pM h^{-1}), with peaks in spring and winter. High uptake rates of glucose were found in the autumn and winter periods (>4 pM h^{-1}), while highest uptake rates of amino acids were found in spring (around 3 pM h^{-1}, Fig. 2).

Year-round percentages of active *Bacteria* as determined by MARFISH- The percentage of Eub+ cells (cells that hybridized with Eub338-II-III probes) that showed uptake activity of the different compounds was more uniform than bulk uptake measurements comparing the three substrates, generally within a general range of 15-35% (Fig. 2). However, substantial changes were found seasonally, especially for amino acids and glucose. The percentage of Eub+ cells active in glucose uptake was high in early spring, decreased in summer, and increased again in autumn and winter. The percentage of Eub+ cells active in the uptake of amino acids was also high in spring, but tended to decrease towards the summer and winter periods, with a minimum in December (9%, Fig. 2). The uptake of ATP showed the most uniform percentages of active Eub+ cells with a minimum in September (16% of active Eub+), and a maximum in December (34%, Fig. 2).

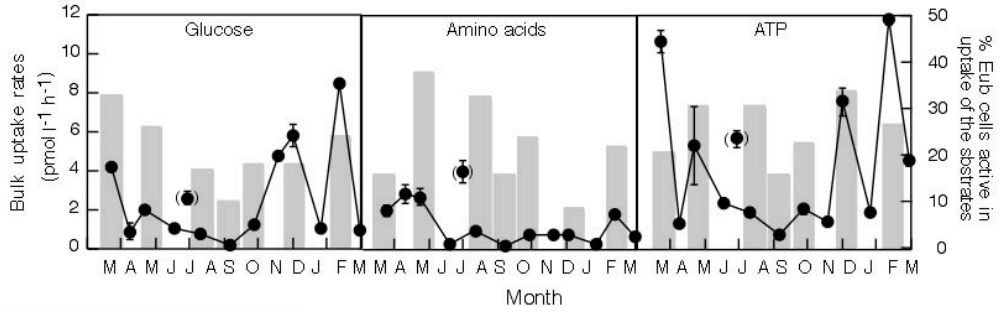

Fig 2- Year-round bulk rates of substrate uptake in Blanes Bay, and percentage of Eub+cells actively incorporating each of the substrates (bars). Samples (bulk uptaker rates) taken on July are presented as outliers, since bacterial assemblage structure and carbon dynamics drastically changed in this sampling (Chapter III, IV).

Seasonality in single cell uptake of glucose, amino acids and ATP by different bacterial phylogenetic groups- The uptake activities of the most abundant bacterial groups in Blanes Bay (which were Alpha- and Gammaproteobacteria and Bacteroidetes, all together comprising, on average, 75% of the Eub+ cells, Chapter III) were analyzed and showed large differences depending on the compound and the time of the year (Fig 3). Alphaproteobacteria showed

significant uptake activity for the three compounds throughout the year. Generally more than 20% of the cells of this group showed activity, i.e., were MAR+ (Fig. 3). On the contrary, Gammaproteobacteria and Bacteroidetes showed relatively low activity (<10% of the cells were MAR+) in the uptake of glucose, and glucose and amino acids, respectively. However, these two broad phylogenetic groups were more active in the uptake of ATP.

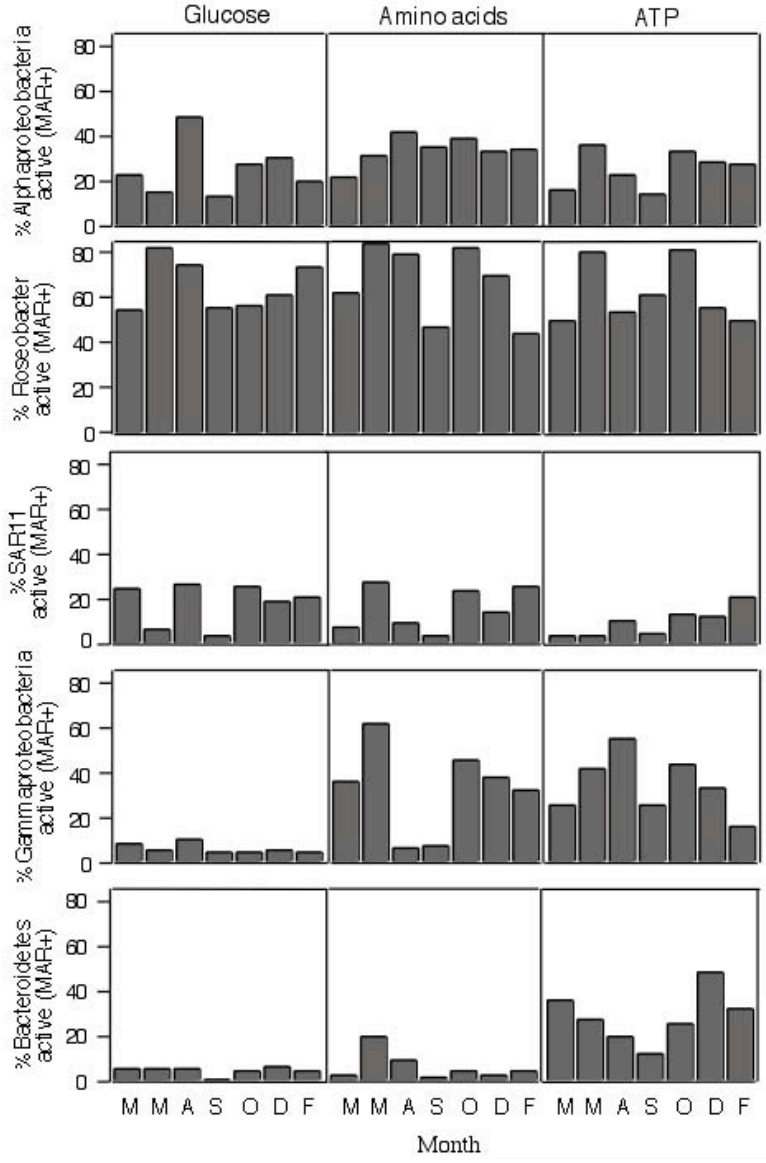

Fig 3- Seasonal dynamics of the percentage of probe positive cells (Alphaproteobacteria, Roseobacter, SAR11, Gammaproteobacteria and Bacteroidetes) active in the uptake of the three substrates (Glucose, amino acids and ATP).

Alphaproteobacteria showed rather uniform percentages of cells active in the uptake of amino acids throughout the year (around 30%), but higher variability was found in the uptake of glucose (with a peak in August, 49% active cells), and ATP (with lower values in summer). When the alphaproteobacterial groups SAR11 and Roseobacter were specifically studied, large differences were found between both populations. Roseobacter consistently showed the highest percentages of active cells in the uptake of all substrates throughout the year (generally over 50% of the Roseobacter cells were MAR+). SAR11 showed larger variability in the uptake of the monomers, but the percentage of active cells rarely exceeded 20%. Generally, higher percentages of active SAR11 cells were found in winter (over 10%), although relatively high percentages were found in August in the uptake of glucose, or May in the uptake of amino acids.

Gammaproteobacteria showed high activities in amino acid uptake in spring and winter, but very low activity during the summer (<10% of active cells). On the contrary, their activity in ATP uptake tended to increase towards the summer. Bacteroidetes showed an opposite trend to Gammaproteobacteria in ATP uptake, with lower uptake rates during the summer.

Uptake activities of bacterial groups versus their in situ abundances- Fig. 4 shows the percentages of cells (of the different groups) active in the uptake of glucose, amino acids and ATP, plotted against their % contribution to total abundance in the samples. Data points on the 1:1 line indicate groups that are participating in the uptake of the substrates proportionally to their proportions in situ. Roseobacter was overrepresented in the uptake of all substrates compared to their abundance in situ, but more markedly in the uptake of glucose and amino acids. SAR11 were close to the 1:1 line or underrepresented when the uptake of glucose or amino acids was analyzed, but it was always underrepresented in the uptake of ATP. Gammaproteobacteria were rather close to the 1:1 line in the uptake of the substrates, but were usually overrepresented in the uptake of amino acids, and underrepresented in the uptake of glucose. Finally, Bacteroidetes were close to the 1:1 line in the uptake of ATP, but underrepresented in the uptake of glucose and amino acids.

Contribution of the different bacterial groups to the total substrate uptake- The seasonal contribution of each of the bacterial groups analyzed to the total uptake of glucose, amino acids and ATP is presented in Fig. 5. Generally, more then 60% of the cells active in the uptake of the compounds could be assigned to the specific groups of bacteria studied, but in some cases, such as in the September sample, most eubacteria active in ATP uptake were

unknown (not detected with the probes used). The percentage of eubacterial cells not identified by the Alpha-, Gammaproteobacteria or Bacteroidetes probes ranged from 9% in September to 45% in February (Chapter III).

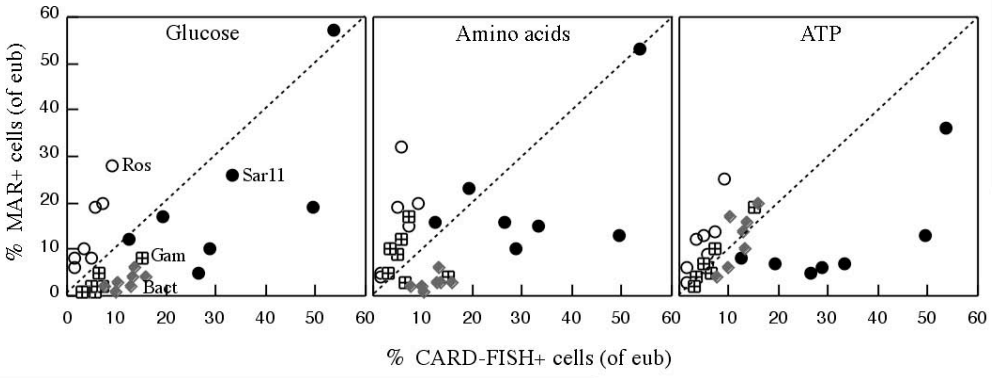

Fig 4- Contribution of various phylogenetic groups to assemblage structure (Roseobacter, open circles; SAR11, solid circles; Gammaproteobacteria, squares; Bacteroidetes, diamonds) versus their participations in the uptake of glucose, amino acids and ATP in Blanes Bay. Percentages were calculated relative to Eubacterial cells (probe Eub338-II-III).

Glucose uptake was dominated by the SAR11 group in two samples (August and February), but lower contributions were found during the rest of the year. In spring (May), Roseobacter dominated the uptake of glucose, and substantial contributions of this group were also found in autumn (October) and winter (December). Alphaproteobacteria not detected with the probes for SAR11 or Roseobacter contributed significant proportions to glucose uptake during the summer and autumn periods. Gammaproteobacteria also contributed more importantly during summer.

The uptake of amino acids was dominated by SAR11 in the post-*bloom* period (February), but uniform contributions of this group to amino acid uptake were found for the rest of the year (around 15% of total amino acids uptake). Roseobacter contributed very importantly in the uptake of amino acids in spring (May) and autumn (October). Similarly to glucose, unknown alphaproteobacteria were found to contribute significantly to amino acid uptake during the summer and winter period. Gammaproteobacteria substantially contributed to the amino acid uptake (around 10%), except for the summer period and december.

SAR11 only contributed significantly to ATP uptake in the post-bloom period (February), with low contributions during the rest of the year. Roseobacter showed high contributions to ATP uptake in March-May, and a lower contribution in the summer. Gammaproteobacteria

showed high uptake of ATP during August, but lower proportions throughout the year. Bacteroidetes, in contrast to the uptake of other compounds, showed high contributions to ATP uptake during the autumn and winter periods, being the dominant group in December.

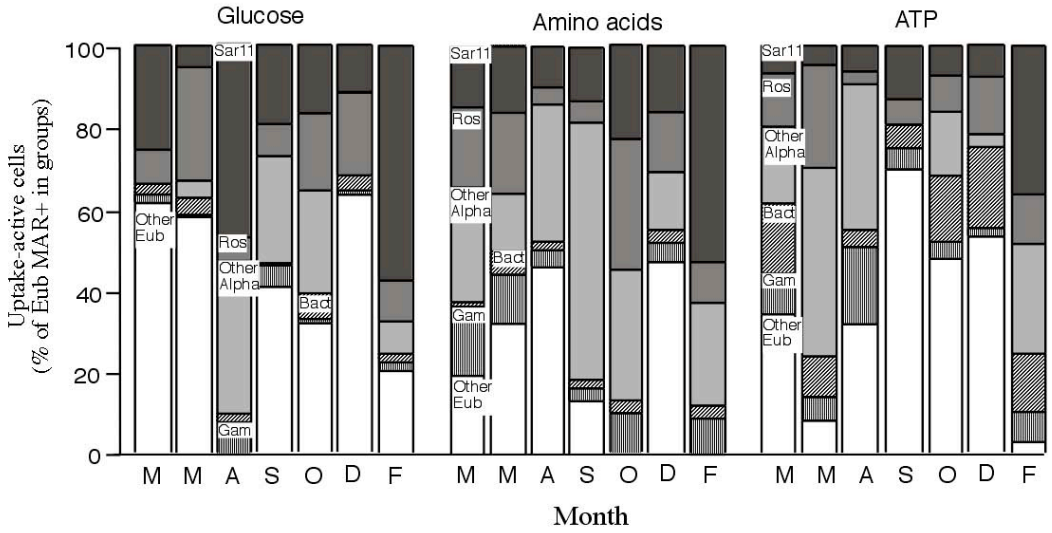

Fig 5- Seasonal contribution of each of the phylogenetic groups analyzed to the total uptake of glucose, amino acids and ATP by eubacteria in Blanes Bay. Percentages were calculated relative to Eubacterial cells (probes Eub338-II-III).

Discussion

Comparison of uptake patterns with previous MARFISH studies- Variability in the patterns of carbon use across phylogenetic groups of bacteria could have implications for carbon cycling. Whether the proportion of active cells differs between the bacterial groups, and whether the activity of bacterial groups is proportional to their in situ abundances are key issues to unveil the partition of carbon flow among groups. Such questions have been assessed in different studies with the MARFISH technique, analyzing the carbon processed by different bacterial groups from the perspective of in situ uptake of representative substrates (at trace concentrations, e.g. Cottrell & Kirchman 2000), or biomass production (using leucine and/or thymidine uptake at saturating concentrations, e.g. Cottrell & Kirchman 2003).

Longnecker et al. (2006) found a parallel increase or decrease in the average abundance of biosynthetically active cells (leucine uptake) by the whole prokaryotic community along a trophic gradient in the California Current System, suggesting that no shifts in the activity of specific groups occurred. However, a great variability in the percentage of active cells within the phylogenetic groups was found in each of the regions compared in their study (shelf, slope, basin). Cottrell & Kirchman (2003, 2004) also found substantial changes in the percentage of active cells (in leucine and thymidine uptake) within different bacterial groups across a gradient in the Delaware estuary. These authors concluded that only 50% of the contribution of the bacterial groups to biomass synthesis could be explained by their relative proportions in the assemblage, by correlating the relative abundance and activity of these groups within the assemblage.

Studies analyzing the uptake patterns of compounds at trace concentrations have also suggested that uptake activities are not uniformly distributed among groups (Cottrell & Kirchman 2000, Elifantz et al. 2005). Since LMW compounds can be easily transported across cell membranes, differences in the uptake patterns of monomers such as glucose or amino acids should be explained by the affinities of the uptake systems of each bacterial group. In this respect, Alonso & Pernthaler (2006a) carried out concentration-dependent experiments, and showed that the contribution of specific groups of bacteria, such as SAR86, to the turnover of particular substrates could be dependent on the concentration in which these substrates were present in the waters.

Here we followed a natural seasonal cycle including different trophic situations. We analyzed the uptake patterns of three monomers that are common components of DOM in marine waters and are rapidly turned over by bacterioplankton: glucose (Rich et al. 1996, 1997), amino acids (Billen & Fontigny 1987) and ATP (Azam & Hodson 1977, Hodson et al. 1981). The rationale for using these three substrates was to study the supply of only carbon (glucose), or carbon plus nitrogen (amino acids) and phosphorus (ATP), across a trophic range from nutrient depleted (in summer) to nutrient replete (winter) periods.

The higher bulk uptake rates of ATP compared to glucose and amino acids throughout the year could be related to the phosphorous requirement of bacteria, because of the strong year-round phosphorous limitation of bacterial production in Blanes Bay (Pinhassi et al. in press). Higher bulk uptake rates of amino acids compared to ATP have been observed in several samples from the Subtropical NE Atlantic Ocean, where bacteria showed carbon (instead of phosphorous) limitation (Alonso-Sáez L & Gasol J.M unpubl.).

The turnover rates of ATP have been shown to closely parallel those of glucose or amino acids (Hodson et al. 1981) and its assimilation is widespread in pure cultures of marine bacteria (Azam & Hodson 1977). These findings are in agreement with the results of our single-cell approach, in which ATP was taken up by all the bacterial groups studied, even if large differences were found for the other compounds (Fig. 3). The uptake of glucose and dissolved free amino acids have been more extensively studied, showing that they can support a large fraction of bacterial growth in marine waters (Billen & Fontigny 1987, Rich et al. 1996, Rich et al. 1997). Although it has been suggested that it is energetically advantageous to use preformed compounds such as amino acids compared to glucose, the energetic cost of transporting amino acids across the membranes can greatly offset this advantage (del Giorgio & Cole 1998). This seems to be the case in our system, where bulk uptake of amino acids was generally lower than that of glucose (Fig. 2). This could be related to the fact that nitrogen was not limiting bacterial production in Blanes Bay throughout the year (Pinhassi et al. in press), or simply reflect differences in the in situ availability of these substrates.

The MARCARDFISH approach allowed the determination of the specific bacterial groups that were more successful in the uptake of these compounds year-round, and possibly driving a large part of the carbon fluxes through the heterotrophic bacterial black box. Percentages of active cells in the uptake of substrates ranged from 20-40%, in agreement with previous autoradiographic studies (as reviewed in Smith & del Giorgio 2003). This implies that 60-80% of the cells either did not take up any of the substrates assayed and were taking up other substrates, or were not active at the time of sampling.

The seasonal measurement of the uptake activities of different groups allowed us to confirm some patterns in the uptake of LMW-compounds shown in previous studies, such as the low activity of Bacteroidetes in amino acid uptake at trace concentrations (Cottrell & Kirchman 2000). Here, we could show that this was a permanent feature year-round. Cottrell & Kirchman (2000) proposed that this group shows higher affinity for HMW compounds. However, we observed here that Bacteroidetes could also significantly take up other LMW compounds such as ATP, probably forced by phosphorous limitation. It has to be noted, though, that the incorporation of nucleotides requires previous extracellular hydrolysis, which could be regarded as a digestion process equivalent to that of POM use.

The consistently low uptake of glucose by Bacteroidetes and Gammaproteobacteria through the year is also in agreement with the results by Alonso & Pernthaler (2006b), that showed that the majority of glucose incorporating-cells at the lower concentrations of the

substrate were members of Roseobacter and SAR11 lineages in the North Sea, whereas the proportion of Bacteroidetes significantly increased at higher levels of available substrate. Gammaproteobacteria also showed low percentages of active cells incorporating glucose in Delaware Bay (Elifantz et al. 2005). However, this group showed much higher uptake activity of amino acids, in agreement with previous studies (Cottrell & Kirchman 2000), suggesting the preference of this group for amino acids rather than glucose as a carbon source

Seasonal changes in single-cell activity of bacterial groups- The most novel contribution of this work is that we showed seasonal changes in the proportions of active cells of specific groups, and also in their contribution to the total uptake of the compounds. Although changes in bulk bacterial production and respiration have been shown seasonally (Lemée et al. 2002, Reinthaler et al. 2005), changes at the single-cell level of bacterial groups have not been systematically explored at the temporal scale.

Different patterns of seasonal activities were found for different phylogenetic groups of bacteria. For example, very low proportions of Gammaproteobacteria and Bacteroidetes took up amino acids or ATP, respectively, during the nutrient-limited season (summer), while high percentages of cells (up to 60% of Gammaproteobacteria) took them up in spring and winter. In contrast, Roseobacter always showed high percentage of active cells in the uptake of the substrates throughout the year. These results could be related with the high ability of this group to take up the three substrates at all concentration ranges (Alonso & Pernthaler 2006a), and therefore, bacteria of the Roseobacter group seem well prepared to cope with different trophic conditions throughout the year. Our results support the view of Alonso & Pernthaler (2006b), who suggest that Roseobacter could be considered as a single ecophysiological unit. Their year-round low contributions to assemblage structure (Chapter III) despite their high activity suggests that other factors, such as grazing, are controlling the in situ abundances of this group (Pernthaler 2005).

The study of the other alphaproteobacterial group, SAR11, is very relevant, given the numerical dominance in marine regions (Morris et al. 2002), including our system, Blanes Bay (Chapter III). SAR11 was very abundant year-round, but its activity was quite variable throughout the year, and the percentage of active cells of this group was much lower than that of Roseobacter. Substantial uptake of glucose and amino acids by SAR11 was found in the Sargasso Sea (Malmstrom et al. 2004). Because of the overrepresentation of SAR11 in the uptake of glucose and amino acids in their samples, these authors concluded that this group

was a major contributor of bacterial biomass production and carbon flux, and outcompeted other bacterial groups for carbon in the Sargasso Sea. However, the percentage of active SAR11 cells found in the Atlantic Ocean (over 80% in amino acid uptake) was significantly higher than in our study (rarely exceeding 20%), and other studies carried out in coastal areas such as the North Sea (Alonso & Pernthaler 2006b, 10-30%) or the Delaware Bay (Elifantz et al. 2005, about 15%). Indeed, we found that this group contributed equally or less than expected based on their abundance to the total uptake of amino acids or glucose (Fig 4), as was found by Elifantz et al. (2005) in Delaware Bay. These authors suggest that these differences could be due to the eutrophic characteristics of Delaware Bay compared to the Atlantic Ocean, and the selection of different phylotypes with diverse metabolism. However, even if Blanes Bay is a coastal site and not directly comparable to the open ocean, it is quite oligotrophic (yearly average chlorophyll is below 0.5 $\mu g\ l^{-1}$) and, therefore, our results suggest that SAR11 is not always a very active component of the carbon flux under nutrient depleted conditions. We suggest that the contribution of this group to LMW-DOM uptake can be highly variable year-round, at least in coastal ecosystems, although it can reach high contributions in some situations (for example, the sample in February during the post-bloom, Fig. 5). Similarly, Alonso & Pernthaler (2006b) have found that this group increased their activity in bloom conditions, compared to a non-bloom situation.

Taxa substitutions within the bacterial groups could explain the seasonal changes in the uptake activities observed. In a fine-scale phylogenetic analysis, Acinas et al (2004) found four microdiverse clusters of the SAR11 lineage by grouping sequences at a 99% similarity level, which could represent different ecological and functional groups (i.e. ecotypes). However, in an exhaustive study on the bacterial assemblage structure in Blanes Bay (Chapter III) including five clone libraries, no marked seasonality was found for SAR11 sequences. This group showed substantial microdiversity (as in other studies, García-Martinez & Rodríguez-Valera 2000, Brown & Furhman 2005), but high similarities between the sequences from spring (May), summer (August), autumn (October) and winter (February 2003, not sampled for MARCARDFISH). Nevertheless, it will be very interesting to look at whether differences in single-cell activity of other groups at a finer phylogenetic level can unveil distinct patterns of uptake activities within this, and other groups. For this purpose, design of more specific probes targeting groups at a lower phylogenetic level, will be needed.

In summary, our results confirm that a) the activity of pelagic bacteria is substantially more dynamic than their population sizes, and b) different groups of bacteria show very distinct

DOM uptake patterns. Furthermore, we show that uptake activities of specific groups can vary seasonally, particularly during the nutrient-limited season (summer). This could be due to different affinities of their uptake systems or phylotype substitution having different metabolisms. Further research on the seasonal activity of the phylogenetic groups of bacteria will be needed in order to better describe their individual contribution to total marine carbon heterotrophic processing.

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Chapter VI

Effect of natural sunlight on bacterial activity and differential sensitivity of natural bacterioplankton groups in NW Mediterranean coastal waters

L. Alonso-Sáez, J. M. Gasol, T. Lefort, J. Hofer & R. Sommaruga

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Abstract

We studied the effects of natural sunlight on heterotrophic marine bacterioplankton in short-term experiments. We used a single-cell level approach involving flow cytometry combined with physiological probes and microautoradiography to determine sunlight effects on the activity and integrity of the cells. After four hours of sunlight exposure, most bacterial cells maintained membrane integrity and viability as assessed by the simultaneous staining with propidium iodide and SybrGreen I. In contrast, a significant inhibition of heterotrophic bacterial activity was detected, measured by 5-cyano-2,3 ditolyl tetrazolium chloride (CTC) reduction and leucine incorporation. We applied microautoradiography combined with catalyzed reporter deposition-fluorescence in situ hybridization (MARCARDFISH) to test the sensitivity of the different bacterial groups naturally occurring in the NW Mediterranean to sunlight. Members of the Gammaproteobacteria and Bacteroidetes groups appeared to be highly resistant to solar radiation, with small changes in activity after exposure. On the contrary, Alphaproteobacteria bacteria were more sensitive to radiation as measured by the cell-specific incorporation of labeled amino acids, leucine, and ATP. Within Alphaproteobacteria, bacteria belonging to the Roseobacter group showed higher resistance than members of the SAR11cluster. The activity of Roseobacter was stimulated by exposure to photosynthetic available radiation (PAR) compared to the dark treatment. Our results suggest that UV radiation can significantly affect the in situ single-cell activity of bacterioplankton, and that naturally dominating phylogenetic bacterial groups have different sensitivity to natural levels of incident solar radiation.

Introduction

Aquatic bacterioplankton has been shown to be sensitive to sunlight radiation, especially to the shortest-wavelength fraction of UV radiation (see, e.g., Herndl et al. 1993, Kaiser and Herndl 1997, Sommaruga et al. 1997, Sommaruga et al. 1999). Solar UV radiation (UVR, 290-400 nm) causes cellular damage on different cell targets including nucleic acids, proteins, and lipids, which may end up in mutations, cell inactivation and death. Because bacteria are considered to be too small to develop efficient photoprotection against UVR (García-Pichel 1994), and their genetic material comprises a significant portion of their cellular volume

(Jeffrey et al. 1996), bacteria are probably among the most susceptible group to photodamage within the plankton.

A relatively large number of studies have assessed the effect of UVR on bulk metabolic activities of natural bacterioplankton assemblages (see compilation in Jeffrey et al. 2000). Results from field studies on marine bacteria indicate that exposure to natural solar UVR results in a decrease in total bacterial abundance (Müller-Niklas et al. 1995, Pakulski et al. 1998), amino acid uptake (Bailey et al. 1983), exoenzyme activities (Müller-Niklas et al. 1995), oxygen consumption (Pakulski et al. 1998), and a significant inhibition of protein and DNA synthesis (Herndl et al. 1993, Sommaruga et al. 1997). However, the effects of UVR on bacterioplankton have seldom been studied at the single-cell level (but see Manz et al. 1992). Thus, we do not know whether the negative effects of UVR on cellular components or activity are uniformly distributed within the bacterioplankton assemblage.

Furthermore, there is little information about the impact of UVR on bacterioplankton community composition. Previous studies have focused on isolates originated from marine bacterioplankton, bacterioneuston (bacteria isolated from the sea surface microlayer), and from other environments, such as marine snow and sediments. The results concur to show interspecific variability in sensitivity to UVR, and also in the subsequent recovery potential of the isolates (Agogué et al. 2005, Arrieta et al. 2000, Joux et al. 1999). Other significant results are that pigmentation seems not to be directly related to resistance of marine isolates, and that bacteria isolated from the surface microlayer do not show higher resistance to UVR than isolates from subsurface waters (Agogué et al. 2005). However, since isolates poorly represent natural communities (Amann et al. 1995), it is uncertain whether the conclusions of these studies can be extrapolated to natural bacterial assemblages. For example, little is known about the response to UVR of SAR11, the potentially more important oceanic bacteria in terms of abundance (Morris et al. 2002).

We present here results from an experimental study designed to assess the effect of natural sunlight on marine bacterioplankton assemblages at the single-cell level. The study was carried out in a highly UV transparent area of the NW Mediterranean Sea. Microautoradiography was combined with Catalyzed Reporter Deposition-Fluorescence In Situ Hybridization (MARCARDFISH), to report the first evidence of different sensitivities of in situ dominating bacterial groups.

Materials and methods

Study area and sample collection. Sampling was carried out in a shallow (20 m depth) oligotrophic coastal station in the Mediterranean Sea, located ~800 m offshore Blanes, Spain (41°39.90'N, 2°48.03'E, the Blanes Microbial Observatory). Water samples were collected with a Niskin Go-flow bottle (5-liter), filtered through a large 200-μm-mesh-size net and transported under dim light to the lab. On 4 August 2003, we measured vertical profiles of PAR and UV radiation to characterize the underwater radiation climate (Sommaruga et al. 2005). Experiments were done with surface samples (0.5-m depth) on the 5 and 11 August 2003 (Exp. 1 and 3 respectively), and subsurface water (5-m depth) on 7 August 2003 (Exp 2). Water was collected at 0800 h in the morning to avoid exposure of the bacteria to sunlight before the experiments.

Underwater profiles of PAR and UVR in the sampling site and the surface incident UVR during the experiments were measured with a multichannel filter radiometer (PUV-501, Biospherical Instruments Inc., San Diego, CA) equipped with a sensitive pressure transducer and a temperature sensor. The integrated incident UV radiation during the experimental period was estimated using the trapezoidal integration rule, and compared to the daily integral light value.

During the spring experiment (13 April 2005, Exp 4), when only the bacterial intergroup sensitivity was assessed, we estimated the UV cumulative exposure from the incident UV radiation measured at one station placed on the coast, about 20 km away (Malgrat de Mar). For this experiment, water was collected at around 1000 h and further processed in the same way as for the summer experiments.

Experimental design. The experimental design to test the effect of different wavebands was similar to that used in Sommaruga et al. (1997, 2005). Briefly, within 3-4 h after sampling, 100 ml spherical quartz glass bottles were filled with the sample. Bottles were exposed for 4 h, centered at solar noon, to the full sunlight spectrum, the full spectrum minus UVR (i.e. PAR only), the full spectrum minus UVB radiation (i.e. PAR+UVA) or kept in the dark. Four replicates were used for each treatment. Quartz bottles with no additional spectral filters were used for full-spectrum treatments. Bottles kept in the dark were wrapped with 3 layers of aluminum foil and exposed apart from the others inside a black plastic bag to avoid reflection. Bottles were wrapped with one layer of a vinyl chloride foil (CI Kasei Co., Tokyo, Japan, 50% transmittance at 405 nm) for PAR-only treatment, or one layer of Mylar-D (150 μm thickness,

50% transmission at 325 nm) for PAR+UVA treatment. In the spring experiment (Exp 4), Courtguard foils (50% transmittance at 383 nm) were used instead of vinyl chloride for PAR-only treatments. The transmittance of the different foils was regularly checked in a double beam spectrophotometer (Uvikon 923).

Bottles were incubated 4 cm under the surface inside a large water bath (200-liter) with walls painted in black and circulating seawater to maintain in situ temperatures within 2°C. After sunlight exposure, subsamples were taken to immediately measure different parameters of bacterial activity.

During Exp. 3, we assessed the effect of algae removal on bacterioplankton activity and the potential for bacterial recovery after radiation. In order to separate eukaryotic phytoplankton and cyanobacteria from bacterioplankton, we filtered the sample through glass fiber filters of 142 mm diameter (AP15-14250 Millipore) with a peristaltic pump. The efficiency of phytoplankton removal after the filtration was checked by flow cytometry. We found that, on average, 91% of bacteria crossed the AP15 filters, while only 10% of *Synechococcus* cells, 37% of *Prochlorococcus* cells, and 20% of phototrophic picoeukaryotes went through the filter.

In order to assess bacterial recovery after exposure, samples from the different radiation treatments in Exp. 3 were placed in the darkness for 24 h, and different parameters, such as bacterial abundance, CTC reduction, and the abundance of high nucleic acid (HNA) content cells (see below), were again measured after the dark period.

Bacterial abundance and heterotrophic production. Samples for bacterial abundance determinations (1.6 ml) were preserved with 1% paraformaldehyde and 0.05% glutaraldehyde (final concentrations), deep frozen in liquid nitrogen and stored at –80ºC. Cell counts were done with a FACSCalibur flow cytometer (Becton Dickinson) after staining with SYTO13 (Gasol & del Giorgio, 2000). Regions were established on the SSC versus FL1 (green fluorescence) plot in order to discriminate cells with HNA content from cells with low nucleic acid (LNA) content, and cell abundance was determined for each subgroup.

Bacterial heterotrophic production was determined using the [^{3}H]-leucine incorporation method (Kirchman et al. 1985), modified as described by Smith & Azam (1992). We used four replicates for each treatment in Exp. 1 and 2, and triplicates in Exp. 3. For each sample, three aliquots (1.2 ml) plus one trichloroacetic acid (TCA)-killed control were incubated with [^{3}H]-leucine (40 nM final conc.) for 1.5 h at in situ temperature.

CTC- Two 0.5 ml sample aliquots were spiked with CTC (5 cyano-2,3 ditolyl tetrazolium chloride, Polysciences, 5 mM final conc.) and incubated for 90 min in the dark, at in situ temperature. The samples were immediately run through the FACSCalibur flow cytometer. An additional 0.5 ml aliquot fixed with paraformaldehyde was used as a background control of CTC fluorescence on dead samples. CTC-formazan is excited by wavelengths between 460 and 530 nm and has bright red fluorescence. CTC+ particles were those that showed red fluorescence (above 630 nm, FL3 in our instrument), higher than the background fluorescence level (see, e.g., Sieracki et al. 1999, del Giorgio et al. 1997). A dual plot of 90° light scatter and red fluorescence was used to separate CTC+ cells from background noise. The FL2 (orange fluorescence) vs FL3 (red fluorescence) plot was used to differentiate the populations of photosynthetic microbes (*Synechococcus*, *Prochlorococcus,* and picoeukaryotes) from the CTC+ particles.

Nucleic acid double staining (NADS). SYBR Green I (Molecular Probes, Eugene, Oreg.) and propidium iodide (PI, Sigma Chemical Co.) were used for the double staining of nucleic acids as described elsewhere (Grégori et al. 2001). Samples were stained with 1:10000 (vol/vol) SYBR Green I and 10 μg ml^{-1} PI commercial solutions. Flow cytometric analysis was done after 20 min of incubation in the dark. SYBR Green I and PI fluoresce green (maximum at 521 nm) and red (maximum at 617 nm), respectively, when associated to nucleic acids and excited with a 488 nm argon laser. A plot of red vs. green fluorescence allowed differentiation of cells considered "live" (i.e., with undamaged membranes), from those considered "dead" (with damaged or compromised membranes). We tested whether this protocol was sensitive enough to detect membrane-level damage as induced by UVR, and found that after 15 min exposure of a coastal sample, initially having 90% "live" cells, at 20 cm from a UVC light source (PHILIPS TUV30W/G30 T8), most cells (95%) were detected as "dead".

Catalyzed Reporter Deposition (CARD)-Fluorescence In Situ Hybridization. We basically followed the protocol described by Pernthaler et al. (2002). Several horseradish peroxidase (HRP)-probes were used to characterize the composition of the microbial community in the original water samples: Eub 338-II-III (target most *Eubacteria* [Amann et al. 1990, Daims et al. 1999]), Alf968 (targets most Alphaproteobacteria [Neef 1997]), Gam42a (targets most Gammaproteobacteria [Manz et al. 1992]), CF319 (targets many groups belonging to the Bacteroidetes group [Manz et al. 1996]), Ros537 (targets members of the *Roseobacter-Sulfitobacter-Silicibacter* group [Eilers et al. 2000]) and SAR11-441R (targets the SAR11

cluster [Morris et al. 2002]). The Eub antisense probe Non338 (Wallner et al. 1993) was used as a negative control. All probes were purchased from biomers.net (Ulm, Germany). Hybridizations were carried out at 35ºC overnight and specific hybridization conditions were established by addition of formamide to the hybridization buffers (20% formamide for Non338 probe, 45% formamide for Alf968 and SAR11-441R probes, and 55% for the other probes). Counterstaining of CARD-FISH preparations was done with 4,6-diamidino-2-phenylindole (DAPI, 1 μg ml^{-1}). Between 500 and 1000 DAPI-positive-cells were counted in a minimum of 10 fields.

MARCARDFISH. We followed the protocol described by Alonso & Pernthaler (2005). Briefly, after sunlight exposure, 20 ml of water from duplicate treatments were incubated for 4 h at in situ temperature with the following tritiated substrates (0.5 nM final. conc.): [^{3}H]mixture of amino acids (Amersham TRK440) for the summer experiment (Exp 1), and [^{3}H]leucine (Amersham TRK754) and [^{3}H]ATP (Amersham TRK747) for the spring experiment (Exp 4). Single samples (for each compound and treatment) were killed with formaldehyde before the addition of the tritiated compounds and were used as controls. After the incubation, samples were fixed overnight with formaldehyde (1.8%) at 4ºC, and gently filtered on 0.2-μm polycarbonate filters (Millipore, GTTP, 25 mm diameter). The filters were then hybridized following the CARDFISH protocol, and subsequently glued onto glass slides with an epoxy adhesive (UHU plus; UHU GmbH, Bühl, Germany). For microautoradiography, the slides were embedded in 46ºC tempered photographic emulsion (KODAK NTB-2) containing 0.1% agarose (gel strength 1%, >1 kg cm^{-2}) in a dark room. Slides were placed on an ice-cold metal bar for about 5 min to allow the emulsion to solidify and subsequently placed inside black boxes at 4 ºC until development. The optimal exposure time was determined for each experiment and compound, and resulted in 7 days for the mixture of amino acids (Exp. 1), 4 days for ATP, and 10 days for leucine (both in Exp. 4). For development, we submerged the exposed slides for 3 min in the developer (KODAK D19), 30 s of rinsing with distilled water, and 3 min in fixer (KODAK Tmax) followed by 5 min of washing with tap water. Slides were then dried in a dessicator overnight, stained with DAPI (1 μg ml^{-1}), and counted in an Olympus BX61 epifluorescence microscope.

Results

Effects of sunlight on bulk and single-cell bacterial activity- We performed three experiments to assess the impact of natural sunlight on bulk bacterial heterotrophic production (BHP) and single-cell bacterial activity during August 2003 (Table 1). During the course of the 6 days from Exp.1 to Exp. 3, a cold water mass entered the system resulting in a strong water column stratification. This intrusion was followed by changes in the in situ BHP and bacterial assemblage composition in surface waters (Table 1).

Table 1- Physical data, bacterial abundance (BA), leucine incorporation rates (LIR) and bacterial assemblage structure described as percentages of hybridized cells with specific probes by CARDFISH, in the in situ starting samples of each experiment. ΔTemp is the difference between the temperature at the surface (the depth sampled) and the bottom of the water column (20 m) to describe the magnitude of water column stratification. UV cum. is the cumulative exposure during the experiment. Eub: Eubacteria (Eub338-II-III); Alph: Alphaproteobacteria (Alf968), Ros: *Roseobacter* (Ros537); SAR11: SAR11 cluster (SAR11-441R); Gam: Gammaproteobacteria (Gam42a), CFB: Bacteroidetes (CF319a). [a] Integrated values during the experiments determined with the multichannel filter radiometer, [b] Estimated from the irradiance values collected at the Malgrat de Mar meteorological station (see Material and methods).

Experiment	Date	Depth	Temp	ΔTemp	UV cum.	BA	LIR
		(m)	*(°C)*	*(°C)*	*KJ m^{-2}*	*cell ml^{-1}*	*pM Leu h^{-1}*
Exp.1	5/08/03	0.5	24	1	9.5[a]	9.2 10^5	41.3
Exp. 2	7/08/03	5	23.6	5	9[a]	1.3 10^6	14.9
Exp. 3	11/08/03	0.5	25.4	9	9.2[a]	1.1 10^6	38.2
Exp. 4	13/04/05	0.5	13	0	5.4[b]	7.3 10^5	39.0

Experiment	Date	Depth	Eub	Alph	Ros	SAR11	Gam	CFB
		(m)	*(% probe positive with respect to DAPI)*					
Exp.1	5/08/03	0.5	79	25	2	30	10	10
Exp. 2	7/08/03	5	84	27	11	24	17	9
Exp. 3	11/08/03	0.5	86	28	11	24	26	20
Exp. 4	13/04/05	0.5	84	39	10	22	10	35

Exposure to sunlight generally inhibited bulk BHP compared to the dark treatment in all the experiments. However, we measured some variability in the response of bacteria to the different wavebands (Table 2). As an example, BHP inhibition after PAR+UVA exposure was less marked compared to the other treatments in Exp. 2 (i.e. PAR-only or full sunlight),

although the difference was not significant (Tukey's test, $p>0.05$). Remarkably, BHP was unaffected by full sunlight exposure in Exp. 3 (whole water fraction, Table 2), although it was significantly inhibited by PAR+UVA treatment.

When assessed at the single-cell level, UVR consistently had a significant negative effect on the percentage of actively respiring cells (CTC+ cells, Fig 1). The percentage of high nucleic-acid-containing cells (%HNA) was also negatively affected by UVR in Exp.1 and 2, but we did not find significant differences in Exp. 3 (Tukey's test, $p>0.05$, Fig 1). The abundance of CTC+ and HNA cells after sunlight exposure decreased by an average (±SE) of 54±10 and 25±4% respectively compared to the dark treatment, while the total bacterial abundance only decreased by 18±4% (Table 3).

Table 2- Bacterial heterotrophic production measured as leucine incorporation rates in the three experiments and in each treatment. Values are expressed as percentage of the dark treatment (± SE). Letters refers to results with a post hoc Tukey's test ($p<0.05$). Different letters indicate significantly different treatment effects. The "filtered water" in the last column refers to water filtered through AP15 (bacterial fraction, see methods).

	5 August 2003		**7 August 2003**		**11 August 2003**			
	Whole water		Whole water		Whole water		Filtered water	
DARK	100	*a*	100	*a*	100	*a*	100	*a*
PAR	88 ± 14	*a*	17 ± 7	*b*	61 ± 12	*ab*	58 ± 5	*b*
PAR+UVA	66 ± 12	*a*	40 ± 4	*b*	46 ± 11	*b*	45 ± 9	*b*
PAR+UVR	52 ± 11	*a*	21 ± 11	*b*	106 ± 4	*a*	79 ± 3	*c*

Effects of sunlight on cell membrane integrity and recovery experiments- The effect of UVR on cell membrane integrity was assessed by the nucleic acid double staining protocol (NADS). We did not detect significant changes in the percentage of cells with intact membranes ("live" cells) in all treatments (Fig 1). The potential recovery of cells after exposure was assessed in Exp. 3. We found that total, CTC, and HNA-cells abundance recovered after 24 h in the darkness, reaching similar values in the samples that had been exposed to UVR than in the samples kept in the dark (Table 3).

Effects of primary producers on bacterial sensitivity to UVR- We tested whether the removal of phytoplankton was a relevant factor influencing bacterial sensitivity to UVR in Exp. 3. The percentage of inhibition of BHP was similar in both samples (with and without primary producers, Table 2). Similarly, the general effect of different solar wavebands on the percentage of "live" (as measured by the NADS protocol) and "active" cells (HNA and CTC+ cells) was unaffected by the removal of phytoplankton (Fig. 1).

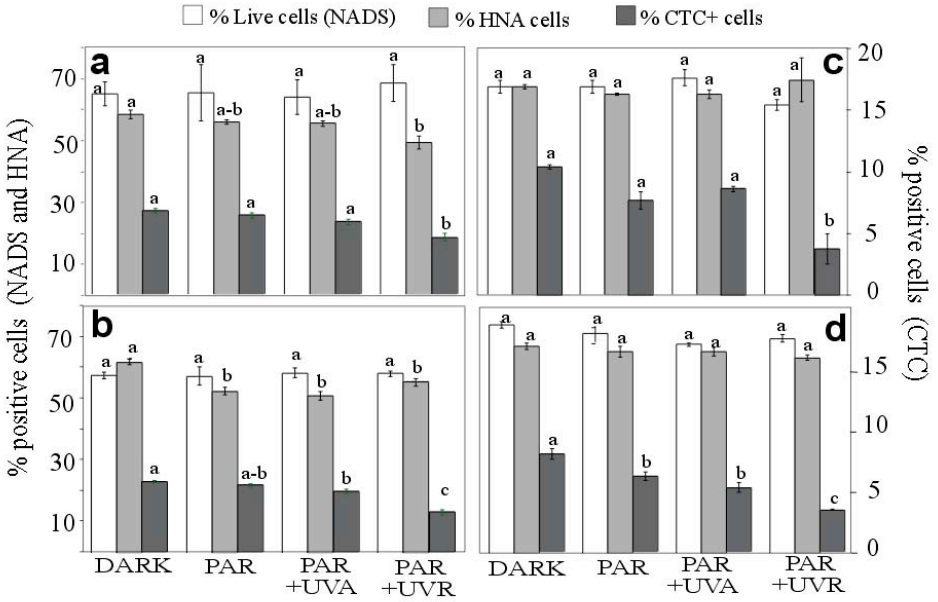

Fig 1- Percentage of active cells measured as NADS Green-positive cells ("live" cells), high nucleic acid content cells (HNA), and CTC positive cells (cells actively respiring) in (a) Exp. 1 (5 August 2003), (b) Exp. 2 (7 August 2003), (c) Exp. 3 (11 August 2003) with unfiltered water, and (d) Exp. 3 with filtered water containing mostly the heterotrophic bacterial fraction.

Differential sensitivity of the dominant bacterial groups- Two experiments were conducted in different seasons to study the sensitivity of different phylogenetic groups of bacteria under relatively higher and lower levels of radiation (Exp. 1 in summer and Exp. 4 in spring, respectively). In the summer experiment, we found a significant reduction in activity of the broad phylogenetic group Alphaproteobacteria (70% inhibition in percentage of active cells in the uptake of amino acids) after sunlight exposure, while no significant reduction was observed in the activity of Gammaproteobacteria or Bacteroidetes (Fig. 2). However, the

results with the Bacteroidetes group were not conclusive, since members of this group did not significantly take up the amino acid mixture.

Table 3- Abundance of total bacteria, HNA bacteria, and CTC+ bacteria after 4 h exposure to the different treatments, in the three experiments performed in August 2003. The values corresponding to the recovery experiment (i.e., 24 h incubation in the dark), carried out with water exposed to the different treatments from Exp. 3 (11 August 2003), are also included.

	Total cells		CTC+ cells		HNA cells	
	10^6 cells ml^{-1} (±SE)		10^6 cells ml^{-1} (±SE)		10^6 cells ml^{-1} (±SE)	
Sample	**Treatment**	**Recovery**	**Treatment**	**Recovery**	**Treatment**	**Recovery**
5 August 2003						
DARK	1.49 (±0.02)	-	0.87 (±0.01)	-	0.10 (±0.003)	-
PAR	1.40 (±0.01)	-	0.78 (±0.02)	-	0.09 (±0.002)	-
PAR+UVA	1.39 (±0.005)	-	0.77 (±0.01)	-	0.08 (±0.003)	-
PAR+UVR	1.32 (±0.03)	-	0.65 (±0.03)	-	0.06 (±0.004)	-
7 August 2003						
DARK	1.89 (±0.05)	-	1.18 (±0.04)	-	0.11 (±0.004)	-
PAR	1.58 (±0.02)	-	0.83 (±0.02	-	0.09 (±0.001)	-
PAR+UVA	1.56 (±0.02)	-	0.80 (±0.02)	-	0.08 (±0.002)	-
PAR+UVR	1.47 (±0.01)	-	0.82 (±0.02)	-	0.05 (±0.004)	-
11 August 2003						
DARK	2.44 (±0.03)	3.28 (±0.13)	1.66 (±0.04)	2.49 (±0.14)	0.26 (±0.005)	0.44 (±0.03)
PAR	2.21 (±0.03)	3.03 (±0.85)	1.45 (±0.02)	2.19 (±0.07)	0.17 (±0.01)	0.37 (±0.02)
PAR+UVA	2.16 (±0.02)	3.43 (±0.13)	1.42 (±0.04)	2.60 (±0.14)	0.19 (±0.005)	0.53 (±0.05)
PAR+UVR	1.94 (±0.17)	4.14 (±0.55)	1.34 (±0.02)	3.29 (±0.64)	0.08 (±0.03)	0.44 (±0.06)

In the spring experiment, we tested the effect of solar radiation (including UVA and UVB separately) on the uptake of two different substrates (leucine and ATP, Fig. 3). The activity of Alphaproteobacteria was significantly inhibited by both UVA and UVB wavebands (55% and 44% inhibition in percentage of active cells taking up leucine and ATP, respectively, after full sunlight exposure). Gammaproteobacteria showed higher inhibition at this time compared to the summer experiment, but the inhibition was only attributable to the UVB waveband (30% and 46% inhibition in percentage of active cells in the uptake of leucine and ATP, respectively,

after full sunlight exposure). The Bacteroidetes showed high resistance to UVB radiation, being only partially affected by solar radiation for ATP uptake (Fig. 3a).

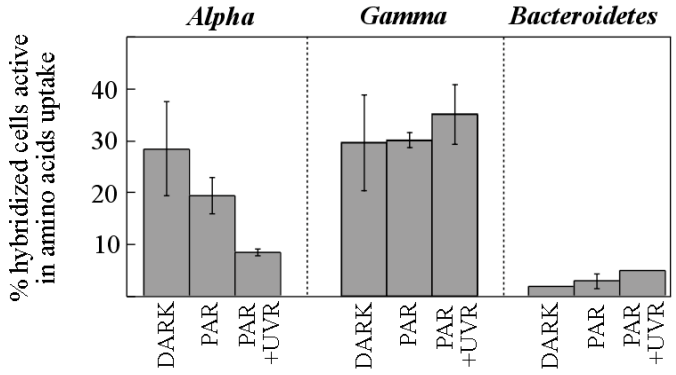

Fig 2- Percentage of positively hybridized cells with probes for Alphaproteobacteria (Alpha, ALF968), Gammaproteobacteria (Gamma, GAM42a), and Bacteroidetes (CF319), taking up a mixture of ^{3}H-labeled amino acids (average ± SD) as measured by MARCARDFISH during Exp. 1 (summer experiment).

During the spring experiment, we also assessed the sensitivity of two more specific groups: SAR11 and Roseobacter. SAR11 were highly inhibited after full sunlight exposure (48% and 67% inhibition in percentage of active cells uptaking leucine and ATP, respectively) although the response to the different wavebands was very different with the two different substrates assessed. Exposure to PAR inhibited the percentage of active cells in leucine uptake, while ATP uptake was only inhibited after full sunlight exposure.

Roseobacter showed lower sensitivity to UVR than the SAR11, and was only significantly inhibited after full sunlight exposure (34% and 49% inhibition in percentage of active cells in the uptake of leucine and ATP, respectively). Remarkably, we found that PAR significantly stimulated the activity of this group compared to the dark treatment (Fig 3b).

Discussion

Solar UVR has the potential to negatively affect bacterioplankton in environments such as the surface layers of the open ocean, where diurnal stratification of the water column is common (Doney et al. 1995), or in highly transparent shallow coastal areas, as our sampling

site (Blanes Bay). At the time of the summer experiments, UVR penetrated the whole water column with 1% of incident UVR at 320 nm reaching the bottom (Sommaruga et al. 2005).

We performed an approach different from that used in previous studies elsewhere, by focusing on the effects of sunlight at the single-cell level, including the differential sensitivity of the bacterial groups that dominate in situ. The most remarkable results found are that the effect of 4 h exposure to UVR did not gravely damage the integrity of the cells but inhibited most markedly the physiologically active cells, and that the inhibitory effect was not the same for all bacterial groups. For example, Alphaproteobacteria (and SAR11 cluster within this group) showed higher sensitivity than Gammaproteobacteria and Bacteroidetes. Considering that Alphaproteobacteria dominate the bacterial assemblage in Blanes Bay all year round (Chapter III), solar radiation could have a strong effect on C-cycling in this environment.

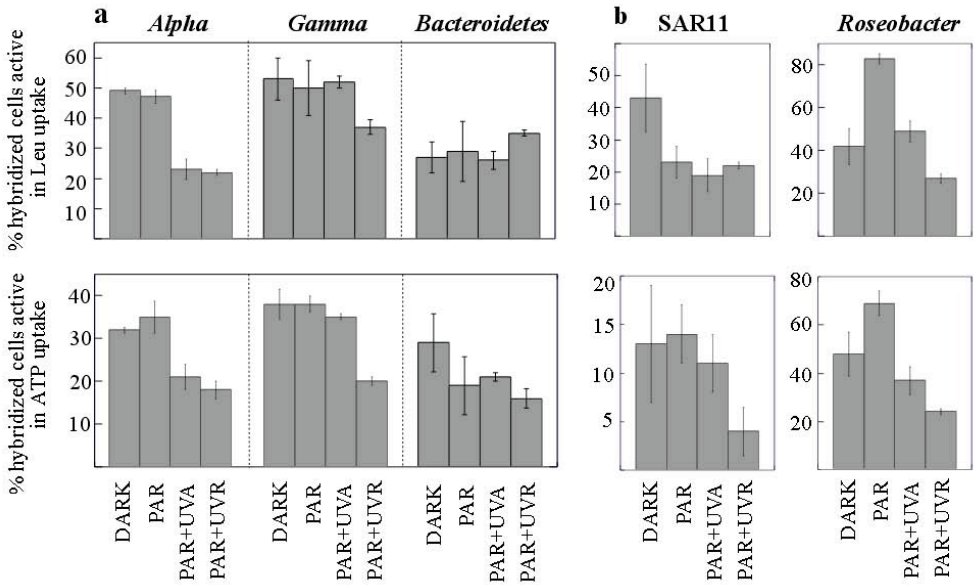

Fig 3- Percentage of positively hybridized cells with probes for: a) Alphaproteobacteria (Alpha, Alf968), Gammaproteobacteria (Gamma, Gam42a) and Bacteroidetes (CF319) groups; and, b) Roseobacter (Ros537) and SAR11 (SAR11-441R) groups, taking up ^{3}H-labeled leucine (upper panels) or ATP (lower panels, averages ± SD) as measured by MARCARDFISH during Exp. 4 (spring experiment).

Effects of UVR on bulk bacterial heterotrophic production- Similarly to previous studies in other marine areas, our experiments provided variable results regarding the extent bulk BHP was affected by different wavebands of the solar spectrum. The main inhibitory effect on bacterial heterotrophic production (BHP) has been assigned to UVA radiation (Sommaruga et al. 1997) or UVB radiation (Aas et al. 1996, Müller-Niklas et al. 1995), but PAR has also been shown to significantly affect BHP (Aas et al. 1996, Sommaruga et al. 1997).

Differences in spectral sensitivity of bacteria, between and within experiments, have been assigned to environmental factors such as nutrient status, dissolved organic matter concentration, presence and composition of the primary producers, and levels of incident solar radiation (Jeffrey et al. 2000). We specifically tested the effect of excluding most primary producers from our samples and, in contrast to other studies (e.g., Aas et al. 1996), we did not find a clear change on bacterial sensitivity at neither bulk nor single-cell level. The effect of algae on bacterioplankton activity could be related to the composition and sensitivity of the phytoplankton assemblage. High sensitivity to UVR can induce cell lysis (Llabrés & Agustí 2006), which would produce a short-term increase in dissolved organic carbon and stimulate the bacterial assemblage. In our summer experiments, the total phytoplankton assemblage was dominated by picophytoplankton (78% of total chlorophyll crossed a 3-μm-filter), mainly by *Synechococcus*. This population has been shown to be resistent to UVR (Llabrés & Agustí 2006, Sommaruga et al. 2005), in agreement with the lack of effect of phytoplankters on bacterioplankton.

Since the methodology used for the three experiments was the same, the variability that we observed was probably due to environmental changes. Indeed, we observed the development of a strong stratification of the water column between the first and the last summer experiment, probably related to an intrusion of offshore deep water from a nearby submarine canyon. Water from the canyon periodically enters this site (Masó & Tintoré 1991, Olivar et al. 1998) and can be enriched with particulate organic matter, since this submarine structure can act as a trap for suspended particles (Granata et al. 1999). Unfortunately, we did not sample particulate or dissolved organic matter (DOM) during these experiments, but changes in DOM supply can affect the response of the bacterial assemblage to different wavebands. For example, UVB radiation has been shown to increase the lability of DOM, particularly of organic matter originating from deep waters (Benner & Biddanda 1998). This could be related to the significant increase in leucine uptake after full sunlight exposure in Exp. 3. The shift in bacterial assemblage composition after stratification of the water column was also probably

related to the different BHP sensitivity, since changes in assemblage structure can determine the sensitivity of a given community to solar radiation.

Effects of sunlight on bacterial assemblage at the single-cell level- In contrast to bulk measurements, UVR showed a consistent negative effect on single-cell activity measurements. These measurements assessed changes in: (1) CTC+ cells, which are usually regarded as highly active cells (Sherr et al. 1999), and (2) high nucleic-acid-containing cells (HNA), which are regarded as active cells, at least in coastal environments (Gasol et al. 1999, Lebaron et al. 2002, Servais et al. 2003). If we assume that the HNA and CTC positive cells represent the "medium-to-high" active cell fractions, then UVR mainly affected the physiologically active cells. This was evident from the higher loss in the abundance of HNA and CTC positive cells after full sunlight exposure than in the total bacterial counts (Table 3). In agreement with this idea, Arrieta et al. (2000), working with isolates, found that the cells that were dividing faster (those taking up thymidine), were also those most inhibited by solar radiation.

We further assessed the effect of UVR on cell membrane integrity using the nucleic acid double staining protocol, which uses a combination of propidium iodide and SYBRGreen I (Grégori et al. 2001). Propidium iodide stains nucleic acids in red, but only enters cells with compromised membranes and, thus, is thought to be an indicator of "dead" cells (Williams et al. 1998). We found that most bacteria maintained membrane integrity after exposure to natural sunlight (Fig. 1). Maranger et al. (2002) used another exclusion stain (TOPRO-1) with lake samples and found a significant increase of damaged cells after UVR exposure, but after several days of exposure.

The lack of structural damage after UVB exposure could be related to the subsequent ability of the cells to recover (Table 3). Similarly, rapid recovery of the cells after exposure to UVB radiation has been shown for marine natural assemblages (Kaiser & Herndl 1997) as well as for bacterial isolates (Arrieta et al. 2000).

Differential sensitivity of in situ dominating groups- Previous studies have reported large differences in the sensitivity to UVB radiation and in the mechanisms of recovery from previous UV stress, for different marine isolates. Clear differences in the inhibition of thymidine and leucine incorporation have been shown among five isolates of the genus *Vibrio* (approx. 40%) compared to two Gram+ isolates (>80%, [Arrieta et al. 2000]). More recently, large interspecific differences were found among 90 marine isolates from the same oceanographic area of our study (Agogué et al. 2005). However, the results from these studies

may not be representative of what occurs in situ, since bacterial isolates do not usually resemble natural bacterial communities (Amann et al. 1995).

Winter et al. (2001) used a different approach to test the effect of UVR on the bacterial assemblage at the DNA, and also RNA level (to discriminate the metabolically active cells), using denaturing gradient gel electrophoresis (DGGE). These authors performed dilution cultures, preincubated in the dark for 20-30 h before starting the experiment, and compared the bacterioplankton community prior and after UVR exposure (up to two days). They concluded that UVR had little effect on bacterial assemblage structure in the North Sea. However, similarly to the isolation techniques, dilution cultures can also introduce a bias in the resulting composition of the bacterial community (Massana et al. 2001), and may not necessarily represent the dominant bacterial groups in situ.

We used the MARCARDFISH method to assess the differential sensitivity of bacterial groups as they occur in situ. We found some variability between seasons (spring and summer), probably due to seasonal changes in species composition of these broad phylogenetic groups. Selection for photoresistant species could occur in the periods of higher solar intensity, as it seemed to be the case in the Gammaproteobacteria group, which showed lower sensitivity to UVR in summer (Fig. 2, 3a).

We also found some differences in the response of the groups analyzed by the inhibition in the uptake of different tracers (leucine versus ATP). Solar UVR can mediate the destruction of the ectoenzymes and uptake systems (Herndl 1997), and the differences we found were probably related to differential damage of the uptake systems for the different substrates. Despite these differences, the general pattern (higher sensitivity to UVR of Alpha- than Gammaproteobacteria and Bacteroidetes) is in agreement with the results of Agogué et al. (2005), working with bacterioneuston isolates from the Mediterranean Sea. These authors found that 43% of Gammaproteobacteria isolates showed high resistance to UV, while only 14% of Alphaproteobacteria were highly resistant isolates. However, opposite to our results, a low percentage of isolates belonging to the Bacteroidetes group were UV-resistant in their study. The higher resistance of the Gammaproteobacteria group is also in agreement with results on the bacterial composition of the sea surface microlayer, which is dominated by two groups of *Gammaproteobacteria* (*Vibrio* and *Pseudoalteromonas* spp., [Franklin et al. 2005]). This suggests that members of this group could be resistant to the high incident levels of solar UVR, typically found in this habitat.

We also found differential sensitivities at higher level of phylogenetic resolution (SAR11 and Roseobacter clusters*)*. Both groups are dominant subgroups of Alphaproteobacteria in this environment, accounting for 22% of total DAPI-positive cells (annual average, Chapter III). The higher sensitivity of SAR11 to UVR could be related to the high A+T content (69%) reported for the genome of a representative member of this group (*Pelagibacter ubique* [Giovanonni et al. 2005]). However, it is remarkable that Agogué et al. (2005) did not find a correlation between the degree of resistance of different bacterial isolates and their G+C content, as had been postulated by Singer & Ames (1970).

Whereas the shortest wavelength of solar radiation (UVR) inhibits bacterial activity, natural sunlight can also act as an energy source for some bacteria. The remarkable enhancement in the activity of the Roseobacter group after exposure to PAR, compared to the dark (Fig. 3b), could be related with the ability of these bacteria to derive energy from light with the use of bacteriochlorophyll-a (BChl a) (Shiba 1991, Shiba et al. 1979). It is well known that some cultured members of the Roseobacter group are aerobic anoxygenic phototrophs (AAPs). Recent reports of abundant AAPs (Kolber et al. 2000), and the discovery of proteorhodopsin (Béjà et al. 2000), suggest that non-chlorophyll a dependent phototrophy may be widespread among marine bacteria. However, Schwalbach et al. (Schwalbach et al. 2005) performed light manipulation experiments with bacterial communities from the California coast and found that members of broad phylogenetic groups typical of marine waters, including Roseobacter, did not respond to light exclusion. These authors used a fingerprint technique (Automated Ribosomal Intergenic Spacer Analysis, ARISA) to determine changes in bacterial assemblage structure after 10 days of incubation under different light regimes. Clearly, this approach is significantly different from our in situ activity measurements (MARCARDFISH), and could explain the differences between both studies.

The large differences in sensitivity found, even among broad phylogenetic groups (as Alpha-, Gammaproteobacteria and Bacteroidetes), can act as a significant determinant of bacterial assemblage structure at high UVR doses. During our summer experiments, the rapid stratification of the water column (within days) was followed by significant changes in bacterial assemblage structure. The groups Gammaproteobacteria and Bacteroidetes increased significantly their proportions in surface waters after stratification (Table 1). Interestingly, these two groups had shown high resistance to UVR in the experiments, suggesting that a selection for UV-resistant groups could occur.

The differential sensitivity to UVR of in situ dominating bacterial groups could be a relevant factor determining their relative biogeochemical role in UV sensitive oceanic regions. Different groups of bacteria have shown remarkable different activities and patterns of DOM utilization (Cottrell & Kirchman 2000). Alphaproteobacteria seem to be responsible for a large part of low-molecular weight DOM uptake, while Bacteroidetes seem to be more specialized in high-molecular weight DOM uptake (Cottrell & Kirchman 2000). The reported higher UVR inactivation of Alphaproteobacteria could lead to temporary shifts in the dominant pathways of DOM use by bacteria, as well as decreases in total DOM uptake and processing, given the dominance of Alphaproteobacteria, and specifically SAR11, in oceanic waters (Morris et al. 2002). However, several other aspects not assessed in our study, such as the effects of UVR on DOM quality or on the release of DOM by primary producers (e.g., Herndl 1997), must also be considered in order to draw a comprehensive picture of the role of UVR on the bacterial carbon processing.

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Discussion

Synthesis of results and general discussion

The goal of this thesis was to relate in situ marine bacterial diversity and their function in carbon cycling. In order to assess such a question, we studied bacterial diversity (**Chapters II and III**) and function (**Chapters I and IV**) in two contrasting marine systems: the coastal NW Mediterranean and the open North Atlantic. Linkages were initially explored using the correlative approach between the multivariate bacterial assemblage structure and carbon metabolism parameters (**Chapters II and IV**), and later through the single-cell technique MARFISH in two coastal studies (**Chapters V and VI**).

Other approaches to analyze linkages between bacterial diversity and function have been used in previous studies, including experimental setups, such as transplant experiments. In such experiments, the effect of environmental conditions on bacterial assemblages and their carbon processing, and whether similar bacterial assemblages perform similar or different carbon metabolism patterns growing in different media are assessed (Langenheder et al. 2005, 2006). Studies exploring the linkage between bacterial diversity and function under in situ conditions (not experimentally) in the sea are scarcer and focused on simple correlations between bacterial richness and bacterial production or respiration (Reinthaler et al. 2005, Winter et al. 2005). However, the linkage between bacterial assemblage structure (not richness) and environmental and biotic conditions has also been recently explored by multivariate techniques, such as multiple regression analysis in marine waters (Fuhrman et al. 2006).

Among the single-cell techniques, approaches different to MARFISH have also been used in several studies, such as flow cytometry cell sorting combined with phylogenetic analysis (Zubkov et al. 2001, Fuchs et al. 2005). Finally, the study of genomics of representative marine bacteria (Giovanonni et al. 2005), or recent advances such as metagenomics, will provide new insights into the carbon metabolism of marine bacterial groups.

Patterns of bacterial carbon processing: seasonal versus spatial gradients

The environmental studies carried out in this thesis included the measurement of two of the most important ecological roles of bacteria in carbon cycling, which are the synthesis of new biomass (bacterial heterotrophic production, BHP) and the remineralization of organic carbon (bacterial respiration, BR). Since the studies were conducted along spatial or temporal

gradients of productivity, a wide range of values of BHP or BR were found (as reviewed in Table 1).

Table 1- Summary of carbon metabolism parameters measured in the studies carried out in the North Atlantic (Chapter I) and the coastal Mediterranean (Chapter IV) . BBM Bacterial biomasa, BHP Bacterial heterotrophic production (using empirical conversión factors), Emp CF_c: Empirical conversion factors, BR: bacterial respiration, BGE: Bacterial growth efficiency. Ranges and averages (±SE) are shown for different seasons (Win, winter, Spr: Spring, Sum: summer, and Aut: Autumn) in the NW Mediterranean (Blanes Bay), and upwelling (upw), coastal (coast) and offshore (off) stations in the NE Atlantic.

Sampling site		**BBM** ($\mu g\ C\ l^{-1}$)		**BHP** ($\mu g\ C\ l^{-1}\ d^{-1}$)		**Emp. CF** (kg C mol Leu^{-1})		**BR** ($\mu g\ C\ l^{-1}\ d^{-1}$)		**BGE** (%)	
		Range	*Avg ± SE*	*Range*	*Avg ± SE*	*Range*	*Avg ± SE*	*Range*	*Avg ± SE*	*Range*	*Avg ± SE*
NW Mediterranean (coastal)	Win	14-29	21 ± 2	0.23-2.62	1.4 ± 0.5	1.65-3.62	2.61 ± 0.55	4.3-36.7	16.7± 7.0	3-27	13 ± 7
	Spr	19-28	24 ± 3	0.23-5.80	2.3 ± 1.7	0.98-2.25	1.52 ± 0.38	7.5-47.9	26.9± 11.7	5-42	18 ± 12
	Sum	8-19	14 ± 3	0.24-1.83	0.9 ± 0.5	1.25-1.35	1.31 ± 0.03	20.7-28.6	24.5± 2.3	8-25	15 ± 5
	Aut	15-20	18 ± 2	0.62-0.74	0.7 ± 0.1	0.98-1.28	1.13 ± 0.15	28.8	28.8	6	6
NE Atlantic (open waters)	Upw	11-68	37 ± 13	0.26-2.15	1.1 ± 0.5	0.25-1.29	0.67 ± 0.23	10.0-30.8	17.5± 4.6	17-56	30 ± 11
	Coast	10-16	13 ± 3	0.08-0.14	0.1 ± 0.03	0.21	0.21 ± 0	9.7-10.6	10.1± 0.4	11-16	13 ± 2
	Off	5-16	9 ± 2	0.03-0.1	0.3 ± 0.1	0.02-0.17	0.11 ± 0.03	2.2-22.2	13.3± 4.3	1-11	7 ± 2

For the measurement of BHP a large effort was devoted at the determination of carbon-to-leucine conversion factors (CFs), which can largely affect bacterial production estimates, especially in oligotrophic waters (**Chapter I**). Empirical CFs were measured using dilution cultures over 3-5 days, in which we determined the increase in biomass and the leucine incorporated. Secondly, we measured bacterial respiration and growth efficiency, which are the two least constrained parameters in current models of marine bacterial carbon flux. Indeed, estimates of BR or BGE had never before been reported for the region of the North Atlantic studied, nor for the coastal Mediterranean at the seasonal scale (**Chapters I and IV**).

Empirical CFs varied up to 64-fold along two transects from the Cape Blanc upwelling offshore (range 0.02-1.29 kg C mol Leu^{-1}). Similarly to other spatial studies along trophic gradients (Pedrós-Alió et al. 1999, Sherr et al. 2001) higher values were found in nutrient rich areas (upwelling), compared to the oligotrophic stations, a fact that largely influenced the BHP estimates (range 0.03-2.15 or 0.6-5.9 μg C l^{-1} d^{-1} using the empirical or a theoretical CF 1.55 kgC mol Leu^{-1}, respectively). Seasonal BHP estimates for the coastal site were higher (range 0.23-5.8 μg C l^{-1} d^{-1}) and the range of empirical CFs was more restricted (only three fold, 0.98-3.62 kg C mol Leu^{-1}). These empirical CFs were generally close to 1.55 kg C mol Leu^{-1}, which is the theoretical factor calculated for marine bacteria based on constant cellular ratios of protein/dry weight and carbon/dry weight (Simon & Azam 1989), and assuming no isotope dilution. Higher values indicate that there was extracellular leucine available, leading to the dilution of the isotope (^{3}H-leucine), which in a coastal site had been calculated to be 2 (Simon & Azam 1989). Higher values of CFs were coincident with peaks of chlorophyll along the seasonal study, and indeed, a positive correlation between both parameters was found. To our knowledge, only another study has measured empirical CFs seasonally (Murrell 2003), and did not found such a relationship.

One of the most interesting results about this objective was the correlation found between empirical CFs and bacterial growth efficiency (**Chapter I**), which had never been reported before. This suggests that BGE and empirical CFs could be measuring similar metabolic processes. We also showed that low empirical CFs could be related to leucine catabolism. Indeed, an opposite gradient of CFs and percentage of leucine respiration was found, supporting this hypothesis (**Chapter I**). A similar strength of the relationship between empirical CFs and BGE was not observed for the coastal site (Fig. 1), probably because of the lower range of CFs found, or a lower importance of leucine catabolism in the coastal area (most CFs were close to the theoretical conversion factor 1.55 kg C mol Leu^{-1}). Furthermore,

values over 1.55 kg C mol Leu^{-1} should not in principle follow this relationship, since isotope dilution can be influencing the results.

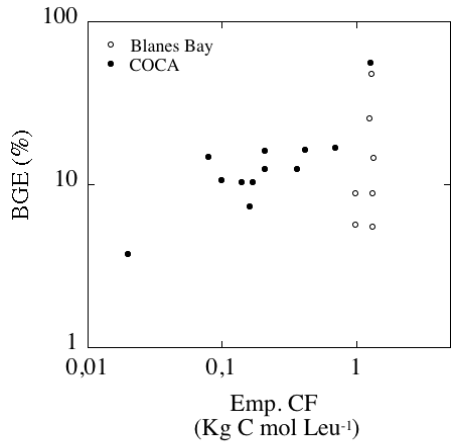

Fig 1- Relationship between empirical conversion factors (Emp CF) and bacterial growth efficiencies (BGE) in samples from Blanes Bay and the COCA cruises.

Compared to BHP, BR showed much more stable values in both studies, within a range of 2-31 μg C l^{-1} d^{-1} in the North Atlantic and 7-48 μg C l^{-1} d^{-1} in Blanes Bay. In general, BR estimates were higher in Blanes Bay compared to N Atlantic open waters. BGE also showed similar ranges through both the oceanic and coastal samples. While maximum BGEs were found in stations closest to the coast, with generally higher chlorophyll concentration in the North Atlantic (range 17-56%), peaks of BGE (around 20%) were also found concomitantly with seasonal peaks of chlorophyll. This suggests that higher BGEs could be due to the release of photosynthetic products by primary producers, and increased lability of DOM during chlorophyll and nutrient rich periods. However, in the seasonal study, the maximum value of BGE was found in May (42%), when chlorophyll was low. This was probably due to an increase in BHP related to the use of high molecular weight (HMW)-DOM, as shown by the enhanced activity of various ectoenzymes.

Bacterial assemblage structure through seasonal and spatial gradients

Bacterial diversity has been shown to be highly stable over large oceanic areas (Acinas et al. 1997, Riemann et al. 1999, 2002). However, changes in bacterial assemblage structure have also been reported seasonally (Schauer et al. 2003, Pinhassi & Hagström 2000) and spatially, in upwelling and frontal systems (Kerkhof et al. 1999, Suzuki et al. 2001, Pinhassi et al. 2003). Since our main objective was to explore the linkage between changes in bacterial diversity and

carbon metabolism, we studied two different gradients where changes in bacterial assemblage composition could occur.

Significant changes were found in the composition of the bacterial assemblages along the spatial (NW African coast upwelling to the subtropical gyre) and seasonal (Blanes Bay) trophic gradients analyzed. In both ecosystems, we combined at least two different approaches for studying bacterial diversity, which are a fingerprint technique (DGGE) and a PCR-independent technique (CARDFISH). DGGE was needed in order to assess the changes in bacterial assemblage structure throughout the samples at a relatively high phylogenetic level, for the analysis of the linkage of bacterial diversity with carbon metabolism. However, this technique is prone to several biases included that of PCR-amplification, which can highly affect the quantitative results. In order to alleviate this problem, a PCR-independent quantification of the most abundant groups was carried out in both studies (i.e. CARDFISH) in order to determine the in situ abundances of broad phylogenetic groups (although necessarily at a lower phylogenetic resolution –see also the annex-).

In the study of Blanes Bay, an exhaustive comparison of these different approaches was carried out, also including results from cloning and sequencing (**Chapter III**). This study showed that the DGGE technique is powerful as a fingerprinting technique, allowing the comparison of a large number of samples. However, the description of the bacterial assemblage provided by the DGGE poorly represented that showed by cloning or CARD-FISH due to several problems, among them, most probably, the high microdiversity of bacterial groups such as the SAR11. Clone libraries and CARDFISH results concurred to show that SAR11 was generally the dominant group in both marine environments, generally ranging between 21-41% of the DAPI counts in the North Atlantic, and 15-37% in Blanes Bay. This is in agreement with previous results in other studies that suggest that this group can be one of the most abundant bacteria in the ocean (Giovanonni & Rappé 2000, Morris et al. 2002). However, their successful hybridization by the FISH technique is quite recent (Morris et al. 2002), and the studies carried out in this thesis (**Chapters II and III**) are some of the few reports of quantitative abundances of this group in natural waters (Morris et al. 2002, Fuchs et al. 2005, Malmstrom et al. 2004, Elifantz et al. 2005).

The other group of Alphaproteobacteria, Roseobacter, was much less abundant (less than 10% in both environments), and remarkably, we found a succession between Roseobacter and SAR11 along the seasonal cycle in Blanes Bay. Although both groups showed high proportions in spring, SAR11 peaked in summer while Roseobacter showed higher proportions in winter,

when the chlorophyll and nutrient concentration was higher. Similarly, along the trophic gradient in the subtropical Atlantic, higher proportions of Roseobacter were found in nutrient enriched stations, such as the Cape Blanc upwelling (8% of DAPI counts), suggesting that members of this group are favored by nutrient enriched conditions across temporal and spatial scales in the sea. Indeed, Roseobacter has been detected in high proportions in association with natural phytoplankton blooms (González et al. 2000, Suzuki et al. 2001). On the contrary, the percent contributions of SAR11 were higher in the nutrient limited season (summer), and in the most oligotrophic stations in the North Atlantic. The second quantitatively most important group, Bacteroidetes, showed rather stable proportions along the year, but higher proportions in upwelling waters.

Links between bacterial diversity and carbon processing: correlation studies

The concept of bacterial carbon processing includes a large number of aggregated functions. In our studies we included three key measurements: BHP (with empirical conversion factors), BR and bacterial biomass. Whether changes in bacterial assemblage structure are influencing carbon processing is a complicated task to elucidate under in situ conditions. One of the reasons is the influence of extrinsic factors, such as abiotic variables (Huston & Mc Bride 2002), which are probably largely influencing both bacterial diversity and carbon functioning. Top-down effects exerted by grazing and viral infection also have a strong impact on bacterial assemblage structure, masking bottom-up effects, if they exist.

The biodiversity-ecosystem paradigm basically proposes that biodiversity is not simply dependent of the environment (i.e. abiotic conditions) but forms a link to ecosystem function that feeds back to the environment (Naeem 2002). We basically wanted to explore whether abiotic conditions affected bacterial assemblage structure, which in turn influenced carbon metabolism, or whether abiotic conditions were affecting both bacterial carbon function and diversity without an important coupling between both parameters. In other words, we wanted to explore whether different bacterial assemblages could perform equally in carbon metabolism (and therefore, a high degree of functional redundancy was expected to dominate within the bacterioplankton assemblages), or whether changes in bacterial assemblage structure were accompanied by changes in carbon metabolism (because specific populations were carrying out different types of carbon metabolism, Fig 2).

To our knowledge only a very recent study conducted by Fuhrman et al. (2006) has assessed such linkages between bacterial assemblage structure and environmental and biotic factors with marine in situ samples. These authors found that a broad range of abiotic and biotic variables significantly influenced and could predict reasonably well (by multiple regression analysis) the distribution and abundance of bacterial taxa through a 4.5 years-seasonal cycle off the California coast. These results suggest a low degree of functional redundancy in this system.

Fig 2- Hypotheses in the relationship between abiotic factors, bacterial diversity and C cycling

In our studies, the relation between environmental, bacterial diversity, and carbon functioning parameters was also explored by multivariate statistical techniques, since all these parameters were composites of several variables. Nonmetric multidimensional scaling and clustering methods consistently showed a stronger relationship between environmental variables and bacterial diversity patterns in both studies, being bacterial carbon metabolism weakly or non-related to any of these parameters. In order to statistically assess the similarity of the stations based on these parameters, we performed a correlation between similarity matrices (Mantel procedure). In both studies the environmental conditions were correlated to bacterial assemblage structure, although in the spatial study in the North Atlantic such relationship was stronger ($r= 0.70$, $p< 0.01$, Fig 3), while in the seasonal study in Blanes Bay it was marginally not significant ($r= 0.20$, $p= 0.06$). On the contrary, substantial changes were found between both studies in the relationship between bacterial diversity and carbon cycling. In the spatial study in North Atlantic oceanic waters a weak but significant relationship was found ($r= 0.36$ $p< 0.05$), while in the seasonal study there was no significant relationship ($r= 0.09$, $p= 0.7$). Bacterial carbon processing seemed not to be related also to the abiotic conditions in this study.

The differences between the results from both studies could be related to the scale at which this coupling was assessed. The degree of forcing created by the upwelling is difficult to measure and compare to the forcing created by seasonality. However, seasonal changes of

bacterial diversity could be too rapid to force concomitant changes in carbon metabolism compared to the Cape Blanc Upwelling, which is a permanent oceanographic feature in which bacterial assemblage structure had time to adapt to the environmental conditions and perform at its optimal patterns of carbon metabolism.

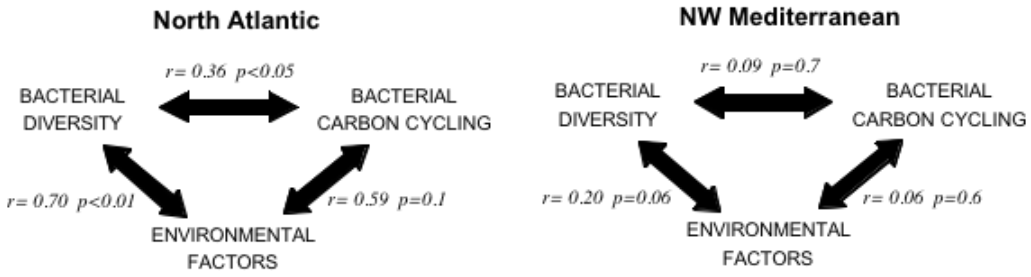

Fig 3- Relationships between abiotic factors, bacterial diversity and bacterial carbon cycling in the studies carried out in the North Atlantic and Blanes Bay (NW Mediterranean), as assessed by correlation between similarity matrices of these multivariate parameters (Mantel procedure).

The absence of a strong relationship between bacterial assemblage structure and carbon processing is in agreement with other studies that have used different approaches (Langenheder et al. 2005, Arrieta et al. 2004). Such lack of coupling could be due to different reasons. First, some bacterial species could be redundant, or at least redundant in respect to the variables measured, such as BHP and BR, which could be too general functions of the system (or too essential functions of the system). As an example, Griffiths et al. (2000) showed that specific microbial functions, such as nitrification and methane oxidation, which are carried out by a limited microbial group, were more sensitive to a decrease in soil microbial diversity than general functions such as respiration or decomposition, carried out by a wide range of organisms. Alternatively, Langenheder (2005) suggested that the low bioavailability of DOM limits the observation of functional differences, masking any clear effect of bacterial assemblage composition on bacterial biomass production and respiration, i.e.: the same total amount of DOM could be consumed in different ways, but producing similar patterns of aggregated functions such as BHP and BR.

Due to the difficulties in unveiling such relationship from the correlation approach, and specially in determining the activity of specific groups, single-cell approaches were thought to be an ideal tool for finding the linkage between bacterial assemblage structure and carbon use. MARCARDFISH was used in two more studies, one extending from the seasonal study in

Blanes, and the second one applying it in an experimental design testing the effects of an environmental force.

Links between bacterial diversity and carbon functioning: single-cell studies

a) Single-cell LMW-DOM incorporation of different bacterial populations.

Several dominant groups of bacterioplankton were chosen, based on results of the diversity study carried out in Blanes (**Chapter III**), to test their single-cell activity as it changed through the year. The three most abundant broad phylogenetic groups (Alpha-, Gammaproteobacteria and Bacteroidetes), and two quantitatively important subgroups within the Alphaproteobacteria: Roseobacter and SAR11 were analyzed. The uptake of three different compounds was considered, in order to determine the uptake of carbon (glucose), carbon with extra nitrogen (amino acids) or extra phosphorus (ATP). Substantial differences in uptake rates of the compounds were observed between the bacterial phylogenetic groups. As an example, Gammaproteobacteria and Bacteroidetes did not take up glucose, while about 20% of SAR11 cells and 60% of Roseobacter were active in the uptake of this compound. Some seasonal differences were also found for groups like Gammaproteobacteria, which substantially took up amino acids throughout the year (about 40% of active cells) but did not significantly take them up during the summer. On the contrary, Roseobacter appeared as a highly active group year round with high percentages of cells active in the uptake of glucose, amino acids and ATP (around 60%). Their low abundance in Blanes Bay, despite their high activity, could be due to the grazing impact exerted by heterotrophic flagellates, which have been shown to exert a higher grazing pressure on active bacteria (del Giorgio et al. 1996).

The lack of correlation between the in situ proportion of the different groups and their proportional activity in carbon use could be related to the weak coupling between bacterial assemblage structure and carbon processing in Blanes Bay, that was earlier discussed. Small changes in the proportion of highly active groups (such as Roseobacter) could have an important impact on carbon functioning, while larger changes in the proportions of other less active groups (e.g. SAR11) could not significantly affect carbon usage. Therefore, if changes in assemblage structure are not reflecting changes in the active bacterial pool, the coupling between overall bacterial assemblage and carbon functioning could be expected to be low.

The results shown here remark the importance of applying single-cell techniques in order to identify the active functional groups of bacteria, in which we should focus for predicting functional characteristics based on bacterial assemblage structure. The seasonal changes in the activity of some of these groups suggests that the phylogenetic level assessed was too broad to find consistent patterns of carbon usage by the groups. Alternatively, environmental changes could highly affect the activity of some groups, which should also be taken into account in depicting their functional characteristics. Further studies should focus on identifying functional groups of bacteria, in whatever level of phylogenetic resolution the changes are significant.

b) Effects of an environmental factor (PAR and UVR) on single-cell activity of bacterial groups.

The single-cell activity of the bacterial groups was further analyzed with an experimental approach, in order to test whether an environmental factor, PAR and particularly UVR, could affect their activity. Remarkably, large differences were found between broad phylogenetic groups, such as Alphaproteobacteria, which was highly sensitive to UV radiation, and Gammaproteobacteria and Bacteroidetes, which showed higher resistance. At a higher phylogenetic resolution level within the Alphaproteobacteria, SAR11 showed higher sensitivity as compared to Roseobacter.

The differences in sensitivity of the broad phylogenetic groups were not coupled with seasonal changes in the abundance of these groups in Blanes Bay (**Chapter III**). For example, SAR11 increased their proportions during the summer, the season with higher irradiances, even if this group showed high sensitivity to UVR (at least in amino acid uptake). Possibly, other factors have higher influence than UVR in determining bacterial assemblage structure, and also recovery of the cells during the night-time could compensate the UVR effect. However, at a much shorter time scale (days), we found changes of bacterial assemblage structure during a stratification process that occurred in August in Blanes Bay, that were in agreement with differences in sensitivity of the groups. Specifically, Gammaproteobacteria and Bacteroidetes, which showed higher UVR resistance, rapidly increased their proportions in stratified waters, in which bacteria were probably subjected to high irradiances. These results suggests that, at least at short time scales, this environmental factor could alter bacterial assemblage composition, which in turn could lead to changes in carbon processing.

Conclusions

The main conclusions that arise from this thesis are:

i) Measurements of bacterial respiration were more uniform and stable than the measurements of bacterial heterotrophic production across the spatial and temporal gradients studied in the open North Atlantic and coastal Mediterranean, respectively. Empirical leucine-to-carbon conversion factors largely influenced the estimation of bacterial heterotrophic production, especially in oligotrophic oceanic waters. These empirical factors were highly correlated to bacterial growth efficiencies through the spatial gradient of the North Atlantic, suggesting that both parameters could be measuring physiologically related mechanisms.

ii) A general linkage between bacterial assemblage structure and in situ carbon metabolism was not found through a seasonal study in a coastal oligotrophic site (Blanes Bay, NW Mediterranean), but a significant relationship was found along a spatial study in the subtropical North Atlantic, from the Cape Blanc upwelling to the central gyre. The permanent nature of the nutrient-rich upwelling could have led to a stable bacterial assemblage, which differed in patterns of carbon metabolism from bacteria inhabiting oligotrophic areas.

iii) The group SAR11 (within the Alphaproteobacteria) was quantitatively dominant in both the oceanic and coastal marine areas studied (up to 40% of total bacterial counts), and showed higher abundances in spring and summer throughout the year in Blanes Bay. However their single-cell activity in processing low molecular weight (LMW) compounds, such as glucose, amino acids and ATP, was relatively low, and usually no more than 20% of the cells were active in the uptake of these compounds in Blanes Bay. Thus, their substantial contribution to substrates uptake was mostly due to their high abundances and not to high specific activities.

iv) The group Roseobacter (within the Alphaproteobacteria) showed very low abundances in both the oceanic and coastal marine areas studied, never exceeding 10% of total bacterial counts. Their abundances tended to increase in the nutrient rich season (winter) in Blanes Bay, and the nutrient rich area (upwelling) in the North Atlantic. Despite their low proportions, this group showed the highest

activity in the uptake of LMW-compounds year round (ca. 60% of cells were active) and could be one of the active populations driving carbon fluxes.

v) The broad phylogenetic group Bacteroidetes was the second quantitatively most abundant group in coastal waters (Blanes bay) with uniform contributions to bacterial assemblage year-round (around 11% of DAPI counts). However, this group showed very low abundances in offshore waters of the North Atlantic, and substantially increased in the upwelling area. Of the monomers tested, this group showed only significant uptake of ATP.

vi) The broad phylogenetic group Gammaproteobacteria was present in low abundances in both systems (less than 10% of bacterial counts), but showed a relatively high single-cell activity in the uptake of amino acids and ATP (around 30% of active cells), except during the summer, when the activity in amino acid uptake drastically dropped.

vii) The broad phylogenetic groups Alpha-, Gammaproteobacteria and Bacteroidetes showed significantly different sensitivities to PAR and ultraviolet radiation (UVR), as assessed at the single-cell activity level. Gammaproteobacteria and Bacteroidetes showed higher resistance to UVR than Alphaproteobacteria, and within this group, SAR11 was more sensitive to radiation than Roseobacter

Annexes

ANNEX I

Fluorescence *in situ* hybridization of pelagic marine bacteria: variability and limitations

Fluorescence in situ hybridization (FISH) techniques have been widely used in marine samples in order to detect specific bacterial groups and follow the spatial and temporal dynamics of bacterial populations. By this technique, rRNA-targeted oligonucleotide probes labeled with fluorochromes are used to hybridize specific bacteria. Fluorescent cells can be further visualized and counted under a microscope or by flow cytometry (Giovanonni et al. 1988, DeLong et al 1989, Amann et al 1990). Although this technique was very successful in cultures and in eutrophic environments, its application to oligotrophic environmental samples usually produces low detection levels (< 60% in most cases).

Since fluorescent probes target bacterial ribosomes, the low detection with universal probes under in situ conditions was attributed to low ribosome content, and thus, low activity of the cells. Theoretically, a minimum content of 1000 to 10000 ribosomes is required for the detection of cells with oligonucleotide probes. If this range is the correct one, most cells (including those with low activity) could be detected by FISH, but in practice, it is not always the case. Even if the ratio rRNA/rDNA has been correlated with the growth rate of cultured bacteria, this relationship is not necessarily general in natural marine bacteria. Jeffrey et al. (1996) showed that the total RNA content did not correlate consistently with bacterial metabolic activity. Furthermore, there are evidences showing that some bacteria might keep a high ribosomal content during starvation (Eilers et al. 2000). These and other observations suggest that ribosome content (and FISH detection) cannot be straightforwardly related to activity (Bouvier & del Giorgio 2003).

Several methodological issues, such as the fixation protocol, can also be related with low detection levels by FISH. Different protocols of fixation have been proposed for marine

and freshwater bacteria (Pernthaler et al. 2004), including different fixatives for specific bacterial groups (such as ethanol for freshwater Actinobacteria, Sekar et al. 2003). Although the type of fixative did not have a significant effect on the proportion of target cells hybridized by Eub338 in a large-scale review (Bouvier & del Giorgio 2003), we experimentally found that this factor can influence the detection rates. For some Mediterranean coastal samples, we found that bacteria fixed with formaldehyde consistently showed lower detection signals than filters fixed with paraformaldehyde, although this was not the case with samples from other locations. In any case, cleaning of the filters with abundant Milli-Q water (or PBS) after filtration to effectively remove the excess of fixative in the samples is strongly recommended.

Even if probes can easily enter the cell, another well-known cause of failure of FISH hybridization is the in situ accessibility of ribosomes to oligonucleotide probes. The three-dimensional structure of the rRNA can hinder the access of the probes to their target site, and several regions of the ribosome can show low accessibility to the probes. Fuchs et al. (1998) studied the accessibility of the rRNA of *Escherichia Coli*, and Behrens et al. (2003) extended this work to different members of the domains Bacteria (*Pirellulla sp. Plantomycetes*), Archaea (*Metallosphaera sedula, Crenarchaeota*) and Eucarya (*Saccharomyces cerevisiae*). Several probes were designed in order to completely cover the 16S rRNA, and accessibility maps were produced with the distribution of relative fluorescence hybridization intensities. Interestingly, Fuchs et al. (1998) showed that a shift of the target region by only a few bases could result in a decline of cell fluorescence from >80% to <10%. Thus, small changes in the probes sequences during their design can have drastic effects on the hybridization intensities.

Low detection levels (sometimes below 20%, Bouvier & del Giorgio 2003) prevented for a long time the widespread application of FISH technique to in situ marine bacterioplankton. The appearance of more sensitive protocols has solved this problem and allows the use of this technique to be applied to oligotrophic marine bacterioplankton.

There are different ways of enhancement of the FISH signal, including the use of multiple probes, "helper" oligonucleotides, peptide nucleic acid (PNA) probes, or polynucleotide probes (reviewed in Zwirglmaier 2005). One of the most successful protocols so far is the catalyzed reporter deposition (CARD)-FISH (Schönhuber et al. 1997, Pernthaler et al. 2002). In this protocol, oligonucleotide probes are linked to a horseradish peroxidase (HRP) enzyme, instead of a fluorochrome. The enzyme catalyzes an amplification reaction by which a high number of fluorescent molecules bind to the membrane cell proteins achieving a brighter and more stable signal. The amplification of the signal makes that FISH counts much less dependent on variations of the physiological state of the cells. Pernthaler et al. (2002) showed that detection with the general probe for Bacteria (Eub338) by this protocol doubled that of the classical FISH protocol with monolabeled probes. Because of its advantages, we used it through the studies carried out in this thesis.

A key step in the CARD-FISH protocol is the permeabilization of the cells. HRP, unlike usual fluorochromes, cannot easily enter the membrane because of their large size. For this reason, cells need to be permeabilized in order to make "holes" in the membrane and allow the entrance of the HRP-probe. Several protocols of permeabilization have been suggested (Pernthaler et al. 2004), being the treatment with lysozyme one of the most widely used. Other protocols include the use of achromopeptidase (suggested for freshwater Actinobacteria, but which performs well with most common marine bacteria), proteinase K (for Archaea) or HCl (Pernthaler et al. 2004). It is highly recommended to carry out preliminary tests in order to find the most suitable permeabilization protocol for a specific set of samples. In this thesis, lysozyme alone, and more frequently lysozyme plus achromopeptidase were used for permeabilization, since in preliminary tests achromopeptidase either did not have a significant effect or enhanced the detection over that obtained with lysozyme alone.

Other than the permeabilization protocol, the (hybridization) incubation time and the concentration of the probes can also significantly affect the detection level. Although in the

original protocol (Pernthaler et al. 2002) two hours of incubation were suggested, we experimentally found that for some probes longer incubation times were needed. Otherwise, the detection of cells drastically dropped. We found that such behavior could differ depending on the probe used. Specifically, the hybridization with Ros537, Gam42a or CF319a probes yielded similar results in hybridizations carried out during 2 h or overnight, and with lower (1:300) or higher (1:100) concentrations of the probes in the hybridization buffer (vol:vol). Yet, the optimal hybridization of cells with Eub338, Alf968, and SAR11-441R probes required higher concentration of the probe (3:300) and overnight hybridization in environmental samples of the Mediterranean. We, thus, recommend adopting as a routine protocol the use of high concentration of probes (1:100) and overnight hybridization in order to ensure an optimal detection level. Such high concentrations may be not necessary in samples in which bacterial cells are known to be actively growing, such as in dilution cultures, as we also observed.

Besides detection rates, a key issue regarding the truthful application of the FISH protocol is the specificity of the probes binding to the target site, which depends on hybridization and washing conditions. The combination of temperature and the concentration of formamide in the hybridization buffer control the stringency (i.e. specificity) during the hybridization step. Formamide decreases the melting temperature by weakening the hydrogen bonds, thus enabling lower temperatures to be used with high stringency. Approximately, an increase of 2% of formamide in the hybridization buffer compensates a decrease of 1ºC in the hybridization temperature. Each probe has its specific stringent conditions, depending, between other factors, on their G+C content and length (Pernthaler et al. 2001). In order to determine the specific conditions for a given probe, a formamide curve can be performed, in which the detection of cells is determined at increasing concentrations of formamide (and at a fixed temperature). Ideally, two curves should be done, one with cultures of the target organism(s) and the second with a culture that has one mismatch with the probe. The optimal conditions should be set to the point

that the target organism is still detected, but the non-target organism is not detected. Given the difficulty in obtaining pure cultures of most in situ dominant bacterial phylotypes, formamide curves are performed with in situ samples when the specificity of a given probe has to be tested. In order to maximize the specific conditions, the most stringent conditions before the detection of the cells sharply decrease, are those chosen.

This approach was applied during this thesis in order to obtain the hybridization conditions of a specific probe for the SAR11 group. Such probe (SAR11-441R) had been designed by Morris et al. (2002) in the first report of in situ abundances of this quantitatively important group in the sea. In their study, a set of four fluorescently labeled probes was used simultaneously in order to enhance their detection. We chose one of this probes (the one that matched most of our clones in Mediterranean waters), and set up the protocol to use it with the CARDFISH methodology (with the collaboration of M. Vila), since it had not been described before in the literature. Several formamide curves (e.g. Fig. 1) were performed with different environmental samples, and the results were compared with the set of monolabeled probes used by Morris et al. (2002). Based on these results, we chose 45% of formamide for hybridizing SAR11 cells with the SAR11-441R probe by CARD-FISH. We also found that the detection level with the HRP-probe SAR11-441R, was similar to that found with the simultaneous use of the four monolabeled probes designed by Morris et al. (2002).

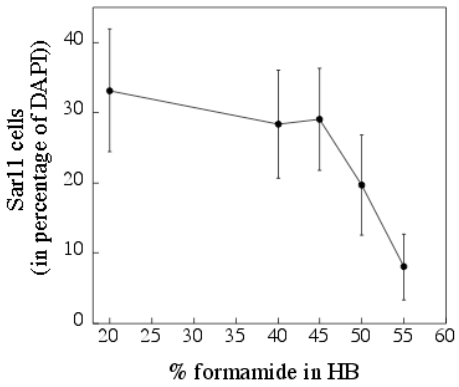

Fig 1- Curve showing the percentage of cells hybridized with the HRP-probe SAR11-441R with increasing percentages of formamide in the hybridization of buffer. This analysis was carried out with a sample from Blanes Bay (March 2003).

Table 1- Bacterial abundance of bacterial groups determined by CARDFISH in seasonal in situ samples and in seawater cultures (SW), unamended or with the addition of inorganic nutrients (SW+nut), carried out in Blanes Bay (NW Mediterranean). Eub338: Eubacteria, Alf968: Alphaproteobacteria, Ros537: Roseobacter, Gam42a: Gammaproteobacteria, Alt1413: Alteromonas, CF319a: Bacteroidetes, Unexpl: Eub cells not covered with broad phylogenetic probes for Alpha, Gammaproteobacteria and Bacteroidetes. Abundaces are shown in percentages (± SE) scaled to DAPI counts. These data presented in Alonso-Sáez L, Pinhassi J, Gasol J.M. *(in prep)*.

Sample	Eub338	Alf968	Ros537	Gam42a	Alt1413	CF319a	Unexpl
Aug *in situ*	74	38	1	8	0	12	16
Aug SW	95	20	4	24	19	11	39
Aug SW+nut	99	29	27	63	58	8	0
Sept *in situ*	59	35	1	3	0	12	9
Sept SW	89	7	1	38	42	2	42
Sept SW+nut	96	12	4	70	63	4	10
Oct *in situ*	63	19	0	1	0	6	37
Oct SW	96	16	13	68	58	4	8
Oct SW+nut	97	18	5	72	60	5	2
Nov *in situ*	78	34	3	4	0	11	29
Nov SW	84	14	1	38	29	8	32
Nov SW+nut	91	14	4	57	51	1	26
Dec *in situ*	85	27	5	3	1	12	43
Dec SW	97	23	13	35	32	38	0
Dec SW+nut	96	24	13	46	36	30	5
Jan *in situ*	73	24	3	2	0	14	33
Jan SW	98	13	11	65	65	6	14
Jan SW+nut	98	20	20	61	56	10	7
Feb *in situ*	84	21	7	4	0	14	45
Feb SW	97	20	18	36	36	20	22
Feb SW+nut	98	15	19	55	46	17	10

In the context of this thesis I applied CARD-FISH to a wide range of environmental samples, from oligotrophic waters of the Mediterranean Sea to highly eutrophic waters from

the Cape Blanc upwelling. Here, I compare these results with other studies in which I collaborated meanwhile (including samples from phytoplankton blooms, Arctic waters, and seawater cultures carried out with water from Blanes Bay) in order to describe the range of detection obtained with this technique.

The bacterial assemblage composition of seasonal samples is compared to the bacterial assemblage that developed in dilution seawater cultures (SW) using the in situ sample as an inoculum (Table 1). The effect of nutrient addition to the dilution cultures (SW+nut) on the bacterial assemblage composition was also analyzed. As it is shown in Table 1, the percentage of cells detectable with the eubacterial probe was significantly higher in the SW cultures compared to the in situ samples. In most of the cultures, >95% of the cells could be detected with the Eub338 probe, compared to usually less than 80% in natural waters. These results agree with the common observation that cells in culture are more easily detected than under in situ conditions, something that has been attributed to the physiological state of the cells. The most profound changes in bacterial assemblage composition of the SW cultures compared to in situ samples was the growth of Gammaproteobacteria in the cultures (from around 5% of DAPI counts in situ, to >60% in the cultures), which could have contributed to the higher detection. However, a relatively high percentage of cells unidentified by the broad phylogenetic probes of Alpha-. Gammaproteobacteria or Bacteroidetes was also present in the SW cultures, although generally less than in the in situ samples.

Higher detection of cells with eubacterial probes was also found in samples taken in more enriched environments compared to the oligotrophic Blanes Bay. As an example, samples taken in natural blooms of several species of the dinoflagellate *Alexandrium* in the Mediterranean, showed high detection levels (average of 83%), compared to the year-average detection rate in Blanes Bay (73% of DAPI counts). The percentage of cells that were detected with the probe Eub338 but not identified with broad phylogenetic probes

(Alpha, Gammaproteobacteria and Bacteroidetes) was generally lower in the blooms (average of 12%) compared to Blanes Bay (average 25%)

Table 2- Bacterial assemblage structure in samples taken in several *bloom* events of *Alexandrium* species in the following Mediterranean harbours: La Fosca (including samples at different stages of the bloom), Arenys and Olbia (including samples at an inner and outer station in the harbour). These data are presented in Garcés E., Vila M, Reñé A., Alonso-Sáez L., Anglés S., Lugliè A., Masó M. and Gasol J.M *Natural bacterioplankton assemblage structure during bloom events of Alexandrium species (Dinophyceae) in NW Mediterranean coastal waters* (Submitted to Aquatic Microbial Ecology). Bacterial groups as in Table 1. Abundances are shown in percentages (± SE) scaled to DAPI counts.

Sample	Eub338	Alf968	Ros537	Gam42a	CF319a	Unexpl
La Fosca Develop *bloom*	80	31	6	8	34	7
La Fosca Decline *bloom*	77	36	9	7	20	14
La Fosca Non *bloom*	82	40	3	4	13	25
Arenys *Inner station*	87	20	20	25	48	0
Arenys *Outer station*	89	7	6	30	48	4
Olbia *Inner station*	79	42	10	12	12	13
Olbia *Outer station*	89	40	6	9	9	31

Finally, Table 3 presents CARDFISH results from a completely different ecosystem compared to the ones sampled in the thesis (NW Mediterranean and Atlantic Ocean), which is the Arctic system. Several samples were analyzed across a gradient from the Mackenzie River to coastal and offshore sites in the Beaufort Sea, in open-water conditions. The results showed dominance of Betaproteobacteria in riverine waters and Alphaproteobacteria in offshore waters, in agreement with other estuarine studies (del Giorgio & Bouvier 2002). An important difference compared to the samples analyzed in this thesis is the appearance of pelagic *Archaea*, which can importantly contribute to bacterial assemblage in Arctic waters as

it has been previously described (e.g. Bano et al. 2004). The detection of Eubacterial cells in this system is very high, over 80%, which together with Archaeal counts made up over 90% of detection of these cells by CARDFISH. Despite the low temperature of the waters, Arctic bacteria seem to be a highly active, as it appears in a study with MARFISH that we are currently processing in our lab .

Table 3- Abundance of bacterial groups in the Mackenzie River (River 1, 2), the coastal (Z1, Z2. Z5) and offshore (65, 66, 49) Beaufort Sea and in Franklin Bay (12) in the Arctic. Arc915: Archaea, Bet42a: Betaproteobacteria, other groups as in Table 1. Abundaces are shown in percentages (± SE) scaled to DAPI counts. Data from Garneau ME, Vincent WF, Alonso-Sáez L, Gratton Y, Lovejoy C (2006) *Prokaryotic community structure and heterotrophic production in a river-influenced coastal arctic ecosystem* Aquatic Microbial Ecology 42: 27-40.

Sample	Arc915	Eub338	Alf968	Bet42a	Gam42a	CF319a	Unexpl
River 1	6	52	2	17	2	4	27
River 2	5	64	2	18	2	2	40
Z1	5	83	33	7	17	15	11
Z2	6	83	33	4	20	17	9
Z5	7	80	15	1	10	16	38
65	2	86	40	1	14	19	12
66	1	88	39	6	17	11	15
49	1	87	36	3	23	16	8
12	2	92	31	4	12	17	28

In summary, a wide range of detection levels can be found across different marine systems. Table 4 summarizes the results of the different studies, in which Archaea always contributed with less than 10% to DAPI counts, and thus the hybridization with Eub338 probe could be considered a good estimate of FISH sensitivity. In situ samples from the oligotrophic station in the NW Mediterranean and open waters of the NE Atlantic showed the lowest detection (with the Eub338 probe), and cells growing in seawater cultures showed the highest detection (close to 100%). Although bacterial activity cannot be straightforwardly related with FISH sensitivity (Bouvier & del Giorgio 2003), differences in the physiologic state of the cells between the samples suggest that this factor influenced

their detection by FISH. Such effect was apparent even if the amplification step of the CARDFISH protocol should reduce the differences between low and highly activity cells.

Table 4- Summary of bacterial assemblage structure in different marine systems as well as in seawater cultures (SW cult) without or with the addition of nutrients (+nut). Bacterial groups as in Table 1. Abundances are shown in percentages (± SE) scaled to DAPI counts.

	Eub338	Alf968	Gam42a	CF319a	Unexpl.
Blanes Bay	73 ± 3	30 ± 7	7 ± 3	11 ± 1	25 ± 4
Atlantic offshore	65 ± 3	37 ± 3	2 ± 1	1 ± 1	24 ± 3
Atlantic upwelling	67 ± 6	34 ± 3	8 ± 1	12 ± 4	10 ± 2
Phyto. blooms	84 ± 2	29 ± 5	15 ± 4	29 ± 7	12 ± 4
Arctic Ocean	86 ± 1	32 ± 3	16 ± 2	16 ± 1	17 ± 4
SW cult	94 ± 2	16 ± 2	43 ± 6	13 ± 5	22 ± 6
SW cult+nut	96 ± 1	19 ± 2	61 ± 3	11 ± 4	9 ± 3

Large differences were also found in bacterial assemblage composition at the level of broad phylogenetic groups (Alpha-, Gammaproteobacteria and Bacteroidetes) between in situ samples and seawater cultures. Alphaproteobacteria were dominant in situ while Gammaproteobacteria dominated the dilution cultures. Some differences were also found between the in situ samples from different marine systems, although much less marked. The abundance of Gammaproteobacteria decreased in the most oligotrophic systems (offshore Atlantic and NW Mediterranean), and the percentage of Eub cells that were not covered by the broad probes was higher, about 25% of DAPI counts. Such increase in unidentified cells could be due to the presence of different bacterial lineages (non identified by the probes) or to the low coverage of the general probes for broad phylogenetic groups, which is the most likely reason.

The minor differences in bacterial assemblage composition found by the general probes between these markedly different systems is an indication of the low power of the FISH

technique to describe bacterial diversity, and the need to use probes which are more specific. In the studies presented in this thesis we used probes for some somehow more specific groups, such as SAR11, Roseobacter or NOR5 (**Chapters II and III**). However, it has to be taken into account that it is not practical to use a high number of probes with this technique given that it is very time-consuming because of the counting effort. As we extensively discussed in **Chapter III**, the FISH technique is not suitable to provide a detailed picture of the phylogenetic groups of bacterioplankton (in the way that the fingerprinting techniques or the clone libraries provide). However, it is a powerful technique for quantifying the abundance of bacterial groups and, if combined with cloning and sequencing, it can be used to appropriately quantify (without the PCR bias) the most important bacterial groups in a given system.

Annex II

Detailed CARD-FISH PROTOCOL

1. Fixation.

- Add buffered particle free paraformaldehyde(*) (final conc 2%) and fix at 4ºC overnight.
- Filter the samples gently (5mm Hg) through a white polycarbonate filter (pore size 0.2 μm GTTP). Use cellulose nitrate support filters beneath the polycarbonate filters to improve the distribution of cells. (Typically 25 ml of sample onto 47mm diameter filters for bacterial concentrations around $1x10^6$)
- Wash twice with 5-10 ml of milli Q water (or PBS).
- Store the filters at –20ºC (or preferably -80ºC) for months to years.

(*) Preparation of paraformaldehyde 4%

Heat 60 ml of milli Q water to 60ºC
Add 4g PFA, stir and dissolve it with a drop of NaOH 2N
Wait until it is completely dissolved (don´t overheat!! and add more NaOH if necessary)
Add 33 ml of 3x PBS
Adjust the pH to 7.2
Fill with milli Q water up to 100ml
Filter by 0.2 μm
Use it freshly prepared, and add to the sample 1:1 (1 vol of sample to 1 vol of paraformaldehyde)

2. Attachment

- Prepare 0.1% (w/w) low-gelling point agarose in milli Q water.
- Boil the agarose before each use in a microwave oven.
- Fill the agarose in a Petri dish and let it cool down to 40-35ºC.
- Dip the filters with both sides into the agarose and put the filters face up onto parafilm on a glass plate. (Try to dip the filters quickly).
- Let the filters dry in the oven (35ºC) for 10-30 minutes.
- Pipette ethanol (96-80% [v/v]) onto the filters and carefully peel them off.
- Let the filters air dry on a paper tissue face up.

3. Permeabilization with lysozyme and achromopeptidase

- Prepare 10 ml of a fresh lysozyme solution
  1 ml EDTA 0.5 M
  1 ml 1 M Tris HCl, pH8
  8 ml milli Q water
  100 mg lysozyme
- Incubate the filters in the lysozyme solution for 60 min at 37ºC.
- Prepare fresh achromopeptidase solution:
  20 µl achromopeptidase
  10 ml achromopeptidase buffer
  (the buffer contains 100µl NaCl 5M, 500 µl TrisHCl 1M, 50 ml Milli Q water, adjust pH to 8, store at 4ºC)
- Put the filters in the achromopeptidase solution right after the lysozyme step and incubate them for 30 min 37ºC.
- Wash the filters first in a Petri dish filled with milli Q water, then in a Petri dish filled with absolute ethanol.
- Let filters dry.

(After permeabilization filters can be stored at –20ºC for several weeks).

4. Preparation of the hybridization buffer

- Pipette in a 50 ml tube:
  3.6 ml NaCl 5M
  0.4 ml 1M Tris HCl
  Add water and formamide depending on the probe to use (Table 1, check probes list at the end of the protocol)
  2 ml blocking reagent
  20 µl SDS
- Add 2 g of dextran sulfate. Heat at 40-60ºC and shake until the dextran sulfate has dissolved completely.

(Small portions of the buffer, typically 900µl in Eppendorf tubes, can then be stored at –20ºC for several months).

Table 1. Volume of formamide and water for the hybridization buffer.

% FORMAMIDE	ml FORMAMIDE	ml MILLIQ
20%	4	10
45%	9	5
50%	10	4
55%	11	3
60%	12	2

5. Hybridization

- Cut filters in sections and label them with a lead pencil.
- Mix hybridization buffer and HRP labeled oligonucleotide probe [50 ng µl-1] (www.biomers.net) at (100:1) in an eppendorf (typically 900µl HB: 9 µl of probe). Place the filters inside.
- Hybridize on a rotation shaker (10 rpm) overnight at 35ºC.

(Lower proportions of probe, i.e. 300:1 can be enough for detecting highly active cells).

6. Preparation of washing buffer

- Pipette in a 50 ml tube:
  0.5 ml EDTA
  1 ml Tris HCl
  Add volume of NaCl depending on % formamide (Table 2)
  Add water to 50 ml.
  25 µl SDS

Table 2. Volumes of NaCl for the washing buffer

% Formamide in HB (35ºC)	µL NaCl 5M (in 50ml WB)
20%	1350
45%	160
50%	90
55%	30
60%	0

- Preheat the WB at 37ºC

7. Washing

- Wash sections after hybridization for 5 minutes in 50 ml of preheated washing buffer (37ºC).

After washing (and during all the protocol) do not let filter sections run dry, this will reduce the activity of the HRP.

8. Catalyzed reported deposition (amplification).

- Prepare the amplification buffer (AB). Pipette in a 50 ml tube:
  2 ml of PBSx20.
  0.4 ml blocking reagent.
  16 ml NaCl.
  Add sterile milliQ water to a final volume of 40 ml.
  Add 4 g dextran sulfate. Heat (40 to 60ºC) and shake until the dextran sulfate has dissolved completely.

(The amplification buffer can be stored in the fridge for several weeks).

- Remove the sections from the washing buffer and incubate them in PBS for 15 minutes at room temperature.
- Prepare fresh 100x H_2O_2 stock by mixing 200µl of 1xPBS with 1 µl of 30% H_2O_2.
- Mix 1 ml of AB + 10 µl of the 100 x H_2O_2 stock + 4 µl of fluorescently labeled tyramide (**) in an eppendorf.
- Put filter sections inside the eppendorf and incubate for 15 minutes in the dark at 46ºC.
- Remove excess liquid by dabbing filters onto blotting paper. Wash sections in PBS for 15 minutes at room temperature in the dark
- Wash sections in deionized water, then in absolute ethanol. Let sections air dry.
- The filter sections can now be counterstained with a mounting mixture of 4 Citifluor:1 Vecta containing DAPI (1 µg/ml).

(**) *Synthesis of tyramide conjugates.*
The active dye stock as well as the tyramide HCl stock must be prepared a few minutes before the reaction.

1.) Synthesis of the active dye stock (Alexa 488):
1 mg succinimidyl ester ($Alexa_{488}$)
100 µl dimethylformamide.

2.) Synthesis of the tyramine HCl stock:
10 µl triethylamine
1 ml dimethylformamide
10 mg tyramine HCl

3.) Reaction
Add active dye ester in 1.1 fold molar excess to tyramine HCl stock solution:
100µl $Alexa_{488}$ stock
25.2 µl tyramine HCl stock

Incubate for 6-12 hours at room temperature in the dark.

Dilute reaction mixture with absolute ethanol to a final concentration of 1 mg active dye per ml. Produce portions of 20 µl and desiccate them in the freeze dryer or under vacuum at room temperature.

For use, tyramides are reconstituted in 20 µl of dimethylformamide containing 20 mg ml^{-1} p-iodophenylboronic acid (IPBA).
(Tyramides in dimethylformamide can be stored in the freezer)

PROBE LIST

PROBE *(source)*	**SEQUENCE** *(5' -> 3')*	**%FA(35°)**	**TARGET**
Eury806 (*Teira 2004*)	CACAGCGTTTACACCTAG	20	Euryarchaea
Cren554 (*Massana 1997*)	TTAGGCCCAATAATCMTCCT	20	Crenarchaea
Eub338 (*Amann 1990*)	GCTGCCTCCCGTAGGAGT	55	Most bacteria
Eub338(II) (*Daims 1999*)	GCAGCCACCCGTAGGTGT	55	Plantomycetales
Eub338(III) (*Daims 1999*)	GCTGCCACCCGTAGGTGT	55	Verrucomicrob.
Non338 (*Wallner* 1993)	ACTCCTACGGGAGGCAGC	20	Control nonsense
Alf968 (*Neef 1997*)	GGTAAGGTTCTGCGCGTT	45	Alphaproteobac.-
SAR11-441R (*Morris 2002*)	TACAGTCATTTTCTTCCCCGAC	45	SAR11 cluster
Ros537 (*Eilers 2001*)	CAACGCTAACCCCCTCC	55	RoseobacterSAR83
Gam42a (*Manz 1992*)	GCCTTCCCACATCGTTT	55	Gammaproteobac-
NOR5-730 (*Eilers 2001*)	TCGAGCCAGGAGGCCGCC	50	NOR5 cluster
Sar86/1245 (*Zubkov 2001*)	TTAGCGTCCGTCTGTAT	55	SAR86 cluster
Alt1413 (*Eilers 2000*)	TTTGCATCCCACTCCCAT	60	Alteromonas
Bet42a (*Manz 1992*)	GCCTTCCCACTTCGTTT	55	Betaproteobac.
CF319a (*Manz 1996*)	TGGTCCGTGTCTCAGTAC	55	Cytophagales

ANNEX III.

Microautoradiography combined with CARDFISH in environmental marine samples

Microautoradiography has been used in microbial ecology studies since the 1960's (Brock & Brock 1968). By this technique the uptake of radiolabeled compounds by microorganisms can directly be visualized under a microscope. Microbial assemblages are incubated with a radiolabeled substrate and, then, cells are put in contact with an autoradiographic emulsion. The exposure of the emulsion to the radioactive emissions produces silver grains deposit around the cells. One of the main advantages of this technique is its great sensitivity, allowing the detection of very low metabolically active cells in the samples (Smith & del Giorgio 2003).

In 1999, two independent studies showed that it was possible to combine this technique with fluorescence in situ hybridization (FISH) in marine samples (Lee et al. 1999, Ouverney & Fuhrman 1999), giving first insights into the in situ single-cell activity of specific bacterial groups. Thereafter, several studies have used the MARFISH methodology in order to explore the carbon processing by different bacterial groups from the perspective of in situ uptake of representative substrates (at trace concentrations), or activity on biomass production (using leucine and/or thymidine uptake at saturating concentrations).

In the initial MARFISH protocol, the transferring of cells from the filter to the emulsion could lead to extensive cell loss. A variation of the initial protocol is still in use in some laboratories (Teira et al. 2005, Herndl et al. 2004). Alonso & Pernthaler (2005), however, suggested a modification of this method, in which cell loss is avoided, that we implemented for the MARFISH studies during this thesis (**Chapters V and VI**). Experimentally, the limitation of this method is that DAPI staining cannot always be performed successfully, possibly because the emulsion is located on top of the cells. Such problem does not apply to CARDFISH hybridized cells, which are easily seen under the microscope. It means, however, that separate treatments must be done for counting the percent contribution of FISH+ cells to the total community and for determining how many of the FISH+ are active in substrate uptake.

Given that, by this protocol, the emulsion is located on the cells, it is also of great importance to check the correct incubation time with the emulsion before developing, since the increasing number of silver grains on highly active cells can hide them after a short time.

Since the activity of the cells can strongly vary depending on the compound added, the concentration, and the environmental conditions, it is highly recommended to perform time-courses for every sample and compound used (unless the environmental and experimental conditions are very similar).

For the MARCARDFISH studies in this thesis (**Chapters V and VI**) we performed time-courses for every sample (seasonal time or experimental treatment) and compound added. Fig. 1 shows the result of the incubation with different substrates corresponding to the sample from August 2003 in Blanes Bay. In general, between 13-19 days of exposure were used for most seasonal samples, except for a sample taken in July, when the bacterial community was highly active, and only 2 days of exposure was needed. Similarly, in other in situ samples from the Arctic currently in process in our lab (in which trace concentrations were also used, 0.5nM), lower exposure times (only 3 days) were necessary to reach the asymptote of the time-course curve.

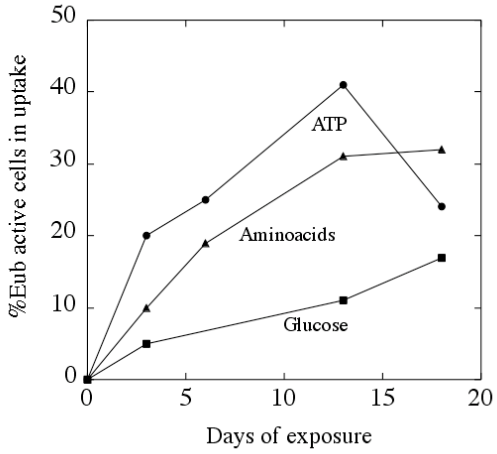

Fig 1- Time-course of the exposure to the photographic emulsion for MARFISH samples (incubated with tritiated glucose, amino acids and ATP at 0.5 nM) taken in August 2003 in Blanes Bay.

MAR-CARD-FISH PROTOCOL

1. Incubation of the sample with radiolabeled compounds

- Fill up the incubation vials (ultra-clean falcon tubes) with sample water (20 ml are typical), and the killed controls with a smaller volume (10 ml).
- Add 2% (final concentration) formaldehyde (or paraformaldahyde) to the killed controls and wait 3-5 minutes.
- Add the radiolabeled compound(*).
- Incubate the vials at in situ temperature (out of light) typically for 4 hours, but sometimes longer (up to 12-24 h) depending on the substrate and concentration.

(*) Its specific activity should be as high as possible. The different compounds can be added in low concentration as tracers (0.5 nM glucose, proteins…) or high concentrations typical of bacterial production measurements (20-40 nM Leu) depending on the process one wants to study.

2. Fixation

- Add formaldehyde (or paraformaldehyde) to 2% (final concentration) and let the sample fix overnight at 4ºC.

3. Filtration

- Set up the filter column with a 0.45 nitrocellulose filter supporting a white 0.22 μm polycarbonate filter. Suck some milli Q water through the nitrocellulose filter before laying the polycarbonate on the top of it.
- Filter (typically) 5 ml of fixed sample (depending on the concentration of cells in the sample). It is recommended to have 3-4 replicate filters for each sample.
- Rinse each filter three times with milli Q water filtered by 0.2 μm.
- Dry the filters on drying paper, label them with a pencil and store them in the freezer.

4. CARD-FISH

- Perform the CARD-FISH protocol as described in Annex 1.

5. Dipping (Autoradiography)

Prior to the dipping, photographic emulsion (KODAK NTB2) has to be aliquoted in Falcon tubes (50ml) containing 10 ml of emulsion each (in the dark). Each falcon tube has to be covered with black tape and several folders of aluminum foil in order to protect them from the light. Store Falcon tubes with emulsion at 4ºC until the dipping. Use one tube (10ml emulsion) for around 25 slides.

- Glue the filters (with the cells face up!) onto slides using epoxy-glue (UHU plus). Up to three pieces of filters can be glued onto one slide).

Before putting the filters, cut one little peace of the corner of each slide in order to be able to detect (in the dark) in which side of the slides the filters are.

Slightly wet the back of the filters with milliQ water (in a Petri dish) before putting them onto the slide with the glue. This helps maintaining the filters attached to the slides once they dry.

- In the dark room, melt the Falcon with emulsion in a 43ºC water bath for 1 hour.
- Cover a metal bar with ice for at least 15 min before the dipping, and dry it before starting
- Set the labeled slides with the filters (in order) in a rack by the water bath.
- In the dark, add 10 ml of agarose solution (0.2% w/v) to the Falcon tube containing the photographic emulsion (1:1 dilution).
- Put the tube back in the water bath to melt and wait for 5 minutes.
- Dip the first slide in the diluted emulsion, by placing it inside the Falcon tube and turning it upside-down smoothly 2 or 3 times.
- Open the Falcon tube and place the slide on the metal bar.
- Repeat it for all the slides and filters.
- Allow the emulsion on the slides to gel on the metal bar for about 7 minutes
- Transfer slides to dark boxes and wrapped them in aluminum foil.

(Use different black boxes for different exposure times)

- Place the dark boxes in the fridge and expose them at 4ºC.

(Exposure times need to be optimized for every experiment)

6. Developing of samples

- Fill up four containers with developer (Kodak D19, diluted 1:1 with milli Q water), milliQ water, fixative (Kodak Tmax, diluted 1:4 with milli Q water) and tap water.
- Turn off the lights.
- Take the slides out of the box and place them in the developer for 3 min.
- Rinse with milliQ water for 30 sec.
- Fix with Kodak fixer for 3 min.
- Rinse with tap water for 10 min.
- Place the slides inside a desiccator overnight, and protected from light.

7. DAPI Staining

- Cover the filters with DAPI (1 μg/ml), and stain for 3 min at 4ºC.
- Clean the slides with abundant milliQ water and ethanol.
- Prepare the *mounting solution*: 4 Vecta Shield: 1 Citifluor (*).
- Pour one drop of the *mounting solution* over each filter and place a coverslip.
- Count under the microscope *(UV-light for DAPI, Blue-light for Alexa488 and transmitted light for silver grains)* .

(*) Alternatively, the mounting solution can be prepared following this protocol:

10 ml glycerol
2 ml Vecta Shield
1 ml PBSx20
Add DAPI to a final conc. 1μg/ml
Adjust pH at 9.5 (with pH-paper).

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