Universidade de Aveiro Departamento de Biologia
2008

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Produção bacteriana em ambientes estuarinos: Condições de incubação e factores de conversão

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requesitos necessários à obtenção do Grau de Mestre em Microbiologia, realizada sob a orientação científica da Professora Doutora Maria Adelaide de Pinho Almeida, Professora Auxiliar do Departamento de Biologia da Univerisdade de Aveiro.

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agradecimentos

À Professora Doutora Adelaide, orientadora da tese, pelo incentivo, pelas suas ideias, pelo interesse, pelas críticas construtivas e pela sua constante disponibilidade.

À Professora Doutora Ângela Cunha pelo sentido crítico e pela simpatia demonstradas ao longo do trabalho.

Ao Doutor Newton Gomes pelo apoio técnico dado na parte molecular do trabalho e pelo sentido crítico.

À Ana Luísa pela sua preciosa ajuda na parte molecular do trabalho, pelas suas brilhantes ideias, pela força dada e acima de tudo pela sua prontidão e uma teimosia superior à do DGGE.

À Sara, ao Francisco, à Catarina, à Yolanda e à Luísa por me terem acompanhado nas colheitas e pela imensa paciência que demonstraram ter naqueles longos tempos de espera.

À Vanessa pelo apoio e força dados nas longas viagens de comboio.

A todos os outros colegas do Laboratório de Microbiologia Ambiental e Aplicada, pela boa disposição e pelo apoio.

Aos meus pais e irmão pela força dada, pela paciência demonstrada, pelo amor e pelo apoio absoluto.

Ao Daniel pela enorme paciência, pelo amor e pelo carinho e pela força constantemente dados e por me ter acompanhado nesta caminhada que nem sempre foi fácil.

palavras-chave

Bacterioplâncton heterotrófico, leucina, taxa de incorporação da leucina, diluição isotópica, factores de conversão, estuário, comunidades bacterianas, produtividade de biomassa bacteriana, condições de incubação, electroforese em gel com gradiente desnaturante, “microbial loop”, ciclo biogeoquímico do carbono

resumo

O bacterioplâncton heterotrófico desempenha um papel muito importante no ciclo biogeoquímico oceânico do carbono. A produtividade de biomassa bacteriana (PBB) converte a matéria orgância dissolvida (MOD) em matéria orgânica particulada (MOP), que fica disponível para os niveis tróficos superiores da cadeia alimentar. A PBB é determinada através da taxa de incorporação da leucina. Existem dois grandes problemas com este método: a utilização de factores de conversão para uma medição correcta da PBB e as condições de incubação sob as quais a leucina é incorporada. De modo a dar resposta a estes problemas amostras de água de duas estações de colheita (zona marinha e zona salobra) do sistema estuarino da Ria de Aveiro foram incubadas sob duas condições principais (condições de campo e de laboratório) e foram determinados factores de conversão específicos (tanto empíricos como semi-teóricos) para ambas as zonas deste sistema estuarino. Relativamente aos ensaios das condições de incubação, descobriu-se que, em condições laboratoriais (luz PAR vs. escuro), a produtividade foi superior quando as amostras são incubadas no escuro e que, para as condições de campo (*in situ* luz vs. *in situ* escuro) não foi observado nenhum padrão de variação. Nas experiências de DGGE encontrou-se, com excepção da água de superfície da zona marinha, que as várias condições de incubação não eram representativas das amostras originais. A PBB deve ser determinada nas condições de *in situ* luz mas, quando isto não é possível, e é necessário determinar a PBB em condições laboratoriais, deve-se realizar as incubações no escuro. Das experiências dos factores de conversão foi determinada uma diluição isotópica média e um factor de conversão semi-teórico médio de 5.07 e 7.51 Kg C mol^{-1} para a zona marinha e de 5.15 e 7.75 Kg C mol^{-1} para a zona salobra, respectivamente. Uma média de um factor de conversão empírico de 20.18 Kg C mol^{-1} foi obtido para a zona marinha e de 10.91 Kg C mol^{-1} para a zona salobra. Os resultados mostram que a PBB tem sido subestimada no sistema estuarino da Ria de Aveiro. As experiências de DGGE realizadas ao longo dos ensaios dos factores de conversão mostram que a consituição da comunidade bacteriana das amostras originais é diferente da comunidade nas amostras filtradas e diluídas utilizadas para a determinação dos factores de conversão empíricos. Também foi observado que a estrutura da comunidade bacteriana muda ao longo dos tempos de incubação e que esta é seleccionada quando se adiciona [^{3}H]leucina às amostras. À luz das nossas descobertas concluímos que a PBB não tem sido correctamente determinada ao longo dos anos e que, para além de ser necessária a determinação de factores de conversão específicos para cada sistema, um novo problema surge quando a PBB é determinada com métodos radioactivos: a selecção da comunidade bacteriana, uma vez que a comunidade que incorpora a [^{3}H]leucina é diferente, tornando-se óbvio que esta afecta grandemente a constituição da comunidade bacteriana.

keywords

Heterotrophic bacterioplankton, leucine, leucine incorporation rate, isotope dilution, conversion factors, estuary, bacterial communities, bacterial biomass productivity, incubation conditions, denaturing gradient gel electrophoresis, microbial loop, biogeochemical carbon cycle

abstract

Heterotrophic bacterioplankton play a very important role in the ocean's biogeochemical carbon cycle. Bacterial biomass production (BBP) converts dissolved organic matter (DOM) into particulate organic matter (POM), which becomes available to the higher trophic levels of the food web. BBP is assessed by leucine incorporation rates. Two major problems are found with this method: the incubation conditions under which the leucine is incorporated and the use of specific conversion factors to an accurate measurement of BBP. In order to give an answer to these problems, water samples from two sampling stations (marine and brackish zone) of the estuarine system Ria de Aveiro were incubated under two main conditions (field and laboratory conditions) and specific conversion factors (both empirical and semitheoretical) were determined in both zones of the estuarine system. Concerning the incubation conditions assays we found that, in laboratory conditions (PAR light vs. dark), BBP was superior when samples were incubated in the dark and that in the field conditions (*in situ* light vs. *in situ* dark) no pattern of variation was observed. In the DGGE experiments it was found that with the exception of surface water of marine zone, the several incubation conditions were not generally representative of the original samples. BBP must be determined *in situ* conditions, but when it is not possible, laboratory incubation for BPP determination must be done in dark. From the experiments of the conversion factors it was determined an average isotope dilution and an average semitheoretical conversion factor of 5.07 and 7.51 Kg C mol^{-1} for the marine station and of 5.15 and 7.75 Kg C mol^{-1} for the brackish water station, respectively. An average empirical conversion factor of 20.18 Kg C mol^{-1} was obtained for the marine zone and of 10.91 Kg C mol^{-1} was obtained for the brackish water station. The results show that BBP has been underestimated in the estuarine system of Ria de Aveiro. The DGGE experiments performed throughout the empirical conversion factor assays show that bacterial assemblages of original samples are different from the filtered and diluted samples used for empirical factor determination. It was also observed that bacterial community structure changes over the incubation periods and that this one is selected when [^{3}H]leucine is added to the samples. In light of our findings we conclude that BBP has not been correctly measured over the years and that besides the necessity to determine the best incubation conditions and the specific conversion factors to each system, a new problem arises when BBP is determined with radioactive methods: the selection of bacterial community since community that uptakes the [^{3}H]leucine, becoming obvious that this one affects greatly bacterial community assemblages .

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LIST OF ACRONYMS AND ABREVIATIONS

PBB	Produtividade de Biomassa Bacteriana
MOD	Matéria Orgânica Dissolvida
MOP	Matéria Orgânica Particulada
PAR	Photosynthetically Active Radiation
DGGE	Denaturing Gradient Gel Electrophoresis
vs.	versus
^{3}H	Tritium
CO	Carbon Monoxide
CO_2	Carbon Dioxide
POC	Particulate Organic Carbon
DOC	Dissolved Organic Carbon
DIC	Dissolved Inorganic Carbon
DOM	Dissolved Organic Matter
POM	Particulate Organic Matter
CO_3^{2-}	Carbonate Ion
HCO_3^-	Bicarbonate Anion
GPP	Gross Primary Production
NPP	Net Primary Production
^{3}H-TdR	Tritiated Thymidine
^{3}H-Leu	Tritiated Leucine
^{3}He	Helium
DNA	Deoxyribonucleic Acid
rDNA	Recombinant Deoxyribonucleic Acid
PCR	Polymerase Chain Reaction
Na_2EDTA	Ethylene Diamine Tetracetic Acid Disodium
UPGMA	Unweighted Pair Group Method using Average linkages
^{14}C	Radiocarbon

BrDU	5-bromo-2’-deoxyuridine
TCA	Trichloroacetic acid
eCF	Empirical Conversion Factor
sCF	Semitheoretical Conversion Factor
DI	Diluição isotopic
ID	Isotope Dilution
GPS	Global Positioning System
UV	Ultraviolet radiation
LIR	Leucine Incorporation Rate
BM_f	Final Biomass
BM_0	Initial Biomass

Chapter 1 Introduction

Role of Bacteria in the Carbon Cycle

For many years bacteria were not considered to be an essential component of the biogeochemical processes in the ocean (Kirchman and Williams, 2000). Nowadays, this vision has changed and microorganisms have very important roles on earth's biogeochemical cycles. The most important biogeochemical cycles are those of the water, carbon and nitrogen.

Carbon, the basic building block of organic matter (Hessen and Anderson, 2008), is the most abundant nutrient in aquatic ecosystems (Quay et al., 1986) and is the currency of choice for the examination of the fate of primary production in the oceans (Kirchman and Williams, 2000). Carbon can exist in reduced forms, like methane and organic matter, and in oxidized forms, like carbon monoxide (CO) and carbon dioxide (CO_2) (Prescott et al., 2005).

The four main reservoirs of carbon are the atmosphere, the oceans, the reserves of fossil fuels and the terrestrial ecosystems, being the oceans the biggest reservoir with approximately 38 000 pg C (Houghton, 2007). Although the ocean's carbon cycle (Figure 1.1) is still far from being understood, it is clear that microorganisms have a very high influence on this part of the carbon cycle (Prescott et al., 2005). The ocean's carbon is exchanged with the atmosphere on a time-scale of several hundred years (Farquhar et al., 2001). The majority of carbon processing occurs in the water surface zone, being the main carbon pools the particulate organic carbon (POC), the dissolved organic carbon (DOC) and, in sediments, the methane hydrate (Prescott et al., 2005). The carbon pool is divided in three major fractions: DOC, POC and DIC (dissolved inorganic carbon) (Lopes et al., 2008).

With the increasing use of fossil fuel, the study of the carbon cycle became a priority. In 1998, with the signing of the Kyoto Protocol, several countries agreed to diminish the emissions of CO_2 and other greenhouse gases to the atmosphere (United Nations Framework Convention on Climate Change, 1998). Since the ocean acts as a major sink for CO_2 (Farquhar et al., 2001), and even the slightest variation in the biogeochemistry of marine carbon could cause deep changes in the atmospheric carbon levels (Lopes et al. 2008), it becomes clear the importance of the ocean's carbon cycle and its monitorization in this days.

It is due to the activities of cyanobacteria, green algae, photosynthetic bacteria and aerobic chemolithoautotrophs that carbon is fixated in the oceans (Prescott et al., 2005). A fraction of this carbon is released as dissolved organic matter (DOM) and recycled via the microbial loop (Anderson and Ducklow, 2001).

The Microbial Loop

The microbial loop (Figure 1.2) is among the new concepts that were added to microbiology and it is, perhaps, one of the most important in microbial ecology. This concept was first introduced in 1974 by Lawrence R. Pomeroy (Pomeroy, 1974) in a paper entitled "The Ocean's Food Web: A Changing Paradigm". In his paper, he draws attention to the role played by bacteria in the ocean's food web. In 1883, the term "microbial loop" is created by Azam et al. (1983) to describe the path of the dissolved organic matter as this one was utilized and transformed by bacteria. Bacteria were then consumed by protozoa which entered the food chain formed by larger creatures (Fenchel, 2008).

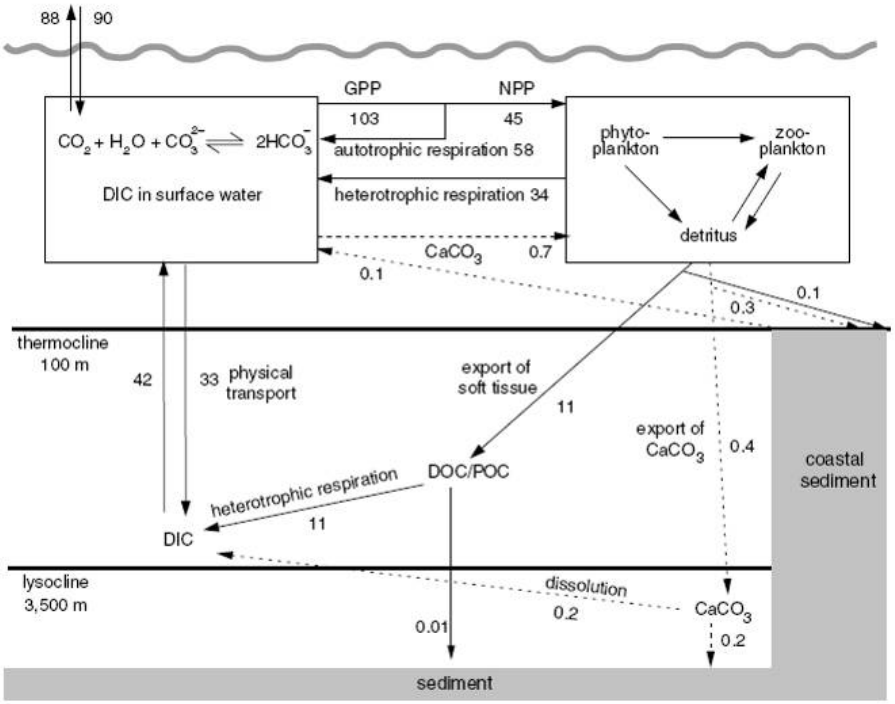

Figure 1.1 - The Ocean's Carbon Cycle. CO_2 is dissolved in the ocean in three main forms (CO_2, $\mathbf{CO_3^{2-}}$,$\mathbf{HCO_3^-}$). Through physical and biological processes dissolved inorganic carbon (DIC) is transported in the ocean. The total amount of carbon produced by photosynthesis is represented by GPP (gross primary production); the balance between GPP and the respiration of the autotrophic component of the system is represented by NPP (net primary production). The sink of dissolved organic carbon (DOC) and particulate organic carbon (POC) from biological origin results in a descending flux known as export production. The organic matter is transported and respired by non-photosynthetic organisms and is upwelled and returned to the atmosphere, remaining only a small amount buried in the deep-sea sediments. Source: Farquhar et al., 2001.

Nowadays, to describe the microbial loop is no longer simple task since it has been enlarged as new players were added, being the most recent the viruses (Fenchel, 2008). It regulates the transfer of energy and nutrients to higher trophic levels and has a high influence in the global carbon and nutrient cycles (Breitbart et al., 2004), since it is in the microbial loop that an immense fraction of carbon is remineralized (Hopkinson and Barbeau, 2008). The concept still has the same foundation: aquatic

bacterial populations utilize dissolved products of primary production recycling the primary production that would be inevitably lost to higher consumers back into biomass (Wommack and Colwell, 2000). The virus role is still uncertain, but it is currently known that they influence the cycling of organic matter (Winter et al., 2004), by infecting and killing bacteria (Breitbart et al., 2004), algae and cyanobacteria (Wommack and Colwell, 2000). When cell lysis occurs, the particulate organic matter is lost from the grazing food chain, but becomes available to heterotrophic bacteria (Bratbak and Heldal, 2000).

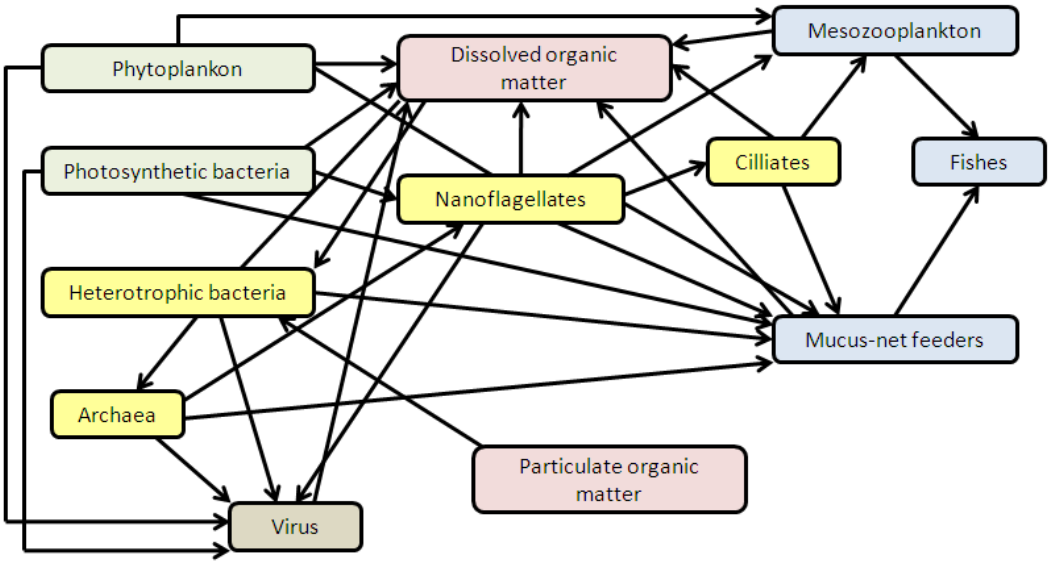

Figure 1.2 - The microbial loop. Source: Adaptation from Pomeroy et al. (2007) and Fenchel (2008).

Ultimately, and as Pomeroy et al. (2007) states, we are the top predator of the food web that has some of its beginning in the microbial loop. To have conscience of its importance, one must know that on average one-half of oceanic primary production is channeled by the microbial loop (Azam, 1998) and one-half of the oxygen we breathe is supplied to us by the microbial loop (Pomeroy et al., 2007).

Heterotrophic Bacterioplankton and Bacterial Biomass Production

This work is focused on one task of one player of the microbial loop: heterotrophic bacteria. The production of new bacterial biomass (bacterial biomass production) and the remineralization of organic carbon and nutrients are the two main tasks performed by them in the cycles of nutrients and carbon (del Giorgio and Cole, 1998). It is well known that heterotrophic bacteria play a vital role in the transformation and mineralization of organic matter in both aquatic and terrestrial environments (Kawasaki and Benner, 2006).

The importance of bacterial biomass production (BBP) was already shown previously: it converts dissolved organic matter (DOM) into particulate organic matter (POM), bacterial cells that become available to higher consumers (Cole and Pace, 1995). The estimation of BBP stands on the base of the estimation of the rates of metabolism and the amounts of organic matter (Riemann and Søndergaard, 1984). So, it becomes clear that the interactions between heterotrophic bacteria and organic matter are of utmost importance in the functioning of all the aquatic ecosystems (Chróst and Siuda, 2006), making the accurate measurement of BBP and the development of reliable methods into a primer objective in microbial ecology (Bååth, 1998).

There are several methods to determine BBP, but the most commonly used over the past 25 years have been the incorporation of [Methyl-^{3}H]thymidine (^{3}H-TdR) into bacterial DNA (Furhman and Azam, 1980) and the incorporation of [^{3}H]leucine (^{3}H-Leu) or [^{14}C]leucine (^{14}C-Leu) into bacterial protein (Kirchman et al, 1985). More recently, a new method based on the incorporation of 5-bromo-2'-deoxyuridine (BrDU) into DNA as an alternative to the use of the [Methyl-^{3}H]thymidine method and can be immunochemically detected with very high sensitivity (Hamazaki et al, 2007). This method is preferred because it doesn't need a separate and specialized laboratory, or any other safety precautions or authorizations to use radioactive material (Steward and Azam, 1999). Nevertheless, the use o ^{3}H-TdR and ^{3}H-Leu or ^{14}C-Leu still are the preferred methods.

To determine BBP in this work it was used the ^{3}H-Leu method, since this technique provides more-direct results than the ^{3}H-TdR because it measures the increase of the major biomass fraction (Fischer and Pusch, 1999). Because leucine constitutes a reasonably fraction of total amino acids and since protein is a major dry weight component, the ^{3}H-Leu method is more sensitive than the ^{3}H-TdR (Simon and Azam, 1989; Buensing and Marxsen, 2005). Besides, the ^{3}H-Leu method is also one order of magnitude more sensitive than the ^{3}H-TdR because the production of a bacterial cell needs 10 times the incorporation of leucine into protein than thymidine into DNA (Fischer and Pusch, 1999; Simon and Azam, 1989; Buensing and Marxsen, 2005). The ^{3}H-Leu incorporation technique can be used as an independent measure of total bacterial production (Kirchman et al., 1986).

As described by Kirchman et al. (1985), this method consists of adding radiolabeled leucine into bacterial protein and measuring the radioactive insoluble fraction in TCA (Kirchman et al., 1985). This insoluble fraction is constituted majorly by proteins (Miranda et al., 2007; Kirchman et al., 1985).

Of course that like most methods, there are some methodological problems that need to be solved if one wants to perform an accurate measurement BBP in a system. There are two major problems: the first is related with the accurate transformation from leucine incorporation rates into protein synthesis by the use of appropriate conversion factors for the system in which measurements of BBP are being done and; the second, concerns the incubation conditions when leucine is being incorporated by the bacterial cells.

The first problem can be divided into two "smaller" problems. The first one is the isotope dilution. In aquatic environments bacteria are surrounded by high concentrations of dissolved free amino acids (Jørgensen, 1992), such as leucine. Thus, if extracellular leucine incorporation happens instead of incorporation of added leucine, the rate of protein synthesis can be underestimated (Kirchman et al., 1986). Intracellular sources of leucine should also be taken into account, although this is very difficult to know (Gasol, 1999). Pollard and Moriarty (1984), described isotope dilution as the proportion in which an added radiolabeled substrate is incorporated in comparison with the exogenous concentration and substrate biosynthesis (Miranda et al. 2007). In order to correct isotope dilution, it has been suggested that leucine should be added in sufficiently high concentrations of leucine into the samples (Kirchman and Ducklow, 1993). Values of isotope dilution can be used to find the semitheoretical conversion factors (Gasol, 1999; Pedrós-Alió et al., 1999), which in turn will be used to determine BBP. So, it becomes clear that to measure BBP more accurately, isotope dilution values should be found for each experiment if one doesn't want underestimated BBP values. van Looij and Riemann (1993) compared high isotope dilution values in oligotrophic to mesotrophic environments with higher values obtained in eutrophic costal environments on which external leucine concentrations are higher.

As mentioned before, the conversion from leucine incorporation rate into the rate of protein synthesis requires the use of appropriate conversion factors (Kirchman et al.,1982). This brings the second "smaller" problem, which is the determination of empirical conversion factors. These conversion factors are different because, in order to calculate them, conversion factors experiments need to be performed (Bjørnsen and Kuparinen, 1991; Kirchman and Ducklow, 1993). The aim of the determination of the empirical conversion factors is to minimize the protein turnover (Gasol, 1999). In these experiments, a diluted sample (1:10) is incubated in the dark and sub-samples are removed in regular periods of time for measuring leucine incorporation rates and bacterial biomass. With these two variables, and using appropriate equations, empirical conversion factors are determined. There are four different ways to calculate the eCF: the derivative method (Kirchman et al., 1982), the modified derivative method (Ducklow and Hill, 1985), the integrative method (Fuhrman and Azam, 1980; Riemann et al., 1987) and the cumulative method (Bjørnsen and Kuparinen, 1991).These conversion factors are not the same as they change from season to season, different environments have different conversion factors and they even change accordingly to growth conditions and substrate composition (Riemann et al., 1990). So, it becomes obvious that using conversion factors determined to other systems is a wrong choice. There are several determined conversion factors (Table 1) for various systems.

Table 1 - Empirical conversion factors in various aquatic systems. eCF stands for empirical conversion factors and sCF stands for semitheoretical conversion factors. Source: Buesing and Marxsen (2005) and Gasol (1999).

System	Conversion Factor (Kg C mol^{-1})		Source
Equatorial Pacific	eCF	2.3	Kirchman et al. (1995); Ducklow et al. (1995)
Pacific Ocean (Oregon Coast, USA)			
Midshelf	eCF	2.5	Sherr et al (1999)
Slope		2.3	
Offshore		1.6	
NW Mediterranean Sea			
Offshore	eCF	0.3	Gasol et al. (1998)
Slope		1.5	
Coastal		2.1	
Southern Ocean	eCF	3.0	Bjørnsen and Kuparinen (1991)
Western Antarctic Waters			
Bransfield Strait	eCF	0.57	Pedrós-Alió et al. (2002)
Bellingshaussen Sea		0.89	
Sargasso, Caribbean and Gulf Stream	eCF	12.8 - 36.4	Rivkin and Anderson (1997)
		1.56	Carlson et al. (1996)
Western Mediterranean			
Coast	eCF	3.25	Pedrós-Alió et al. (1999)
Slope		1.5	
Open sea		0.29	
North Sea (Belgium)	eCF	3.95	Servais (1990)
Okefenokee Swamp (Georgia, USA)	eCF	8.6	Moran and Hodson (1992)
Eutrophic lakes (Northern Zealend, Denmark)	eCF	2.11	Jørgensen (1992)
Freshwater sediment (Breitenbach, Germany)	eCF	1.45	Buesing and Marxsen (2005)
	sCF	1.44	
Equatorial Atlantic Ocean	eCF	0.73	Pérez et al. (2005)
Lagoinha (eutrophicated lagoon, Brazil)	sCF	1.3	Miranda et al. (2007)
Paint Creek (Michigan, USA)	sCF	1.59 - 4.00	Gillies et al. (2006)
A Coruña (Galicia, Spain)	sCF	1.2 – 2.1	Valencia et al. (2003)
Seawater culture	sCF	1.55	Simon and Azam (1989)
Lake water	sCF	1.36	Jørgensen (1992)
River Sediments (River Spree, Germany)	sCF	1.7 – 25.81	Fischer and Pusch (1999)

eCF – empirical conversion factor
sCF – semitheoretical conversion factor

One of the problems that exist in long term incubations, such as experiments to estimate bacterial growth efficiency or to determine the empirical conversion factors is that in these experiments the grazing of bacterioplankton by protists is diminished because water samples are diluted or filtrated (Massana et al., 2001). As a result, the bacterial populations present in the samples of the experiments are not going to be the same as those of in situ (Gasol et al., 2008; Suzuki, 1999), turning these methods less reliable.

A supplementary problem, also raised in this study is the negative effects on bacterioplankton caused by the tritium applied on the radioactively marking of leucine used in the BBP method. To my knowledge no recent works have been done on this matter. However, there are a few studies done between the 60's and the 80's linking bacterial death and mutagenic effects of tritium. As an example Person and Brockrath Jr. (1964) referred that killing efficiencies caused by the decay of ^{3}H-Leu were lethal and mutagenic, although in a smaller way than the effects caused by the decay of ^{3}H-TdR. Higo and Yakamoto (1985) considered that the biological effects of tritium exposure should be of great concern. These two studies were made with *Escherichia coli*. Nevertheless, several studies have been done relating the exposure of tritium with several types of cancer (Fairlie, 2007). It is important to be aware of tritium's physics. Tritium is the radioactive isotope of hydrogen, and has a radiobiological half-life of 12.3 years and decays to the stable isotope helium (^{3}He) emitting a beta particle. Because the average track length of tritium beta particle is of the same magnitude as the diameter of the human chromosome this turns the DNA an important target (Fairlie, 2007). So, one must wonder: if tritium is so harmful to the human cell, how harmful is he to bacterial cells?

The other major problem with the ^{3}H-Leu incorporation technique, as referred, the incubation conditions used to incubate the samples. After adding the appropriate concentration of ^{3}H-Leu into the samples an incubation is necessary in order to give time for the bacterial cells to incorporate that leucine (Kirchman et al., 1985). Normally, those incubations are performed in the dark (Morán et al., 2001), but questions about this matter arose approximately 10 years ago. In order to answer these questions several authors performed some studies to evaluate the effects of several incubation conditions, such as PAR light, ultraviolet radiation and dark (Morán et al., 2001; Sommaruga et al., 1997; Pakulski et al., 1998; Aas et al., 1996; Jeffrey et al., 1996, Michelou et al., 2007). Aas et al. (1996) and Michelou et al. (2007) found an increase in the leucine incorporation rate when samples were incubated under PAR light. On the contrary, Sommaruga et al. (2007) found inhibitory effects of light in leucine incorporation rates. Other authors, such as Morán et al. (2001) found both inhibitory and stimulatory effects of PAR light in the leucine incorporation rate. There are several suggestions to justify these differences and variations on these studies, such as the presence of photoheterotrophs in the water samples, indirect effects due to enhanced phytoplanktonic release of DOM caused by light stress, cyanobacteria responsible for the uptake of labeled substrates in light incubations, the negative impact caused by light on viruses and protistan grazers when compared with bacteria, photochemical transformations of DOM, the trophic state of an ecosystem or even the community structure of the samples (revision of Gasol et al., 2008). Incubations in the dark avoid the problem of reproducing *in situ* levels of light and avoid the possible stimulatory effect of primary production (Morán et al., 2001). Nowadays, because we are facing the stratospheric ozone depletion, the importance of solar radiation and its consequence on aquatic systems has increased majorly (Pakulski et al., 1998).

Thesis Outline

The aim of this work is to find specific conversion factors and to determine the effects of incubation conditions for BBP, in the estuarine system of Ria de Aveiro. Another objective is to assess if the bacterial community structure changes over the course of the conversion factors experiments or if it changes when samples are incubated under different incubation conditions. Finally, we will also try to see if the incubations performed with [^{3}H]Leucine have some influence on the bacterial.

Chapter 2 describes the several assays performed at field and laboratory conditions in order to assess the incubation conditions under which leucine should be incorporated. In this chapter it will be also described the changes on bacterial community structure between the different incubation conditions tested, using denaturing gradient gel electrophoresis (DGGE).

Chapter 3 describes a series of assays performed in order to determine isotope dilution (and semitheoretical conversion factors) and empirical conversion factors, for the estuarine system of Ria de Aveiro. In this chapter it will also be found two assays performed with DGGE, in order to assess the changes of bacterial community assemblages along the empirical factor experiments and between several treatments.

Chapter 4 discusses the results obtained and the main conclusions of this work and also makes suggestions for future work.

Chapter 2 Influence of Light Incubation Conditions for the Estimation of Bacterial Biomass Productivity in an Estuarine System

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Abstract:

The role of heterotrophic bacterioplankton in the ocean's carbon cycle has been well described over the years. The incorporation of leucine into bacterial proteins is a measure for bacterial biomass productivity (BBP). One of the problems with this method is the incubation conditions. In this work it was tested two different incubation conditions for BBP assessment: field conditions (*in situ* light vs. *in situ* dark) and laboratory conditions (PAR light vs. dark). A significant difference was observed between the field and the laboratory incubation conditions. In the laboratory conditions a well defined pattern of variation was detected but, in field conditions, no clear pattern was observed. Variations in field conditions are perhaps due to the physical and chemical characteristics of the water column and of the weather conditions. Bacterial community composition is substantially different in the various incubation conditions used for BBP determination. These incubation conditions alter considerably the bacterial community composition in both surface and bottom layers. In order to obtain more accurate values, BBP should be measured at *in situ* conditions. When this is not possible and BBP determination must be done in laboratory conditions, incubations should be carried out in dark conditions.

Keywords: Heterotrophic bacterioplankton, bacterial biomass productivity, incubation conditions, bacterial communities, denaturing gradient gel electrophoresis

Introduction:

Heterotrophic bacterioplankton plays a vital function in the dissolved organic matter incorporation ("microbial loop") and in the mineralization of organic carbon and nutrients, being a key in the organic matter processing in aquatic ecosystems (Ghiglione et al., 2007; Ram et al. 2007; Valencia et al. 2003). In coastal waters they may utilize as much as 40% of the carbon fixed by primary producers (Cho and Azam, 1990; Ducklow and Carlson, 1992; Cole et al., 1988). Heterotrophic bacterioplankton converts dissolved organic carbon (DOC) into particulate organic carbon (POC) (i.e. bacterial biomass) and making it available to higher trophic levels of the food web (Cole and Pace, 1995), processing roughly as much energy as the classical grazing food chain (Riemann and Søndergaard, 1986). Bacterial biomass can reach frequently 10 to 30% of the living carbon biomass (Cho and Azam, 1990) Moreover, they also respire organic carbon to inorganic carbon (i.e. bacterial respiration) (del Giorgio and Cole, 1998), making bacterial respiration the major fraction of the total measured respiration in most aquatic systems (Williams, 1981). True estimates of the

contribution of the microbial loop to the aquatic carbon cycle can only be achieved upon an accurate estimation of the fraction of organic matter incorporated, assimilated and respired by heterotrophic bacterioplankton (Morán et al., 2001).

Due to the major role of organic carbon transformation in aquatic trophic net, measurements of bacterial biomass productivity [BBP] need to be precise (Buensing and Marxen, 2005). This measurement has been traditionally done by incorporation of radiolabeled substrates into bacterial cells (Valencia et al., 2003) followed by conversion of the incorporating rates into carbon units (Morán et al, 2001). The two main methods to estimate BBP are the incorporation of radiolabeled thymidine (TdR) into bacterial DNA (Fuhrman and Azam, 1980; Fuhrman and Azam, 1982) and the incorporation of radiolabeled leucine (Leu) into bacterial proteins (Chin-Leo and Kirchman, 1988; Simon and Azam, 1989), which is considered to be a finer technique by many researchers (Buensing and Marxsen, 2005). However, both these techniques share some limitations. One of those limitations is the dark incubations of the samples for BBP determination (Morán et al. 2001; Aas et al, 1996; Pakulski et al., 1998) intended to avoid the problem of reproducing the levels of ambient light (Morán et al. 2001), remove the stimulatory effects of primary production and eliminate the harmful effects of solar radiation on bacteria (Aas et al., 1996). Furthermore, upon exposure of sunlight, highly variable results have been reported (Sommaruga et al, 1997), probably as a result of photodegradation or photoalteration of the radiolabeled substrates before they are incorporated by bacteria. Recently, Gasol et al. (2008) makes a compilation of studies done by other authors, where in some there is stimulation of leucine incorporation rates by PAR light irradiation and in others, by the contrary, there's a strong inhibition.

In order to assess if typical dark incubation affect the determination of BBP, different incubation conditions to calculate bacterial productivity were tested, namely, in the dark, upon exposure to photosynthetically active radiation (PAR) (380 to 700 nm), *in situ* (PAR solar radiation) and *in situ* dark. Changes in the composition of bacterial communities during the incubation period were also monitored for the different conditions tested by denaturing gradient gel electrophoresis (DGGE).

Materials and Methods

Study Site:

Ria de Aveiro (Figure 2.1) is a coastal lagoon located at the northwest coast of Portugal. It has a maximum length of 45 km and maximum width of 10 km, and is connected with the Atlantic Ocean by a narrow artificial channel, named Barra, that has a 1.3 km of length and a medium depth of 20 m. This lagoon consists of a high complex system of channels: it has four long major channels (S. Jacinto, Ovar, Mira and Ílhavo) that ramify into other smaller channels and is supplied in freshwater by two

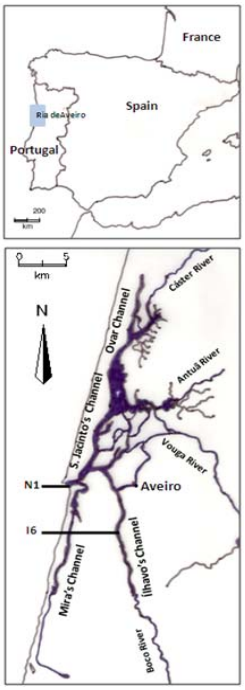

Figure 2.1- The estuarine system of Ria de Aveiro. The arrows indicate the two sampling stations (N1 and I6).

main rivers (Vouga and the Antuã river) and two minor rivers (Boco and the Cáster river). In general, Ria de Aveiro is very shallow with depths ranging from 1 to 3 m. During spring high tides, it can cover an area of 83 km^2 and at spring low tide it covers an area of 66 km^2 (Leandro et al., 2007).

The water circulation is mainly regulated by tides and, occasionally, by elevated river discharges due heavy rain periods. In the central area of the lagoon, close to the mouth, the estimated residence time is lower than 2 days, promoting a high marine influence in this area while in the upper areas of the lagoon the residence time can be higher than 1 week (Leandro et al., 2007).

This estuary suffers high anthropogenic pressure by harboring navigation and recreation facilities, supporting extensive agricultural fields and a various number of small and medium industries along its borders.

Water samples were collected at two sampling stations: N1 (N 40^o 38' 42", W 08^o 45' 25"), located in Barra, at the mouth of the estuary, representative a deep marine zone, with a maximum depth of 20 m; and I6 (N 40^o 35' 41", W 08^o 41' 21"), located in Ílhavo's Channel, representing the shallow waters of a brackish zone, with a maximum depth of 2 m.

Sampling

Samples were collected with a Van Dorn Bottle at low tide, in four different dates (11th of October, 2007; 28th of November, 2007; 22nd of April, 2008 and 4th July, 2008), and at the depths of 0.5 m and 18 m below surface for N1 and 0.5 m and 1.5 m below surface for I6. The surface depths are represented by letter B (N1B and I6B) and, the bottom depths by letter E (N1E and I6E). The location of both sampling sites was kept in all four sampling dates, with the resource of a GPS (Magellan GPS 315).

Physical and Chemical Characterization of Water Column and Weather Conditions

Physical and chemical characteristics were determined on the field. Irradiation was obtained with a light meter (LI-COR Model LI-250). Dissolved oxygen concentration was determined with an oxygen meter (WTW Oxi 197). Temperature and salinity were measured with a conductivity meter (WTW Cond 330i/SET) and the pH was determined with a pH meter (WTW 196).

In order to characterize the conditions in the collection days it was done an intensive research on the weather conditions. Percentage of cloud cover, air temperature, wind velocity and wave high were obtained from the archives of the website WindGURU. Precipitation was gently provided by the Physic's Department of the University of Aveiro. Ultraviolet radiation index was acquired in the archives of the website of TEMIS (Tropospheric Emission Monitoring Internet Service).

Experimental Setup

After collection, part of the samples was processed directly to determine BBP in field conditions and the other part was transported to the laboratory, and processed within the maximum of 2 hours after collection for BBP in laboratory conditions and within 6 hours for DGGE analysis.

Field conditions **–** Samples were dispensed into Nalgene tubes (Nalgene 3137 Oak Ridge Centrifuge Tubes), that only let PAR light to pass, and put in polyethylene transparent bags (*in situ* light condition). The bags were than tide up into a rope, and send into the water at the corresponding sampling depth, at each sampling site. Surface samples were also dispensed into 20 ml scintillation vials, previously covered with aluminum paper (*in situ* dark condition), and put in the same polyethylene transparent bags as described for *in situ light* condition. This last condition was not tested for the bottom samples, since virtually no light reaches the bottom.

Laboratory conditions **–** Samples were also dispensed into Nalgene tubes and put in polyethylene transparent bags. After these, the bags were placed into an open glass container (47.5 x 29 x 31.5 cm), with water at *in situ* temperature, and samples were irradiated with PAR radiation (13 lamps OSRAM21 of 18 W each one), with a fluence rate of 40 W m^{-2}, measured with a light meter (LI-COR Model LI-250) (PAR light condition). Samples were also dispensed into 20 ml scintillation vials and kept in the dark, at in situ temperature (dark conditions).

Bacterial Biomass Productivity (BBP)

BBP was determined using 10 ml triplicate sub-samples plus a control fixed with 475 µl of formaldehyde. Samples were incubated at a previously determined saturating concentration (83.2 nM) of ^{3}H-Leucine (Amersham, specific activity 63.0 Ci/nmol) plus cold leucine, for one hour at the different incubation conditions described in the experimental setup.

Incubations were stopped with 475 µl of formaldehyde. After 15 minutes incubation on ice, it was added 1 ml of ice cold trichloroacetic acid (TCA) (20%) to the sub-samples and to the control, followed by 15 minutes incubation. Subsamples and control were than filtered through 0.2 µm polycarbonate membranes (Poretics) and rinsed two times with 2 ml of ice cold TCA (5%) and 5 ml of ice cold ethanol (90%). The membranes were then placed into 5 ml scintillation vials to which was added 4.5 ml of scintillation cocktail UniverSol (ICN Biomedicals, USA). After a period of 3 days, the radioactivity was measured in a Beckman LS 6000 IC liquid scintillation counter. The conversion of leucine incorporation rates to carbon units was accomplished according to Simon and Azam (1989).

DNA extraction, PCR and DGGE

DGGE was only performed in the last sampling date. For this assay, samples were dispensed into laboratory bottles (SHOTT DURAN) of 500 ml, and were incubated along with the BBP tubes.

Triplicate sub-samples of 250 ml were incubated with the same saturating concentration of ^{3}H-Leucine plus cold leucine as the BBP samples, for one hour at *in situ* light condition and laboratory conditions. DGGE was also performed from the original sample, i.e. sample without incubation. The V6-V8 region of 16S rDNA was amplified by PCR from DNA extracts using the primer pair F968GC and R1401 (Nubel et al., 1996). Amplification was performed in 25µL reaction mixtures containing 1 x PCR buffer (PCR buffer without $MgCl_2$: PCR buffer with KCl_2, 1:1), 2.75 Mm $MgCl_2$, 0.2 mM of each nucleotide, 0.1 µM of each primer, 1 U of Taq Polymerase (all reagents were purchased from MBI Fermentas, Vilnius, Lithuania). The template DNA amount was approximately 50 to 100 ng per PCR. Acetamide (50%; 5uL) was added to the reaction mixture to facilitate the denaturation of double-stranded DNA and to circumvent the formation of secondary structures. The reactions were carried out in a MultiGene Gradient Thermal Cycler from MIDSCI. After 5 min of denaturation at 94ºC and 35 thermal cycles of 1 min at 95ºC, 1 min at 53ºC and 2 min at 72ºC, PCR was finished by an extension step at 72ºC for 10 min. Amplification was confirmed by electrophoresis in 1.5% (wt/volt) agarose gels and ethidium bromide staining.

Samples containing approximately equal amounts of PCR amplicons were analysed by DGGE with a denaturing gradient of 40 to 80% of the denaturant and DGGE was performed with 0.5x TAE buffer (20 mM Tris-acetate, pH 7.4, 10 mM sodium acetate, 0.5 mM Na_2EDTA) at 60ºC at a constant voltage of 150V for 16h. The gels were air dried after silver staining according to Heuer et al. (1999) and scanned transmissively. The bacterial community profiles of the images of DGGE gels were analyzed with GelCompar 4.0 program (Applied Maths) as described by Smalla et al. (2001). The images were processed using the rolling disk method with an intensity of 8 (relative units) to subtract the background. After this, a dendogram was constructed using the Pearson correlation index (r) calculated for each pair of lanes within each gel and the cluster analysis was calculated through the unweighted pair group method using average linkages (UPGMA).

Statistical Methods

To verify if there were any significant differences between the incubation conditions (*in situ* light vs. *in situ* dark and PAR light vs. dark) it was applied the t-test (SPSS 16.0) after verifying if there was a normal distribution of data using the Kolmogorv-Smirnov test. With a P-value below 0.05 the null hypothesis (no significant differences between the incubation conditions) was rejected in favor of the alternative hypothesis (there is a significant difference between the incubation conditions). PRIMER v5 was used for data analysis. To verify which environmental (physical and chemical) characteristics best explained the BBP patterns at *in situ* condition, it was performed the BioEnv function (PRIMER 5.0). BioEnv uses the Spearman Rank Correlation Coefficient and, although it doesn't prove that a relationship exists, it is strong evidence that it might exists.

Results:

Physical and Chemical Characterization of Water Column and Weather Conditions

At the four sampling dates low tide was always approximately at the same hour (Table 2). The two first sampling dates presented a clear sky and no precipitation. On the other hand, on April and July, there was a cloud cover of 89 and 77%, respectively and there was some precipitation (Table 2). April highlights by having the highest precipitation value 15 days prior to collection. Samples from I6 station, in April presented a clayey aspect. Ultraviolet index was not very high in neither of the sampling dates, being the lowest value in November (1) and the highest in July (4) (Table 2). The highest value of air temperature was of registered in July and the lowest in November (Table 2). Wind velocity and wave high were higher in July and April and lower in November (Table 2).

Table 2 - Sampling date conditions.

Date	11 Oct 2007	28 Nov 2007	22 Apr 2008	4 July 2008
Low Tide Hour[a]	10 h 43 m	11 h 46 m	11 h 47 m	11 h 25 m
Cloud Cover (%)	0	0	89	77
Air Temperature (ºC)	19.5	11.0	14.5	20.5
Wave High [b] (m)	1.35	0.25	1.40	1.45
Wind Velocity (m s^{-2})	3.50	1.50	5.00	5.00
Precipitation [c] (mm)	30.5	61.8	235.8	5.0
Precipitation Day [d] (mm)	0	0	0,3	3.0
UV index [e]	2	1	3	4

[a] At I6 sampling station low tide hour has an adition of 1.5 hours relatively to N1 sampling station.
[b] Measurements for N1 Station.
[c] Total precipitation 15 days prior to collection.
[d] Precipitation in collection day.
[e] Erythemal UV index.

Salinity values ranged from 20.0 to 35.7 PSU (average 31.94 PSU) at N1 station and from 3.0 to 33.3 (24.17 PSU) at I6 station (Table 3). This variation is consistent with heavy rain periods in the 15 days prior to collection in the third sampling date, where salinity registered the lowest values in Ria de Aveiro hydrographic basin. The average water temperature was 16.96 ºC (range 13.6 to 18.7 ºC) at N1 station and 19.01 ºC (range 12.0 to 22.5 ºC) at I6 station (Table 3). The values of dissolved oxygen also registered a considerable variation, ranging from 1.07 to 7.72 mg l^{-1} (average 4.39 mg l^{-1}) at N1 station and from 0.82 to 6.79 mg l^{-1} (average 3.79 mg l^{-1}) at I6 station (Table 3). Like salinity, the lowest values of dissolved oxygen were also obtained in the third sampling date. pH values were relatively consistent, with an average of 8.20 (range 8.06 to 8.34) at N1 station and of 7.83 (range 7.71 to 7.95) at I6 station (Table 3). As for air irradiation, the highest values were obtained in the second and in the first sampling date for N1 and I6, respectively (Table 3).

Bacterial biomass production and community structure variations in field and laboratory conditions

In laboratory conditions (Figure 2.2), BBP showed a clear pattern of variation with the highest values in the dark but, in field conditions (Figure 2.3) this pattern of variation was not so distinct. At N1 station, water samples from 0.5 m depth showed higher BBP values when they were incubated at *in situ* light conditions than at *in situ* dark conditions. However, in the surface samples of I6 station this pattern of variation was generally the opposite with higher values of BBP when incubations were performed at *in situ* dark conditions (Figure 2.3). For the laboratory incubation conditions (PAR light vs. dark) the differences were significant for most of all the sampling dates and sampling sites. The differences between the field incubation conditions (*in situ* light vs. *in situ* dark) were also significantly different ($P<0.05$) with the exception of the surface sample of the N1 sampling station in November.

In field conditions (Figure 2.3) and for N1 sampling station it was observed that, in 90% of the cases, values of BBP were higher at surface samples than in the bottom. At I6 sampling station this pattern of variation was only observed in 60% of the cases. In laboratory conditions (Figure 2.2), and at N1 station BBP was only superior at the surface samples in 50% of the cases where as at I6 station this was observed at almost 65% of the cases.

In the field experiments, BBP was superior at I6 station than at N1, for *in situ* dark incubation conditions and at both depths, and also generally higher for *in situ* light conditions. In laboratory conditions, BBP was higher twice the times at I6 station than at N1 station, for both conditions. BBP was in general almost three times superior in the laboratory than in the field experiments.

For N1, at 0.5 m below surface, the environmental characteristics that best explained the BBP pattern were the pH and the wind velocity with a correlation coefficient of 1.00. For N1, at 18 m below surface, the environmental characteristics that best explained the BBP pattern were the water temperature, water irradiation and pH, with a correlation coefficient of 0.771. The environmental characteristics that best explained the BBP pattern for I6, at 0.5 m below surface were the water irradiation, oxygen and pH, with a correlation coefficient of 0.657. For I6, at 1.5 m below surface, the environmental characteristic that best explained the BBP patterns were salinity, oxygen and pH, with a correlation coefficient of 0.943.

From the cluster analysis of the DGGE gel, at N1 sampling station (Figure 2.4) for the surface water, it is clear that there are two main groups with 50% of similarity: one containing the sample incubated under PAR light and the other containing the rest of the samples. In this last cluster it is clear an isolation of the sample incubated in the dark relatively to the original sample and *in situ* light incubated samples. For the bottom surface of N1 station it is also evident the presence of two groups but with only 10% of similarity: one with the original sample and the other with the remaining

samples. In this last cluster samples incubated at *in situ* light conditions are isolated from those incubated in laboratory conditions (80% of similarity).

Table 3 - Physical and Chemical Characterization of the Water Column

Station	Date	Depth (m)	Salinity (PSU)	Irradiation Water (W m^{-2})	Irradiation Air (W m^{-2})	Temperature (ºC)	Dissolved Oxygen (mg l^{-1})	pH
N1	11 Oct 2007	0.5	35.4	64.90	199.80	18.7	7.72	8.06
		18	35.7	0.22		18.4	7.58	8.21
	28 Nov 2007	0.5	34.7	71.10	202.10	13.6	3.46	8.14
		18	35.1	0.50		13.6	3.08	8.15
	22 Apr 2008	0.5	20.0	74.34	183.06	17.4	1.11	8.29
		18	23.0	0.04		17.7	1.07	8.33
	4 July 2008	0.5	33.4	31.00	99.99	18.4	5.41	8.12
		18	33.4	0		17.9	5.7	8.34
I6	11 Oct 2007	0.5	32.9	132.38	375.1	21.1	5.83	7.81
		1.5	33.3	8.54		20.0	6.79	7.83
	28 Nov 2007	0.5	29.3	7.69	188.98	12.7	2.93	7.95
		1.5	29.7	4.44		12.0	2.88	7.92
	22 Apr 2008	0.5	3.0	44.76	202.9	20.9	0.82	7.71
		1.5	3.0	0.34		20.4	0.9	7.83
	4 July 2008	0.5	31.1	31.24	61.77	22.5	5.35	7.79
		1.5	31.1	0		22.5	4.82	7.81

Still at N1 station, from the analysis of the DGGE gel (Figure 2.5), there is the presence of two main groups: one holding the surface sample and the other holding the bottom sample.

In the DGGE gel (Figure 2.6), for I6 sampling station at 0.5 m below surface (Figure 2.8) it is clear the presence of two main groups: one containing the original sample and the other containing the samples incubated at *in situ* light condition, under PAR light and in the dark. At 1.5 m below surface the DGGE gel analysis shows also two distinct groups: one holding the samples incubated in laboratory conditions (PAR light and dark) and the other containing the original sample and samples incubated at *in situ* light conditions.

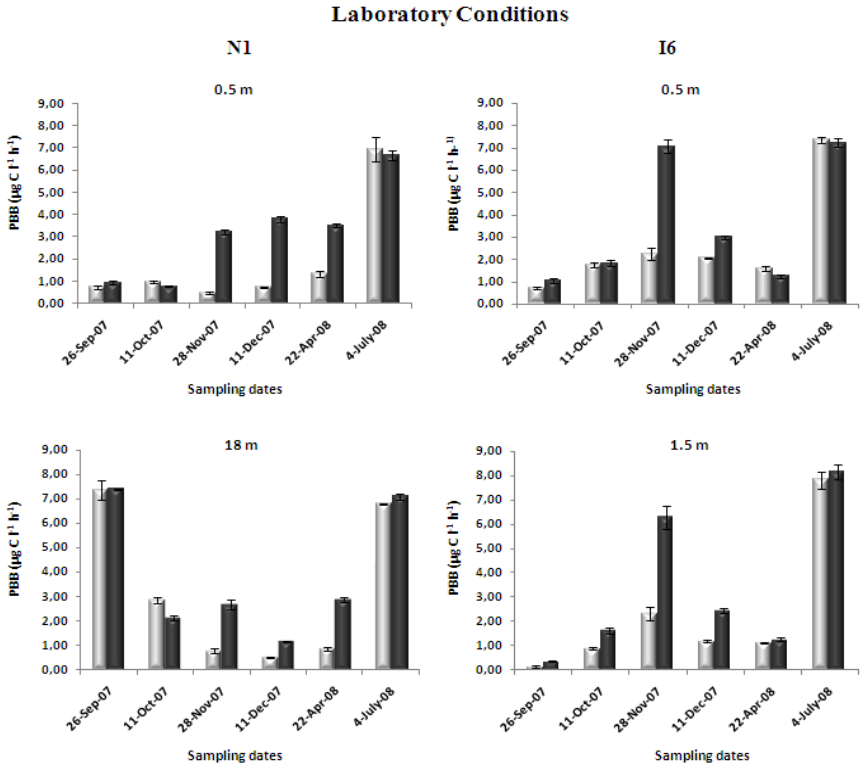

Figure 2.2- BBP for the six sampling dates and in the two sampling stations for laboratory incubation conditions. Grey bars represent the PAR light incubation condition and black bars represent dark incubation condition.

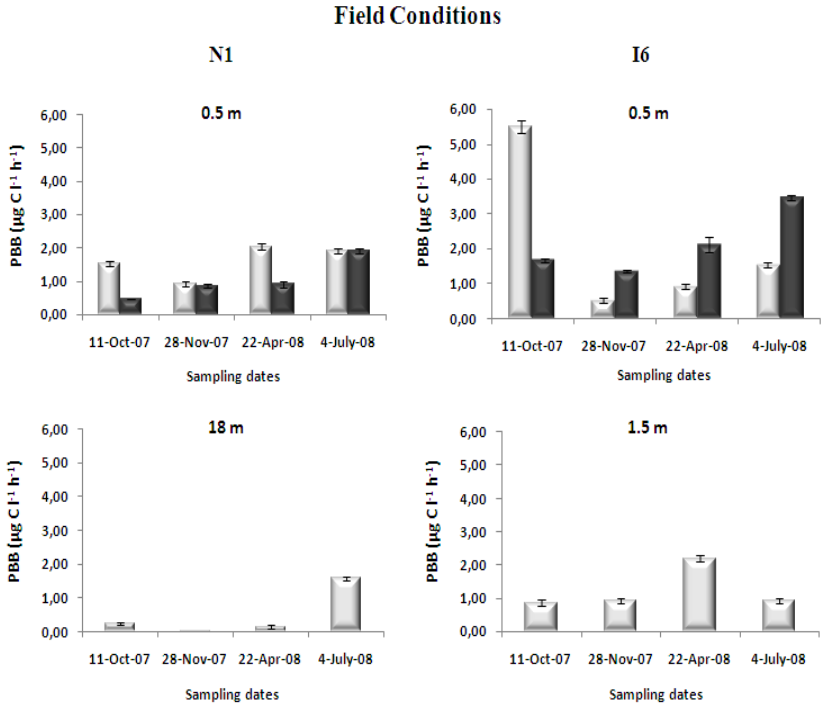

Figure 2.3 - BBP for the four sampling dates at the two sampling stations for field incubation conditions. Grey bars represent the *in situ* light incubation condition and black bars represent *in situ* dark incubation condition. At N1, for 18 m depth values obtained for BBP at the November campaign were very low (0.012 µg C L^{-1} h^{-1}).

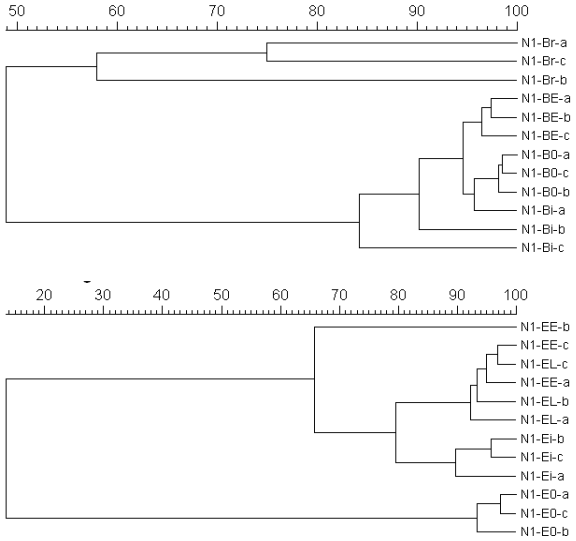

Figure 2.4 - Relationships of community structures between incubation conditions in the two depths of N1 sampling station. The several incubation conditions are represented by the letters: *i* (*in situ* light incubation condition), *L/r* (PAR light incubation condition), *E* (dark incubation condition) and O (original sample without incubation).

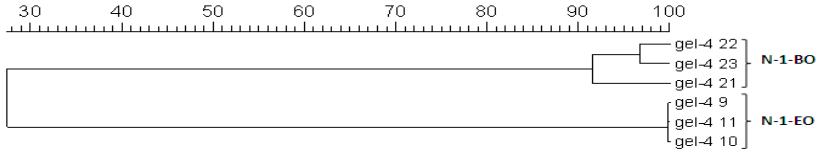

Figure 2.5 - Relationship of community structures between the original samples from depth B (0.5 me below surface) and depth E (18 m below surface), at N1 sampling station.

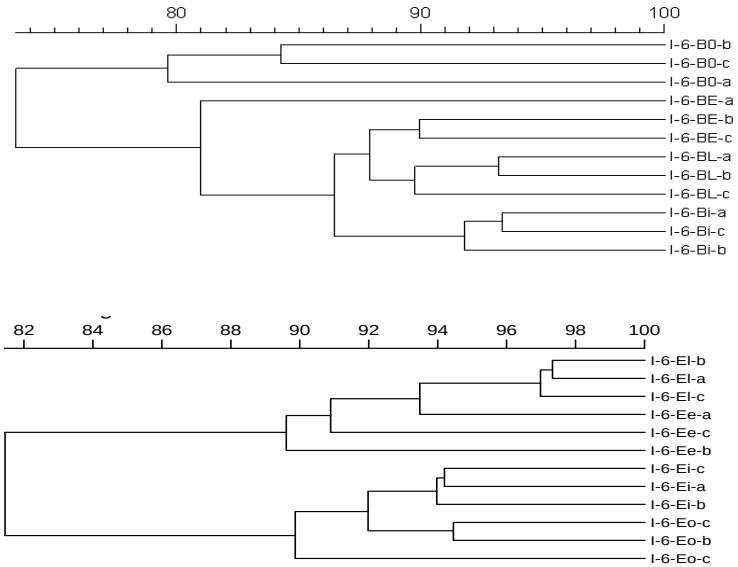

Figure 2.6 - Relationship of community structures structures between incubation conditions in the two dephts of I6 sampling station. The several incubation conditions are represented by the letters: *i* (*in situ* light incubation condition), *L* (PAR light incubation condition), *E* (dark incubation condition) and O (original sample without incubation).

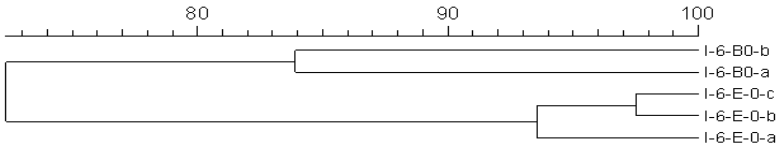

Figure 2.7 - Relationship of community structures between the original samples from depth B (0.5 m below surface) and depth E (1.5 m below surface), at I6 station.

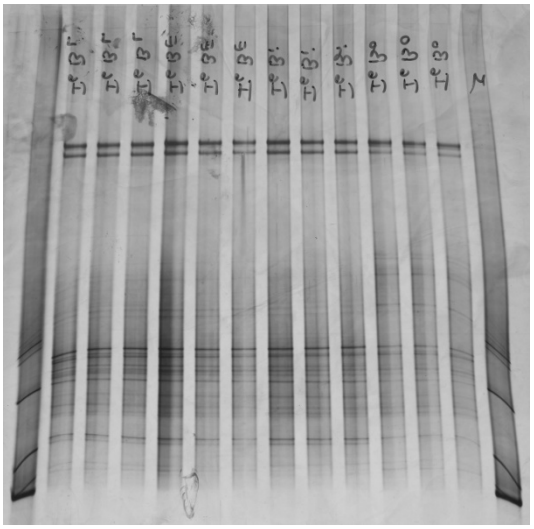

Figure 2.8 - Structure of bacterial communities in the surface water sample at I6 station .The several incubation conditions are represented by the letters: *i* (*in situ* light incubation condition), *L* (PAR light incubation condition), *E* (dark incubation condition) and O (original sample without incubation).

In the DGGE gel (Figure 2.7), there is the presence of two distinct groups each one holding the two different depths (0.5 m and 1.5 m below surface) for I6 sampling station.

Discussion

Since bacteria play a most important role in the organic matter transformation in aquatic systems (Ghiglione et al., 2007; Ram et al. 2007; Valencia et al. 2003), bacterial biomass productivity (BBP) must be precise (Buensing and Marxen, 2005).

Our results showed that BBP determined in field experiments is significantly ($p < 0.05$) different from that determined in laboratory, being this last one the condition frequently used to assess BBP in aquatic environments. In general, values of BBP were higher in laboratory conditions. Consequently, for an accurate determination of BBP in aquatic systems, water samples must be incubated at *in situ* light condition. If BBP is to be determined in laboratory, theoretically a better assessment should be achieved if incubations are done in PAR light conditions for surface samples and in dark conditions for bottom samples. However, the results of this study do not allow to take this conclusion and it is difficult to decide which laboratory conditions must be used. Under these conditions both surface and bottom water samples presented, in a general way, higher values of BBP when incubated under dark conditions relatively to PAR conditions, in the two sampling stations.

Higher values of leucine incorporation rates (LIR) were already detected in dark incubation conditions relatively to PAR radiation in other studies (Sommaruga et al., 1997; Morán et al., 2001). These authors explained the lower values of LIR when incubations were made under PAR light irradiation with photodynamic processes that reduced the transport of leucine to the cell, causing a decrease on leucine uptake under those conditions. These same authors also justify the higher values of LIR when incubations are performed in the dark as a result of the possible lack of competition for amino acids by phytoplankton.

The stimulation of BBP in dark laboratory condition is high enough to attenuate the differences observed between the two sampling stations when samples are incubated in field conditions, as well as the differences between surface and bottom samples, observed mainly in the marine zone.

In order to clarify the best way to determine BBP, bacterial community structure was analyzed during the different incubation conditions. The results confirm that incubation of surface waters at *in situ* light condition is the best way to estimate bacterial BBP. At N1 station, for surface water sample this condition is the most similar (similarity of 90%) to the original water sample (without leucine and without incubation). For bottom waters of the marine zone, however, the *in situ* light condition is not representative of original water. In laboratory condition, the best way to determine BBP, at water surface is dark incubation. Bacterial community structure is similar (similarity of 94%) from that of the original sample and of the *in situ* light condition. For the bottom samples, bacterial community structure under PAR light and dark condition are similar, but quite different from the original sample (similarity of 14%). Nevertheless, since at bottom bacterial communities are not exposed to light (only 0.095 and 1.198% of light reaches the bottom at N1 and I6, respectively), the chosen incubation should be the dark condition.

At I6 sampling station, for the surface water, none of the conditions tested is representative of the original sample. The similarity between this one and the several incubation conditions is of 84%. The laboratory incubation conditions are more similar (98% of similarity) between each other than with the *in situ* light incubation condition (96% of similarity), but they are all quite close. However, for the bottom samples the in situ light condition is representative of the original sample (similarity of 92%). For this depth the laboratory conditions were quite different from the original sample and the sample incubated at *in situ* light condition.

The high differences of bacterial composition between the surface and bottom for the original samples, namely in the marine zone (similarity of 28% for N1), can explain the differences in BBP observed in both surface and bottom samples, which are also superior in the marine zone. The differences observed between the two communities, particularly in the marine zone, results of the prevalent environmental characteristics such as the water column depth, turbidity, organic matter

quality and quantity, water irradiation, among others. In the brackish water zone, the turbid shallow water column make BBP more stable down the water column.

The elevated values of BBP at in situ light conditions in the surface samples of the marine zone compared to those of the bottom samples suggest that bacterial activity at surface can be stimulated by photodegradation of recalcitrant organic matter as observed before in this estuarine system (Almeida et al., 2001; Santos et al., 2007). On the other hand, these superior values of BBP can also be explained by the high activity of photoheterotrophs and cyanobacteria responsible for the uptake of the substrates under light incubation conditions (Gasol et al., 2008) or as well by the incorporation of amino acids, such as leucine, by phytoplankton under light conditions (Paerl, 1991; Morán et al., 2001). These three factors can also explain the lower values of BBP in the samples collected at 18 m, where photodegradation does not occur and the activity of photoheterotrophs, cyanobacteria and phytoplankton is lower, since the percentage of light that reaches this depth is practically zero.

At I6 station, where the water is shallow and not so clear, photodegradation is not so evident. On the other hand, the high organic matter concentration in this station masks the photodegradation effect. Moreover, the shallow water column allows sediment ressuspention that can also hinder the possible effect of photodegradation. In this station the presence of photoheterotrophs, cyanobacteria and phytoplankton can explain the cases on which BBP was higher in the surface probably when the water column is more transparent.

In resume, we can conclude that in order to obtain values of BBP more close to the reality, this one should be measured at *in situ* conditions allowing bacteria to incorporate leucine in their environment. However, one practical problem with this *in situ* incubation condition is when the collection point is in Open Ocean or in turbulent waters. In light of our results, when BBP assays must be done in laboratory conditions, we think that it will be best to do BBP incubations in the dark. Although this incubation condition can cause a stimulation of BBP, we avoid problems such as the incorporation of leucine by photoheterotrophs, cyanobacteria and phytoplankton which can lead to an increase in BBP values, or, on the other hand, avoid the photodynamic processes that reduce the leucine transportation into the cell which can cause a decrease in BBP values, as well as the false PAR light irradiation that is used in laboratory, that varies very much from the field conditions.

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Chapter 3 Bacterial biomass production in aquatic systems: specific conversion factors and bacterial community changes

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Abstract

Heterotrophic bacterioplankton play an important role in the aquatic's carbon cycle. They produce bacterial biomass that will be consumed to higher trophic levels of the food web. Thus, it becomes of major importance the accurate measurement o bacterial biomass production. In order to do so, semitheoretical and empirical conversion factors must be obtained for each system. The bacterial biomass production (BBP) determined with empirical and semitheoretical conversion factors was superior then that determined with theoretical conversion factors, for two distinct zones of the estuarine system of Ria de Aveiro. Empirical conversion factors ranged between 9.26 and 29.81 Kg C mol^{-1} in the marine zone and between 4.25 and 16.88 Kg C mol^{-1} in the brackish zone. Semitheoretical conversion factors ranged from 5.06 and 8.96 Kg C mol^{-1} in the marine water zone and from 5.28 to 9.34 Kg C mol^{-1} in the brackish water zone. During the determination of the empirical conversion factors bacterial community structure were also analyzed and it was observed a strong variation between the original sample and the incubated samples, in both zones. In these experiments it was observed that the sampling preparation led to a change in bacterial community structure. In addition to the determination of specific conversion factors a new problem arises which is the selection of bacterial community when BBP is determined with radioactive methods. In light of our findings, this probably means that, over the years, BBP has not been correctly measured since the original community is greatly changed by factors such as filtration/dilution, incubation and radioactivity.

Keywords: Heterotrophic bacterioplankton, leucine incorporation rate, isotope dilution, conversion factors, estuary, bacterial communities, DGGE

Introduction

After viruses, heterotrophic bacterioplankton is the most abundant and important component in the aquatic systems and are the major mineralizers of organic carbon (C) and nutrients (Ram and Chandramohan, 2007; Pace and Cole, 1996). They play a key role in the biological transfer of carbon through the microbial loop (Ghiglione et al., 2007), constitute a significant fraction of total biomass (Valencia et al., 2003) and execute two main tasks in the transformation of organic matter: they produce new bacterial biomass (bacterial secondary production) and they respire organic C to inorganic C (bacterial respiration) (del Giorgio and Cole, 1998).

Bacterial biomass production (BBP) by heterotrophic bacteria is, from an ecological point of view, secondary production and reflects the overall bacterial response to the prevailing ecological conditions. Through BBP, dissolved organic carbon (DOC), which would be lost to other members of the food web, is converted into particulate organic carbon (POC) in the form of bacterial cells, which become potentially available to the consumers of the higher trophic levels of the food web (Cole and Pace, 1995). Estimations of BBP are useful because it allows the estimation of the rates of metabolism and the amounts of organic matter metabolized by aquatic bacteria (Riemann and Søndergaard, 1984). Therefore, the development of reliable methods to accurately measure BBP becomes an important objective in microbial ecology (Bååth, 1998).

Since the 80's, several techniques have been used to estimate BBP, but the incorporation of [Methyl-^{3}H]thymidine into bacterial DNA (Fuhrman and Azam, 1980), the incorporation of [^{3}H]leucine or [^{14}C]leucine into bacterial protein (Kirchman et al, 1985), are the most commonly used. More recently, the incorporation of 5-bromo-2'-deoxyuridine (BrDU) into DNA (Nelson and Carlson, 2005) has also been used, as an alternative to the [Methyl-^{3}H]thymidine incorporation (Hamasaki et al., 2007). According to Simon and Azam (1989), the leucine technique is extremely sensitive for measuring bacterial production since protein represents a very constant percentage of bacterial carbon (Buesing and Marxsen, 2005; Simon and Azam, 1989). In addition, it is also more sensitive one order of magnitude, because over time, bacterial cells incorporate 10 times more leucine than thymidine (Fischer and Pusch, 1999; Simon and Azam, 1989).

However, these techniques are not free of negative aspects. The method used in these assays is the incorporation of [^{3}H]leucine. One of the main problems with this method is the determination of specific conversion factors that translate the leucine incorporation rates into bacterial carbon production must be specific to each system in order to obtain the most reliable values as possible. Therefore, the determination of conversion factors is extremely essential. There are three ways of converting leucine incorporation rates into bacterial production rates.

The first one, the theoretical approach is the most commonly used and was described by Simon and Azam (1989).

The second, the semitheoretical approach, substitutes the value of isotope dilution (ID) of the theoretical approach by the value obtained in ID experiments (Pedrós-Alió et al., 1999). Another important aspect is the isotope dilution which is used to determine the specific activity of [^{3}H]leucine incorporated into protein in environmental samples (Bird, 1999). In a simple way, to determine the ID a constant amount of [^{3}H]leucine and progressively higher amounts of cold leucine are added to a sample and then a plot with the different concentrations of leucine versus the leucine incorporation rate is made (Kirchman and Hodson, 1986; Gillies et al., 2006; van Looij and Riemann, 1993; Fischer and Pusch, 1999; Bååth, 1998). To minimize ID one must add high concentrations of leucine in order

to inhibit *de novo* synthesis of this amino acid (Simon and Azam, 1989; Bastviken and Tranvik, 2001; Fischer and Pusch, 1999; van Looij and Riemann, 1993;, Jørgensen, 1992).

And the third one, the empirical approach, where conversion factors are determined by comparison of leucine incorporation rates with the increase in bacterial biomass over a period of time (Buensing and Marxsen, 2005). Accordingly to Riemann et al. (1990), these conversion factors will vary between environments, over the seasons, and will depend on growth conditions and substrate composition. For example, several authors have determined conversion factors for other systems, such as Pedrós-Alió et al. (1999) for marine systems with values of 3.25 Kg C mol^{-1}, JØrgensen (1992) to freshwater systems with values of 2.11 Kg C mol^{-1}, Buensing and Marxsen (2005) to freshwater sediments with values of 1.445 Kg C mol^{-1}.Therefore, using conversion factors determined previously to other systems can induce a wrong BBP determination.

In long term incubations, such as the empirical conversion factor experiments, there has been found changes in bacterial composition during the incubation and between treatments (Massana et al., 2001) and it was also found that filtration and consequent removal of bacterivores caused a shift in bacterial communities after two days incubation (Suzuki, 1999).

The aim of this paper is to determine specific conversion factors to the estuarine system of Ria de Aveiro as well as to evaluate the effect of sample treatment and incubation during conversion factor experiments on bacterial communities using denaturing gradient gel electrophoresis (DGGE). In addition, DGGE was also performed to access if the bacterial communities were the same or different when incubations were performed with [^{3}H]leucine or with unlabelled leucine.

Materials and Methods

Study Site:

Ria de Aveiro (Figure 1.1) is an estuarine system located in the northwest coast of Portugal. Considered a coastal lagoon, it has a maximum length of 45 km, maximum width of 10. It is connected with the Atlantic Ocean through Barra, which is a thin artificial channel with 1.3 km of length and an average depth of 20 m. Ria de Aveiro is made up of a complex system of four main channels (S. Jacinto, Ovar, Mira and Ílhavo) that ramify into other smaller channels. The freshwater supply is made by two major rivers (Vouga and the Antuã river) and by two minor rivers (Boco and the Cáster river).

Two sampling stations were chosen for the present study: N1 (N 40^o 38’ 42”, W 08^o 45’ 25”), located in Barra, at the mouth of the estuary, representing the deep marine zone; and I6 (N 40^o 35’ 41”, W 08^o 41’ 21”), located in Ílhavo’s Channel, representing the shallow waters of a brackish water zone.

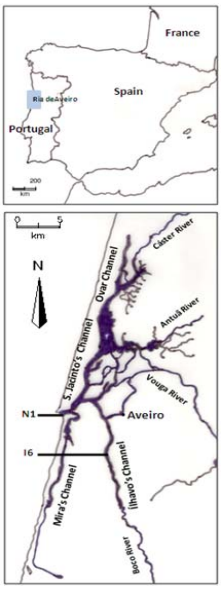

Figure 3.1 - The estuarine system of Ria de Aveiro. The arrows indicate the two sampling stations (N1 and I6).

By supporting extensive agricultural fields, a various number of small and medium industries along its borders and by harboring navigation and recreational facilities, Ria de Aveiro is subjected to extremely elevated anthropogenic pressure. As a result, it is considered to be in a "moderate low" overall eutrophic condition, and on which the trophic status of the inner part is higher than that of the outer part (Lopes et al., 2007).

Sampling

Samples for the conversion factors experiments, were collected with a Van Dorn Bottle in three different dates (31st of October, 2007; 27th of February, 2007 and 15nd of April, 2008), at low tide and in 0.5 m below water surface, for both sampling stations.

Samples for the isotope dilution experiments were also collected with a Van Dorn Bottle at low tide, in three different dates (11th of December, 2007; 21st of January, 2008 and 18th of October, 2008) and in 0.5 m below water surface, for both sampling stations.

Conversion Factors Experiments

Empirical conversion factors (eCF) between leucine and carbon production were performed according to Bjørnsen and Kuparinen (1991) and to Kirchman and Ducklow (1993). Water samples were collected from each sampling site. From those samples, 900 ml were filtered through 0.2 µm polycarbonate membranes (Poretics), to which it were added 100 ml of unfiltered sample, allowing a dilution of 1:10. The diluted samples were then incubated in acid-clean bottles (SHOTT DURAN), covered with aluminum paper, for 36 h at room temperature with agitation. In regular periods of time (4 h), subsamples were taken to access leucine incorporation rate (LIR) and bacterial biomass. In order to calculate eCF, data were computed following the integrative method (Riemann et al., 1987):

$$eCF = \frac{BM_f - BM_0}{\int (LIRdt)}$$

where eCF is the empirical conversion factor, BM_f and BM_0 are the final and initial bacterial biomasses determined by nonlinear regression and $\int$(LIRdt) is the leucine incorporation rate integrated over the course of the experiment. Although incubation may be stopped when bacterial number decreases or stabilizes, we performed the experiment always during the 36 h. The conversion from biovolume to biomass was accomplished by using a conversion factor determined by Norland (1993):

$$\text{pg C cell}^{-1} = 0.12 \cdot (\mu\text{m}^3 \text{ cell}^{-1})^{0.7}$$

Isotope Dilution Experiments

Saturation curves were performed in order to assess isotope dilution. This was accomplished with the protocol described above and by adding the same concentration of [^{3}H]leucine and varying concentrations of cold leucine. The concentrations used to perform the saturation curves ranged from 23.2 to 203.2 nM l^{-1} at N1 station and from 83.2 to 323.2 nM l^{-1} at I6 station. The LIR obtained were plotted against the respective concentrations and the resulting incorporation velocities were fitted to the hyperbolic function of Michaelis-Menten enzyme kinetics by using nonlinear regression (SigmaPlot 11.0, Systat Software Inc.). The fitted parameters were used to determine Vmax (maximal incorporation rate) which was used to calculate isotope dilution:

$$ID = \frac{V_{max}}{V_{meas}}$$

where V_{meas} is the incorporation rate at the concentration used in the routine assays (van Looij and Riemann, 1993).

Using the values of DI, semitheoretical conversion factors (sFC) were calculated (Pedrós-Alió et al., 1999):

$$sCF = PM \cdot (\frac{1}{L_p}) \cdot C_{cp} \cdot DI$$

where, PM is the molecular weight of leucine (0.1312 kg mol-1), L_p is the leucine content of cellular protein (0.073), C_{cp} is the ratio of cellular carbon to protein (0.86) and DI is the isotope dilution previously determined (Simon and Azam, 1989).

LIR and bacterial biomass determination

LIR was determined using 10 ml triplicate sub-samples plus a control fixed with 475 µl of formaldehyde. Samples were incubated with ^{3}H-Leucine (Amersham, specific activity 63.0 Ci/nmol) at a previously determined saturating concentration (83.2 nM). After one hour, incubations were stopped with 475 µl of formaldehyde. Following 15 minutes incubation on ice, 1 ml of ice cold trichloroacetic acid (TCA) (20%) was added to the sub-samples and to the control, and incubated for 10 minutes. Subsamples and control were filtered through 0.2 µm polycarbonate membranes (Poretics) and washed two times with 2 ml of ice cold TCA (5%) and 5 ml of ice cold ethanol (90%). Membranes were then placed into 5 ml scintillation vials and 4.5 ml of scintillation cocktail UniverSol (ICN Biomedicals, USA) was added. Radioactivity was measured after a period of 3 days in a Beckman LS 6000 IC liquid scintillation counter.

Bacterial biomass and total bacterial number were determined by using the acridine orange technique (Hobbie et al., 1977). In a simple way, bacterial cells were collected on 0.2 µm black

polycarbonate membranes (Poretics) and stained with 0.03% acridine orange. Cell counting and measurements were performed using an epifluorescence microscope (Leica DMLS), using a blue filter. Ten fields were counted and 200 cells were measured.

DNA extraction, PCR and DGGE

For DGGE 4 different types of samples were chosen. The first type was the original sample without filtration or incubation (O); the second type was the filtered/diluted sample used in the conversion factors experiment (dilution of 1:10) (A); the third type was similar to the second but unlabelled leucine was added as if it was to perform LIR assays (B); and in the fourth type was similar to the third but it was added unlabelled leucine plus [^{3}H]leucine (C). From the sample types A, B and C, sub-samples were removed in pre-determined periods of time: 0, 1, 16 and 32 hours.

Genomic DNA was isolated after filtering 250 mL of the sampled water through 0.22-mm polycarbonate filters. Collected cells were resuspended in 2 mL of TE buffer [10mM Tris-Cl, 1mM ethylenediamine tetraacetic acid (EDTA), pH 8.0] and centrifuged. After resuspension in 200 μL TE, 1 mg mL^{-1} lysozyme solution was added to induce cell lysis and incubated at 37ªC for 1 h according to the procedure described by Henriques et al (2004). DNA extraction was performed using the genomic DNA purification kit (MBI Fermentas, MBI Fermentas, Vilnius, Lithuania). DNA was resuspended in TE buffer and stored at -20ºC until analysis. The yield and quality of DNA were checked after electrophoresis on 0.8% (w/v) agarose gel.

PCR amplification of an approximately 400 bp 16S rDNA fragment (V6-V8 region) was performed using the primer set F968GC and R1401 (Nubel et al., 1996). Positive and negative (without DNA) controls were always included in PCR amplification experiments. The reaction was carried in a MultiGene Gradient Thermal Cycler from MIDSCI.. The 25 μL reaction mixture contained approximately 50 to 100 ng of extracted DNA, 1 x PCR buffer (PCR buffer without $MgCl_2$: PCR buffer with KCl_2, 1:1), 2.75 Mm $MgCl_2$, 0.2 mM of each nucleotide, 0.1 uM of each primer, 1 U of Taq Polymerase (all reagents purchased from MBI Fermentas, Vilnius, Lithuania) . Acetamide (50%, 0.5μ) was also added to the reaction mixture. The PCR protocol included a 5 min initial denaturation at 94ºC, 35 cycles of 95ºC for 1 min, 53ºC for 1 min and 72ºC for 2 min, and a final extension for 10 min at 72ºC. After PCR amplification, 5μL of the PCR product was electrophoresed on 1.5% (w/v) agarose gel, and then checked with ethidium bromide staining.

DGGE was performed with the Dcode System (USA, Bio-Rad Co.). PCR products were loaded onto 6-9% (w/v) polyacrylamide gel in 1xTAE buffer (20 mmol/L Tris, 10 mmol/L acetate, 0.5 mmol/L EDTA (pH 7.4)). The 6-9% polyacrilamide gel (bisacrylamide:acrylamide = 37.5:1) was made with a denaturing gradient ranging from 40 to 70% (100% denaturant contains 7 M urea, 40% (v/v) formamide). Electrophoresis was performed at 60ºC for 16h at 130V. Following electrophoresis,

the gels were incubated for silver staining. The solutions used were 10% (v/v) ethanol plus 0.1% acetic acid for fixation, 0.25g silver nitrate for staining, freshly prepared developing solution containing 0.4% (v/v) formaldehyde, 1.2% (w/v) NaOH, and finally, 0.75% sodium carbonate solution to stop the development. After silver stainning (Heuer et al., 1999), gels were air dried and scanned transmissively. GelCompar 4.0 program (Applied Maths) was used to analyze bacterial community profiles of the images of DGGE gels as described by Smalla et al. (2001). Using the rolling disk method with an intensity of 8 (relative units) to subtract the background, images were processed. A dendogram was then constructed using the Pearson correlation index (r) calculated for each pair of lanes within each gel and the cluster analysis was calculated through the unweighted pair group method using average linkages (UPGMA).

Results

Determination of empirical conversion factors (eCF)

The difference between BM_f and BM_0 was, in general, higher at I6 station, and the values of BM_f and BM_0 were also higher that at station N1 (Figure 3.2 and Table 4). At N1 this difference was higher in April, while at I6 this difference was higher in October. An exception is the February campaign, where although BM_0 has a higher value at I6, BM_f is lower than in N1, resulting in a difference between BM_f and BM_0 higher at N1. Values for $\int$(LIRdt) were always superior at I6 station and were registered in October for both sampling stations.

In general, values of eCF were superior for N1station relatively to I6 station. For N1, the average eCF was of 20.18 Kg C mol^{-1} with the highest value achieved in April (29.81 Kg C mol^{-1}) and the lowest in October (9.36 Kg C mol^{-1}). For I6 the average eCF was of 10.91 Kg C mol^{-1}, the highest value was obtained in April (16.88 Kg C mol^{-1}) and the lowest in February (4.25 Kg C mol^{-1}).

Determination of isotope dilution and semitheoretical conversion factors (sCF)

Values for ID and sCF (Table 5) were generally higher at N1 sampling station. For this station, ID was higher in the October campaign (6.143), and, as a consequence, values of sCF (9.495 Kg C mol^{-1}) were also higher in October. The average ID in this station was of 5.072 and the average sCF was of 7.840 Kg C mol^{-1}. On the contrary, for I6 station, the highest values for ID (6.041) and sCF (9.339 Kg C mol^{-1}) were obtained in the April campaign. For this station the average ID was of 5.015 and the average sCF was of 7.752.

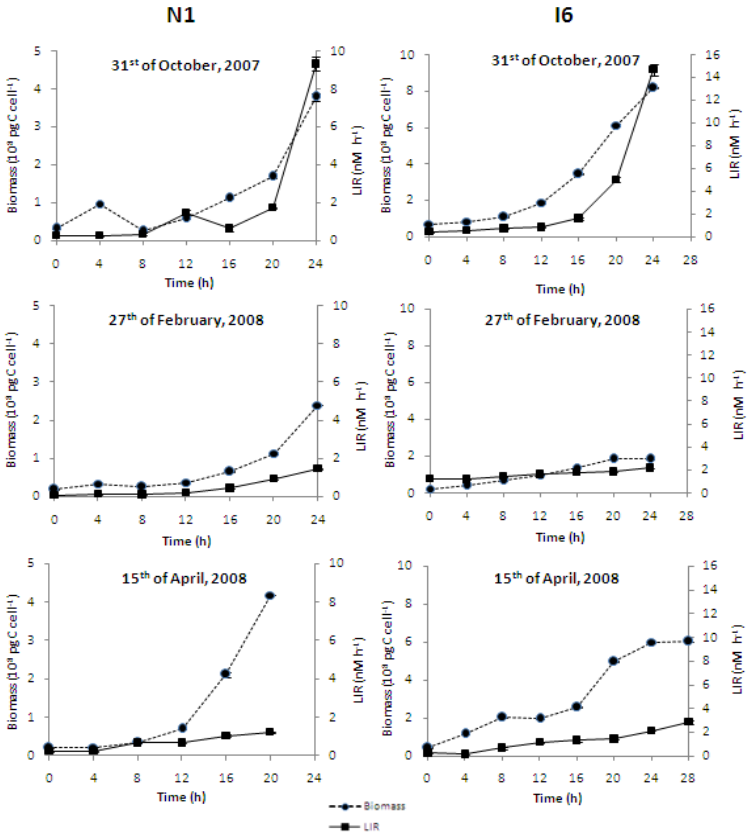

Figure 3.2 - Results of biomass (10^8 pg C $cell^{-1}$) and leucine incorporatio rate (LIR) (nM h^{-1}) over time, used to calculate eCF for N1 and I6 station in the three different sampling dates.

Table 4 - eCF calculation for N1 and I6, in the three sampling dates.

Sampling station	Date	BM_f (10^8 pg C $cell^{-1}$)	BM_0 (10^8 pg C $cell^{-1}$)	∫(LIRdt) (mol h^{-1})	eCF (Kg C mol^{-1})
N1	31-Oct-2007	3.791	0.324	3.70 x 10^{-8}	9.36
	27-Feb-2008	2.401	0.198	1.03 x 10^{-8}	21.37
	15-Apr-2008	4.190	0.202	1.34 x 10^{-8}	29.81
I6	31-Oct-2007	8.202	0.701	6.47 x 10^{-8}	11.59
	27-Feb-2008	1.859	0.225	3.85 x 10^{-8}	4.25
	15-Apr-2008	6.039	0.442	3.32 x 10^{-8}	16.88

Table 5 - Isotope dilution and the respective sCF, for both N1 and I6 station, in the three sampling dates.

Sampling station	Date	V_{meas}	V_{max}	Isotope dilution (Vmax/Vmeas)	r^2	sCF(Kg C mol^{-1})
N1	21-Jan-2008	0.832	4.824	5.798	0.94	8.962
	22-Apr-2008	2.188	7.168	3.275	0.93	5.063
	18-Oct-2008	1.449	8.900	6.143	0.93	9.495
I6	21-Jan-2008	7.770	26.541	3.416	0.96	5.279
	22-Apr-2008	2.120	12.812	6.041	0.89	9.339
	18-Oct-2008	2.253	12.590	5.988	0.92	8.637

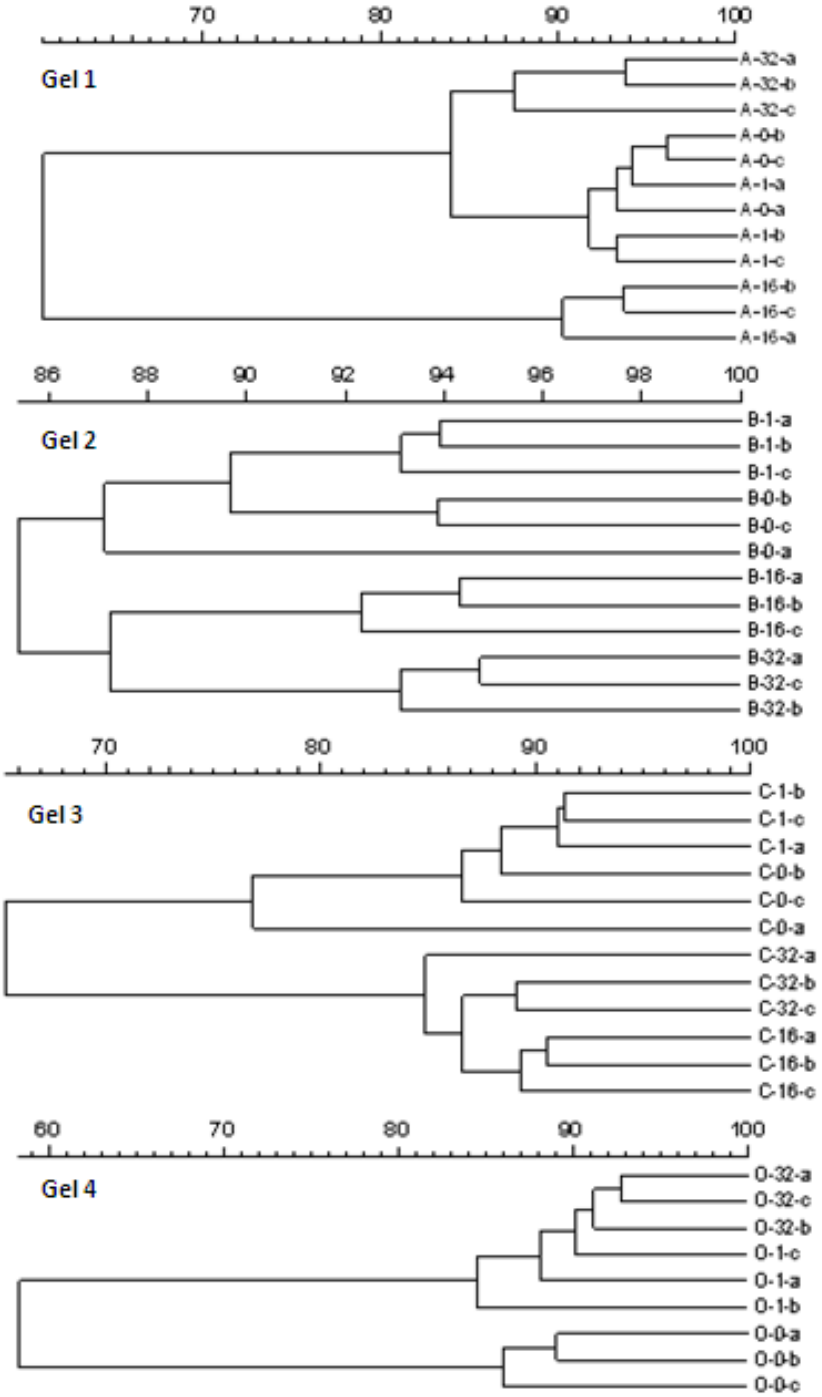

Figure 3.3 - DGGE gel comparing and respective dendograms showing the different conditions at N1 station over the several incubation times. (A) Sample without leucine; (B) Sample with cold leucine; (C) Sample with [^{3}H]leucine and cold leucine; and (O) Original sample, without filtration..

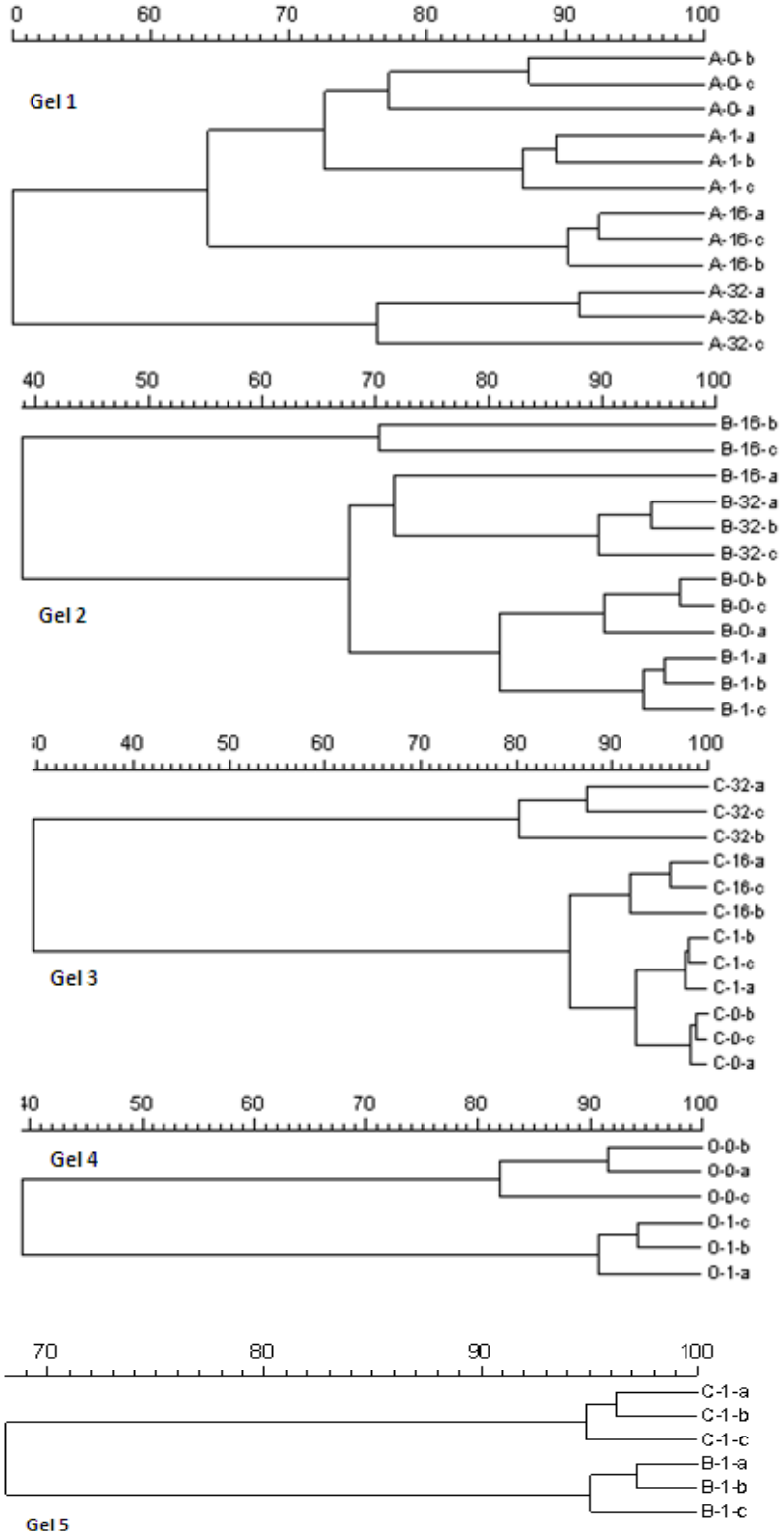

Figure 3.4- DGGE gel comparing and respective dendograms showing the different conditions at I6 station over the several incubation times. (A) Sample without leucine; (B) Sample with cold leucine; (C) Sample with [^{3}H]leucine and cold leucine; and (O) Original sample, without filtration.

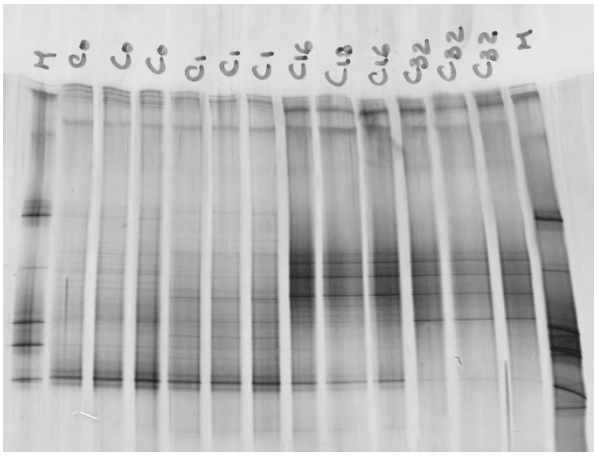

Figure 3.5 – Structure of bacterial communities for the sample type C (sample incubated with both unlabelled and labeled leucine), over the incubation times (0, 1, 16 and 32 hours), at I6 station.

DGGE analysis

From the cluster analysis of the DGGE gel of the original sample of N1 sampling station (Figure 3.3, Gel 4), there is the presence of two groups: one containing the original sample without leucine and the other containing the original sample incubated for 1 and 32 hours. In Gel 1 (Figure 3.3), correspondent to the diluted and filtrated sample there are two groups present: one holding the times 0, 1 and 32 hours, and the other holding the time 16 hours. In Gel 2 (Figure 3.3), correspondent to the sample type with unlabelled leucine, two groups are also present: one containing the incubation times 0 and 1 hour and the other containing the times 16 and 32 hours. In Gel 3 (Figure 3.3), correspondent to the sample type with unlabelled leucine plus [^{3}H]leucine the first two times of incubation (0 and 1 hour) are in the same group and the two last times (16 and 32 hours) are together in the another group.

From the cluster analysis of the DGGE gel number one, correspondent to the filtrated and diluted sample, we have two principal groups: the first holds the times 0, 1 and 16 hours and the second holds the time 32 hours. In the second DGGE gel, (Figure 3.4, Gel 2 sampling station I6), correspondent to the sample type with unlabelled leucine, it is clear the presence of two major groups: the first group contains three main smaller branches with the times 0, 1 and 32 hours on each branch; the second group contains the samples with 16 hours of incubation. In the third DGGE gel (Figure 3.4, Gel 3 sampling station I6), correspondent to the sample type incubated with both unlabelled leucine and [^{3}H]leucine, we have also two main groups: one holding the times 0, 1, 16 hours in separate branches; the other group contains the time 0 and 32 hours. The fourth gel (Figure 3.4, Gel 4 sampling station I6), correspondent to the original sample, has two groups: the first contains the original sample without any kind of treatment and leucine free; the second contains the original sample also without treatment but with one hour of incubation with unlabelled leucine and [^{3}H]leucine, similar to BBP assays. In order to compare the sample B (unlabelled leucine) with the sample C (unlabelled leucine

plus [^{3}H]leucine) another DGGE gel (Figure 3.4, gel 5) was performed. From the analysis of this gel, it is clear the presence of two groups: the first holds the time 1 hour from the sample with unlabelled leucine and the second holds the time 1 hour from the sample with both unlabelled leucine plus [^{3}H]leucine.

Discussion:

The current methods for measuring BBP are simple to perform and are a convenient way to follow bacterial growth over time (Bell and Kuparinen, 1984), or over changing geographical or local conditions (Pace and Cole, 1994). Although today one of the biggest concerns is the determination of specific conversion factors that translate the leucine incorporation rates into bacterial carbon production, the changes occurring in bacterial community structure during incubation of conversion factors experiments, as far as we know, have never been contemplated in other studies. However, in light of our results, bacterial community structure may have an enormous influence on leucine incorporation rates during conversion factors experiments.

Concerning the conversion factors, it is well known that they are specific to each system and must be determined frequently if one wants to have a trustworthy determination of BBP (Riemann et al., 1990; Bååth, 1998). In spite of that, there are not a lot of studies on which conversion factors have been determined, although this number has increased in the recent years. In most studies, it is the theoretical conversion factor determined by Simon and Azam (1989) that it is used to determine BBP in aquatic systems. According to our results, we recommend that BBP should be calculated using the semitheoretical conversion factors derived from isotope dilution. The reasons are quite simple. Firstly, in the empirical method, to transform biovolume in biomass one must use a conversion factor. The problem is that there are many conversion factors to do so such as 0.22 g C cm^{-3}(Bratbak, 1985), 0.38 g C cm^{-3} (Lee and Fuhrman, 1987), 15 fg C cell^{-1} (Caron et al., 1995), 0.35 pg C cell^{-1} (Bjørnsen, 1986) or even the usage of formulas such as $0.12 \cdot V^{0.7}$ in pg C cell^{-1} (Norland, 1993) or $435 \cdot V^{0.86}$ fg C cell^{-1} (Loferer-Krößbacher, 1998), where V is the biovolume. Thus, depending on the conversion factor used our empirical conversion factor will be different even if it is determined for the same site and in the same day. Hence, there is the need to get to a consensus concerning this matter and then, empirical conversion factors can be compared and applied. Secondly, to determine empirical conversion factors, it is necessary a sample preparation that includes filtration and dilution which leads to huge changes in the composition of bacterial community.

In the estuarine system of Ria de Aveiro, BBP is very different when specific conversion factors determined by the empirical method are used relatively to values obtained by the theoretical or the semitheoretical approaches. BBP in this estuary is underestimated when the theoretical approach is used.

Since values for isotope dilution were approximately 5, differences for BBP measurements without isotope dilution can be almost five times lower. Using an average sCF of 7.840 Kg C mol^{-1} for N1 station, differences can go from 2.89 to 14.08 µg C L^{-1} h^{-1} and with an average sCF of 7.752 Kg C mol^{-1} for I6 station differences can go from 3.63 to 17.46 µg C L^{-1} h^{-1}. Fisher and Pusch (1999) found values for isotope diluition (ID) in the same range of ours (1.1 to 16.7) in river sediments, but, in general, our values of ID are very high when we compare them with other studies. Pedrós-Alió et al. (2002) found values of isotope dilution between 1.0 and 1.4 in the Antarctic Peninsula; Gillies et al. (2006) found values between 1.03 and 2.12 in Paint Creek (inland emergent marsh wetland); Buensing and Marsen (2005) obtained an isotope dilution of 1.025; and Miranda et al. (2007) found values of 1.3 for a eutrophicated freshwater ecosystem. Given the eutrophic state of Ria de Aveiro, high values of isotope dilution would be expected since in eutrophic environments is more difficult to saturate the system, unless high leucine concentrations (more than 200 nM) are used (van Looij and Riemann, 1993).

As in other systems, the value of ID in the estuarine system of Ria de Aveiro have suffered a variation over the time, however no pattern of variation was observed. For both study areas, it is observed different values of ID in the three sampling dates, being the highest values 2 times higher than the lowest. If we consider a long term temporal scale, comparing our ID values with the ones obtained in this estuarine system around ten years ago (Almeida et al., 2001), when ID was equal to one, meaning that there was no ID, nowadays we have values almost five times higher.

If differences to the BBP values are enormous when isotope dilution is used, these differences are much huger when values of the empirical conversion factors are used. Values obtained for the empirical conversion factors were very high, especially at N1 station. Using these empirical conversion factors BBP can vary from 2.89 to 36.23 µg C L^{-1} h^{-1} for N1 station and from 3.63 to 24.58 µg C L^{-1} h^{-1} for I6 sampling station. Values of eCF as high as ours (12.8 and 36.4 Kg C mol^{-1}) were obtained, as far as we know, only by Rivkin and Anderson (1997) obtained for the Sargasso Sea and the Caribbean sea and for the Gulf Stream. For estuaries, the only work that we have knowledge of is the one performed by Kirchman and Hoch (1988) in the Delaware Bay estuary, in which they obtained a conversion factor that ranged between 0.78 and 1.97 Kg C mol^{-1}, using the conversion factor of Lee and Fuhrman (1987) to transform biovolume in biomass.

The advantages of ID use in comparison to the empirical conversion factors stand on the fact that there is no need for conversion factors to transform biovolume into biomass, which makes this method patternized and comparable to other ID's from other studies and on its simplicity and quickness, since ID experiments can be done in the same time as BBP assays. Empirical conversion factors experiments are no patternized since there are four different approaches for their determination

and there is no conformity in the biomass conversion factors. Besides, these experiments take a lot of time.

The DGGE analysis shows a variation on the community structure along the incubation times. At N1 station, the differences in bacterial community assemblages are patent in all sample types. For the samples filtered and diluted, without leucine (type A treatment), the similarity between the incubation times 0, 1 and 32 hours were more similar between each other (84% of similarity) than that of these incubation times with the incubation times 16 hours (71% of similarity). For the type treatments B (sample incubated with unlabelled leucine) and C (sample incubated with both unlabelled leucine and [^{3}H]leucine), the similarity between the two first times of incubation (0 and 1 hour) and the two last times of incubation was of 86% for type B treatment and 76% for type C treatment. At I6 station, the differences in the bacterial community structure over time are patent in the sample filtered and diluted, without leucine (type A treatment) and in the sample filtered and diluted, with both unlabelled and labeled leucine (type C treatment), varying the similarity between the times 0 and 1 hour by 73 and 92%, but similarity between these first two times and the time 16 hours is 64- 85%. In general, the similarities decrease between these first three times and the 32 hour time, reaching values of about 50%. These findings are consistent with other studies, (Massana et al., 2001; Suzuki, 1999) on which changes were found in bacterial composition over incubations times and in sample treatments. Second, even the addition of unlabelled leucine, used to reach the saturation concentration in BBP assays in an inexpensive way, also causes a change in the bacterial community structure in both sampling stations. The similarity between the samples with unlabelled leucine (type B treatment) and with both unlabelled and [^{3}H]leucine (type B treatment) is 68% for I6. Third, and perhaps the most surprisingly, is the effect that [^{3}H]leucine has on bacterial community assemblages affecting them significantly. The similarity between bacterial community structure of samples incubated solely with unlabelled leucine is different from that incubated with both unlabelled and labeled leucine. Besides, it is also clear that the similarity between original sample and original samples incubated for one hour with unlabelled and [^{3}H]leucine (type C treatment), like the ones used in the determination of BBP, is low, being 58% for N1 and 40% for I6. This shows the effect that [^{3}H]leucine has over the bacterial communities with only 1 hour of incubation. Moreover, these differences occur very rapidly, after 1 hour, since the bacterial community of the original samples incubated during 1 hour is quite similar when original samples are incubated for 32 hours with leucine (95% similarity). There are a few studies from the 50's, linking bacterial death to the mutagenic effects of tritium (Person and Brockrath Jr., 1964; Higo and Yakamoto; 1985).

In summary, water samples used for the assessment of empirical conversion factors are manipulated, consequently, bacterial community structure is altered which affects the determination of those conversion factors that need to be as accurate as possible in order to obtain reliable values of

BBP. On the other hand, the simple addition of cold leucine and of cold plus [^{3}H]leucine causes an alteration on bacterial community assemblages, affecting directly the determination of empirical conversion factors. So, besides the determination of specific conversion factors to each system, a new problem arises: the selection of bacterial community groups when BBP is determined with radioactive methods. This means that over the years BBP has not been correctly measured since there is a selection of the bacterial community groups that can growth in the presence of [^{3}H]leucine, contrarily to the other groups that are greatly affected, becoming extinct, by radioactivity.

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Chapter 4 Discussion

Heterotrophic bacterioplankton play a vital role in the organic matter transformation in the aquatic systems (Ghiglione et al., 2007; Ram et al., 2007; Valencia et al., 2003). Through bacterial biomass production (BBP), this organic matter becomes available to higher consumers of the food web (Cole and Pace, 1995).

The leucine incorporation technique, as a method for BBP determination, has some flaws, such as the incubation conditions in which BBP assays should be performed and the determination of specific conversion factors that transform the leucine incorporation rate into the rate of protein synthesis (Kirchman et al., 1982).

Concerning the results of experiments of incubation conditions, we can conclude that BBP musb be determinated *in situ* conditions. In laboratory conditions the difference between sampling stations and between surface and bottom waters are attenuated and the BBP estimation is significantly higher than that obtained in field conditions. However, when it is impossible to determine BBP *in situ* light conditions, such as for Open Ocean and turbulent waters, it is better to do the determination in dark laboratory conditions, avoiding the problematic of artificial PAR light incubation.

In the experimental assays performed in field conditions for surface samples, no consistent pattern of variation was observed. Although for N1 station BBP is generally superior at *in situ* light condition than at *in situ* dark conditions, for the I6 station the opposite is observed. In the laboratory assays, on the other side, BBP was always superior in dark incubation conditions than under PAR radiation. This was previously detected in other studies by Sommaruga et al. (1997) and by Morán et al. (2001). In order to explain this superior values in the dark conditions, these authors suggested that when samples are under PAR light conditions some sort of photodynamic processes can occur causing a reduction in the transport of leucine into the cell, or even by the lack of competition for amino acids by phytoplankton. Other studies showed also that dark incubation avoids the incorporation of substrates (leucine) by photoheterotrophs and cyanobacteria when samples are incubated under PAR light (Gasol et al., 2008). Besides, by making incubations in the dark the reproduction of ambient PAR light is also avoided.

When comparing BBP in the field experiments with laboratory experiments, BBP is generally superior in laboratory (3 times on average), which is usually the condition used to assess BBP. This points to an overestimation of BBP in laboratory conditions, demonstrating that the better incubation condition should be the *in situ* light condition. On the other hand, the higher values observed at *in situ* light conditions for surface water of the deeper sampling station

relatively to bottom waters is not observed in laboratory incubation as well as the difference between the two sampling stations, showing the importance of the environmental factors that are impossible to reproduce in laboratory conditions.

Nevertheless, the *in situ* light incubation condition is the correct way to determine BBP but, the DGGE analysis shows that only bacterial community structure of surface water of N1 sampling station and of the bottom water of I6 incubated at *in situ* light conditions is more similar to the original samples (without leucine and without incubation). For the other samples (bottom samples of N1 station and surface samples of I6 station) this does not happen. In fact, these original samples are in different groups from the incubated samples, with similarities between 14 and 84%. However, the addition of leucine and the incubation period used to determine BBP in situ (as well in the other conditions of incubation) can alter bacterial community structure, explaining the low values of similarity with the original water samples. The bacterial community of surface water of station N1 seems to be the most affected by laboratory incubation.

The DGGE results show that bacterial community structure is different between the two depths at each sampling station, being the major difference (28% similarity) in the marine zone where the difference between depths is much higher than that of the brackish zone as BBP. This means that the higher values of BBP of surface water relatively to bottom water at the sampling station N1 could, in part, be due to differences in bacterial community structure in both layers. At N1 station, where organic matter is much lower than at I6 station (Almeida et al., 2001; Santos et al., 2007) and where the water column is clear, surface bacterial community seems to be stimulated by photodegradation of recalcitrant organic matter that reach the marine zone, that can change the structure of bacterial community. On the other hand, along the clear water column of this sampling station, photoheterotrophs and cyanobacteria groups can also be stimulated affecting also bacterial assemblage at surface.

Specific conversion factors for Ria de Aveiro, when compared to the literature ones, are higher than most values for both empirical conversion factors (eCF), isotope dilution (ID) and, consequently, than semitheoretical conversion factors (sCF). For eCF, values as high as ours were only found by Rivkin and Anderson (1997) for the Sargasso and the Caribbean Sea and for the Gulf Stream. For ID and sCF Fisher and Pusch (1999) found values similar to ours for river sediments.

Values found for both types of conversion factors demonstrate that BBP in the estuarine system of Ria de Aveiro is quite different from the values found with the theoretical approach (Simon and Azam, 1989), which is usually used, causing an underestimation of BBP.

The average eCF was higher in the marine zone. Values of DI and as consequence sCF were also higher in the marine zone. This was not to be expected since the brackish water zone has a

higher trophic index and values of ID are supposed to higher in eutrophic environments because it is more difficult find a saturation concentration of leucine in these systems.

The usage of ID and, as a consequence, the usage of sCF is more recommended than the eCF. This choice is very simple to explain since the determination of eCF needs conversion factors to transform bacterial biovolume into bacterial biomass. There are several of these conversion factors (see references Bratbak, 1985; Lee and Fuhrman, 1987; Caron et al., 1995; BjØrnsen, 1986; Norland, 1993; Loferer-Krößbacher, 1998). Even if the same calculation method used to assess eCF was to be used these values would change a lot, depending on the conversion factor used to convert biovolume into biomass. Besides, there are also four different calculation methods to determine eCF. These two variations don't allow a true comparison between the several eCF found in literature and a comparison between our values and those of literature. The usage of the ID and sCF approach is more simple to be performed and it's calculation is patternized, allowing us to compare our values with the ones found in literature. Besides, this approach is quicker and it can be done when BBP assays must be perfomed. This allows making a correct correction on BBP values.

On the other hand, the simple addition of cold leucine, used to reach the saturation concentration in BBP assays in an inexpensive way, causes an alteration on bacterial community assemblages, affecting directly the determination of empirical conversion factors. Moreover, and perhaps the most surprisingly, is the effect that [^{3}H]leucine has on bacterial community assemblages affecting them significantly.

Differences in bacterial community structure were found along the incubation times. These findings are consistent with those of Massana et al. (2001) and Suzuki (1999), who found changes in bacterial community structure along the incubation times. Generally, these differences were lower between the first three incubation times (0 and 1 hours), and higher between those and the two last incubation times (16 and 32 hours), indicating an accentuation of differences along the incubation times.

On the other hand, the addition of labeled leucine also causes a change in the bacterial community structure in both sampling stations. Differences between the bacterial assemblages of samples incubated only with unlabelled leucine and samples incubated with both unlabelled and labeled leucine were also verified (similarity of 68% for N1 sampling station). Since [^{3}H]leucine is somehow expensive, unlabelled leucine is used in order to achieve the desired concentration in an expensive way. It is supposed that bacteria don't make distinction between these two types of leucine. Our results show that the bacterial community structure is not the same between these two treatments, demonstrating that bacterial groups that incorporate unlabelled leucine are not entirely the same as those that incorporate the two types of leucine. This can be the result of the damaging effect that [^{3}H]leucine has over some of the groups present in the samples. Really, significantly

differences in the bacterial community structure between original samples and the original samples incubated with leucine for one hour, with a similarity of 58% at N1 station and of 40% at I6 station. It is obvious that some sort of damage occur in some of the groups present in the original community assemblages and that only the most resistant groups incorporate the [^{3}H]leucine. There are a few studies from the 60's and the 80's that show the killing efficiencies of tritium (^{3}H) in *Escherichia coli* (Person and Brockrath Jr., 1964; Higo and Yakamoto, 1985). This should be expected, since tritium beta particle is known to be a causing agent of cancer in mammals, due to its small magnitude (Fairlie, 2007). For N1 sampling station, besides the incubation with radioactive leucine for one hour, the original sample was also incubated for a period of 32 hours. Surprisingly, the differences between the original samples incubated for one hour and original samples incubated for 32 hours were not big, showing a similarity of 88%. The results show that the bacterial community structure doesn't alter that much along the longest incubation period with [^{3}H]leucine and that the biggest differences occur with only one hour of incubation in their presence.

In order to an accurate assessment of BBP it is clear, from this study, that this technique must overcome its flaws, otherwise we will never achieve correct values of BBP. Somehow, a decision concerning the incubation conditions must be taken. If one cannot perform the incubations at *in situ* light conditions than it has to be decided if, in laboratory conditions, PAR light is to be used or, on the opposite, incubations should remain in the dark. Knowing from the eCF experiments that [^{3}H]leucine can cause a change in bacterial assemblages it is quite possible that the differences between the original samples (without leucine and incubation) and the several incubation conditions (that use both types of leucine) can be explained by the harsh effect of tritium in bacteria. Literature shows higher values of BBP when samples are incubated in the dark but, there are also studies that show the opposite in which BBP is higher when incubations are performed under PAR light. (Aas et al., 1996; Michelou et al., 2007). Still, we think that, in order to avoid the several possible problems associated with PAR light incubation conditions, BBP should be performed in the dark.

It is also clear, from the eCF experiments that a selection of bacterial assemblages is made by the simple usage of [^{3}H] leucine. The whole technique is based on the incorporation of [3H]leucine but, if tritium is harmful for some bacterial groups than we are no measuring the BBP from all the groups present in the original sample. So, a new problem arises: the selection of bacterial community when BBP is determined with radioactive methods. This means that over the years BBP has not been correctly measured since there is a selection of the bacterial community that uptakes the [^{3}H]leucine.

One way to bypass the [^{3}H]leucine problem a non-radioactive method should be used. The alternative is the method of incorporation of 5-bromo-2'-deoxyuridine (BrDU) into DNA, which is radioactive free but a generic of the [^{3}H]thymidine technique. Nevertheless, this method shares some of the incubation problems as the [^{3}H]leucine incorporation technique since the determination of conversion factors continues to be necessary for an accurate BBP determination.

Still much work needs to be done in order to solve all the problems associated with the [^{3}H]leucine technique. In the future we intend to go deeper on the matter of the harmful effects of tritiated leucine to bacterial cells. Our experiments will cover membrane integration assays, *recA* expression, damages caused in L-leucine specific receptor and the activity of leucine aminopeptidase. We also intend to perform assays to see the differences of BBP during the day and during the night, in different incubation conditions (ultraviolet, PAR light, sun light and dark), and in different meteorological conditions. In this assays DGGE will be performed to assess bacterial assemblages in all conditions.

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