UNIVERSIDADE FEDERAL DE SÃO CARLOS
CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA E RECURSOS NATURAIS

EFEITO DA QUALIDADE E COMPLEXIDADE DA MATÉRIA ORGÂNICA DISSOLVIDA DE ORIGEM FITOPLANCTÔNICA NA DIVERSIDADE E ATIVIDADE DE COMUNIDADES BACTERIANAS

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Prof. Dr. Armando Augusto Henriques Vieira

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RESUMO

Bactérias são os organismos mais abundantes e a principal força motriz para o ciclo de carbono e de nutrientes em ambientes aquáticos. Variações na atividade metabólica e composição das comunidades bacterianas heterotróficas que resultam das interações com matéria orgânica dissolvida (MOD) têm capacidade de alterar ciclos biogeoquímicos globais. Considerando um contexto de mudanças ambientais globais, o efeito da quantidade e qualidade da MOD na composição e atividade das comunidades bacterianas foi avaliado em experimentos em microcosmos. Embora permitam ir além de uma análise puramente descritiva das comunidades microbianas e realizar manipulações, experimentos em meso e microcosmos têm limitações. Estes experimentos necessitam iniciar com a mesma comunidade, o que limita o número de manipulações concomitantes possíveis. Além disso, cultivos em microcosmo normalmente resultam em mudanças drásticas na composição das comunidades. Assim, um método de criopreservação de comunidades bacterianas naturais completas, que permita a estocagem e utilização em diferentes tempos de uma mesma comunidade inicial, foi avaliado, bem como o efeito de diferentes MODs sobre a manutenção da diversidade nessas comunidades cultivadas após criopreservação. Os resultados mostraram que tanto quantidade quanto qualidade de MOD correlacionaram-se com mudanças na comunidade bacteriana, mas a qualidade exerceu um efeito rápido por competição, com sua intensidade e duração moduladas pela quantidade. No geral, também foi possível manter a diversidade e composição de comunidades bacterianas naturais após criopreservação, embora uma fração da diversidade seja perdida, especialmente em comunidades oriundas de ambientes mais estáveis e oligotróficos. Isto é problemático pois se espera que em um contexto de aquecimento global a temperatura dos oceanos e corpos d’água

doce aumentem, algo que poderia levar a mudanças na quantidade e qualidade da MOD destes ambientes. Isto potencialmente alteraria a composição e metabolismo das comunidades bacterianas heterotróficas, causando impactos nos ciclos globais do carbono e nutrientes que ainda não são completamente compreendidos.

Palavras-chave: Matéria Orgânica Dissolvida; MOD; Bactérias Heterotróficas; Comunidade Bacteriana; Ambiente Aquático; Criopreservação; Efeito-garrafa.

TITLE: EFFECT OF QUALITY AND COMPLEXITY OF PHYTOPLANKTONIC DISSOLVED ORGANIC MATTER ON THE DIVERSITY AND ACTIVITY OF BACTERIAL COMMUNITIES

ABSTRACT

Bacteria are the world's most abundant organisms and the foremost drivers of carbon and nutrient cycles in aquatic environments. Changes in the metabolic activity and composition of heterotrophic bacterial communities in response to interactions with dissolved organic matter (DOM) can potentially alter global biogeochemical cycles. In a context of global environmental changes, effects of the quantity and quality of DOM on the composition and activity of bacterial communities were investigated in experimental microcosms. Although such experiments allow a less descriptive analysis of these communities, they have limitations. Initial experimental communities need to be the same, limiting the number of manipulations that can be performed. Furthermore, microcosm cultivation usually results in drastic compositional changes in the communities. Thus, we also evaluated a cryopreservation method for whole natural communities which allows experiments with a unique initial community to be performed separately, as well as the effects of varying DOMs to keep the diversity of the cryopreserved communities. The results showed that both DOM quantity and quality were related to changes in the communities, with quality exerting an early response effect driven by competition and the intensity and duration of the effect modulated by quantity. Moreover, diversity and community composition of the bacterial communities were generally maintained after cryopreservation, although part of the diversity was lost, something more evident for communities from more stable and oligotrophic environments. In a global warming context, increases in the temperature of oceans and freshwater bodies are

expected, which could cause changes in the quantity and quality of the available DOM within these environments. This outcome could alter composition and metabolism of heterotrophic bacterial communities, causing not fully understood impacts on the carbon and nutrients global cycles.

Keywords: Dissolved Organic Matter; DOM; Heterotrophic Bacteria; Bacterial Community; Aquatic environment; Cryopreservation; Bottle-effect.

Introdução

Bactérias são organismos-chave nos ciclos biogeoquímicos globais. Elas são a principal força motriz para o ciclo de carbono e nutrientes em ambientes aquáticos

(Amado et al., 2013; Tranvik et al., 2009), sendo parte do que é descrito como o 'microbial loop' (Azam et al., 1983; Pomeroy, 1971) – um caminho alternativo para a energia e nutrientes na rede trófica clássica, com as bactérias compondo sua base. Consequentemente, bactérias heterotróficas são os decompositores mais importantes de matéria orgânica em ambientes aquáticos, sendo responsáveis por considerável parte da produção e respiração planctônica (Cotner and Biddanda, 2002). Considerando este papel crucial no ecossistema aquático, é imperativo entender como a comunidade microbiana conduz e modula estes ciclos, especialmente frente às mudanças ambientais, isto é, mudanças climáticas e aumento da poluição da água, para que possamos entender, modelar e possivelmente mitigar corretamente eventuais efeitos resultantes (Fuhrman, 2009; Moran et al., 2016).

A matéria orgânica dissolvida (MOD) de ecossistemas aquáticos naturais é uma mistura complexa de compostos carbônicos reduzidos, (Kujawinski, 2011), o que a torna uma das mais complexas misturas do planeta (Logue et al., 2016). Tanto a quantidade quanto a qualidade da MOD podem variar com sua origem e processamento (Amado et al., 2013; Bertilsson and Jones, 2003; Bittar et al., 2015; Giroldo and Henriques Vieira, 2005; Stubbins et al., 2010), e esta MOD afeta a diversidade e o funcionamento de comunidades microbianas. Como consequência das inúmeras interações possíveis entre MOD e assembleias microbianas, é necessário analisar tanto a MOD e as comunidades microbianas em grande detalhe para entender como estas comunidades são estruturadas.

Com estas questões em mente, investigamos o efeito de matéria orgânica dissolvida (MOD) sobre comunidades de bactérias heterotróficas aquáticas, usando tanto comunidades de água doce e marinhas como modelo. O tema central dessas

investigações foi entender qual é o efeito de diferentes qualidades de MOD sobre as comunidades, e tentar responder se a qualidade ou quantidade da MOD tem maior influência sobre a estrutura desses organismos.

Em ambientes aquáticos, a MOD é o principal elo entre fitoplâncton e bacterioplâncton. Portanto, é crucial compreender como a diversidade fitoplanctônica modula a qualidade e quantidade da MOD, uma vez que a MOD varia de acordo com a espécie fitoplanctônica que a produz (Giroldo and Vieira, 2005; Grossart et al., 2005), e como diferentes qualidades e quantidades de MOD afetam a composição e metabolismo das comunidades bacterianas, tanto com respostas positivas ou negativas para os diferentes integrantes de uma mesma comunidade (Bagatini et al., 2014; Bertilsson et al., 2007; Dinasquet et al., 2013; Gómez-Consarnau et al., 2012; McCarren et al., 2010; Tada and Suzuki, 2016). Desta forma, o Capítulo 1 tem por objetivo analisar as diferenças, através de técnica de espectrofotometria de absorbância de luz ultravioleta à luz visível, na MOD excretada por duas espécies dominantes de fitoplâncton em uma represa hipertrófica tropical e avaliar, em um experimento em microcosmos, o efeito da quantidade e qualidade destas MODs sobre a comunidade bacteriana natural deste ambiente.

Meso/microcosmo experimentais estão entre as poucas ferramentas disponíveis para ecólogos microbianos que permitem ir além de uma análise puramente descritiva das assembleias microbianas e realizar testes experimentais. Para estes experimentos, tipicamente se coleta determinado volume de água dos ambientes e incuba-se este volume em condições que imitam as condições naturais deste ambiente, manipulando-se, então, fatores selecionados. Estas investigações são comumente afligidas pelo ‘bottle-effect’, ou efeito-garrafa, (Hammes et al.,

2010), um conceito de difícil definição que reflete a possibilidade de que um efeito ou resposta observados em cultivos em microcosmos são consequências do confinamento em si e não da manipulação realizada (Vekeman and Heylen, 2017). Além disso, para que comparações entre experimentos sejam completamente adequadas, a mesma comunidade bacteriana inicial deve ser submetida a todos os tratamentos (Fukami, 2015; Kerckhof et al., 2014; Vekeman and Heylen, 2017), e isto limita (e limitou em nosso Capítulo 1) o tipo de experimentos que podem ser feitos e, principalmente, a quantidade de fatores e interações que podem ser investigadas.

Entretanto, recentemente se descobriu, em um experimento com mesocosmos, que a adição de matéria orgânica dissolvida (MOD) com conteúdo celular de micróbios de ecossistemas aquáticos naturais possibilita o desenvolvimento de comunidades altamente diversificadas que não são muito diferentes da comunidade natural original (Shen et al., 2018). Mais especificamente, foi possível cultivar taxa bacterianos como *Pelagibacter* sp (SAR11) bactérias de água doce do grupo Actinobacteria, clados de difícil cultivo em condições laboratoriais (Giovannoni, 2016; Giovannoni et al., 2014; Rappé et al., 2002). Assim, se fosse possível acoplar tanto um método de criopreservação, que permitisse congelar a comunidade completa e ressuscitá-la em um momento posterior, com um método de cultivo que contornasse os efeitos do efeito-garrafa, as possibilidades de experimentação com micro/mesocosmos seriam expandidas.

Com esta questão em mente, e em parceria com a Dra. Sara Beier, Pós-doutoranda associada ao Laboratoire d'Océanographie Microbienne (Lomic) do Observatoire Oceanológique de Banyuls-sur-Mer, França, e responsável pela pesquisa bem sucedida no cultivo de taxa de difícil cultivo em laboratório (Shen et

al., 2018), testamos um método de criopreservação de comunidades naturais inteiras acoplado a um método de cultivo em microcosmos, usando adição de MOD de origem natural ao meio de cultivo, que nos permitisse recuperar uma diversidade de bactérias heterotróficas o mais próxima possível do ambiente de onde as amostras foram coletadas.

Neste teste, consideramos o efeito que a adição de MOD natural ao meio de cultura usado para ressuscitar estas comunidades tem sobre a composição e crescimento destas comunidades cultivadas em microcosmo. Estas MODs foram obtidas dos mesmos locais das comunidades naturais a partir de lisados celulares das comunidades microbianas totais que ali estavam presentes, retidas em membrana de 0,22 µm. Estas MODs foram usadas sozinhas ou com subsequente adição de compostos que, acredita-se, facilitam o crescimento de cepas bacterianas de difícil cultivo em microcosmos. Água natural, oriunda da mesma localidade da comunidade testada e esterilizada por filtração, também foi utilizada como meio de cultura em um dos testes. Sendo bem-sucedidos nestes testes, ilustramos possíveis aplicações desta metodologia, avaliando concomitantemente o efeito sobre a comunidade bacteriana de uma MOD de um determinado local isoladamente e em mistura com MOD obtida em outra localidade (resultando em um aumento de complexidade da MOD) e os efeitos da coalescência de duas comunidades bacterianas.

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CAPÍTULO 1 - EFFECT OF QUANTITY AND COMPLEXITY OF DISSOLVED ORGANIC MATTER ON FRESHWATER BACTERIOPLANKTON CITOMETRIC DIVERSITY

To be submitted to Water Research

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Abstract

Heterotrophic bacterial communities are the key players in degrading and cycling dissolved organic matter (DOM), reacting to DOM availability and characteristics by changing both their metabolic rate and composition, and in turn also causing changes in the DOM. However, it is unclear if these communities are

more influenced by DOM quantity or quality, a pressing question in a context of global environmental changes. To shed light on this question, we tested a freshwater bacterial community growing on three quality levels across two quantity levels of phytoplanktonic DOM, obtained from exudates of axenic cultures of the bloom-dominant Cyanobacteria, *Microcystis aeruginosa*, and of a transient Cryptophyte grower, *Cryptomonas obovata*. Phytoplanktonic organisms and bacterial community were isolated from the same site, a tropical hypertrophic freshwater reservoir dominated by Cyanobacteria blooms throughout the year. We used flow cytometry to investigate the bacterial community and the UV-visible absorbance spectra to describe the DOM, and we tried to establish correlations between changes in both community and DOM composition. We found that the DOM quality effects on community composition were exerted as early response and modulated in duration and intensity by the DOM quantity. Longer bacterial growth lag phase and lower productivity at early stages of incubation were caused by DOM from *M. aeruginosa*, but all treatments caused decrease of diversity levels at early stages (24 hours) and partial recovery at further experimental time, with treatments converging to similar levels of diversity and a less dissimilar bacterial community composition. We hypothesized that a sequence of competition and facilitation dictates the assembling processes of the community and is controlled by Quality, which exerts a competition effect at early incubation stages that, as the DOMs were modified, changed to facilitation of growth of more diverse bacteria.

Key words: DOM, phytoplankton, microbial ecology, competition, facilitation, community assembly

Introduction

The Earth's carbon budget hangs in the carbon balance between the aquatic, atmospheric and land continuums. These dynamic systems are primarily connected by water fluxes (Ward et al., 2017) and large aquatic systems, such as the oceans, are the major reservoirs of carbon (Hedges, 1992). Carbon in the marine system is present mainly as dissolved organic matter (DOM) and parallel the amount of carbon in the atmosphere as CO_2 (Moran et al., 2016). Through heterotrophic bacteria, DOM might be regarded as one of the major pipelines in the flow of energy and cycle of nutrients in aquatic environments, a concept known as the microbial loop (Azam et al., 1983; Pomeroy, 1971).

Inland aquatic environments were historically seen as "passive pipes" in global carbon cycles, acting only to drain the terrestrial DOM lixiviated from the catchments of streams and rivers towards the ocean, where DOM biological transformation would occur (Cole et al., 2007). This view greatly overlooked the biochemical processes that take place in freshwater environments (Cole et al., 2007; Logue et al., 2016), with few studies aimed to investigate such questions in freshwater, especially in tropical environments (Sarmento, 2012), leaving a lacuna in our knowledge of such important environments for carbon cycling (Abril et al., 2013).

The quantity and quality of DOM in the environment vary widely depending on its origin and various degrees of processing (Bertilsson and Jones, 2003; Bittar et al., 2015; Giroldo and Henriques Vieira, 2005; Sarmento et al., 2013; Stubbins et al., 2010). While allochthonous (i.e. lixiviated) DOM is normally more refractory to bacterial consumption, autochthonous (usually phytoplanktonic or macrophitic derived) DOM are more labile and readily utilized by the microbial community (Bertilsson and Jones, 2003; Medeiros et al., 2017), therefore constituting a pool that

rapidly cycles through the microbial loop. Furthermore, eutrophication is common in many tropical freshwater systems, where high temperatures throughout the year favors such events (Mowe et al., 2015), which could increase DOM autochthonous availability and that has the potential to severely impact the microbial loop and the carbon cycle.

Ecological studies usually focus on the processes and changes/transformations occurring either in the community or in DOM (Howe et al., 2016; Osterholz et al., 2016). However, the interaction between DOM and bacteria requires the study of both in order to have a more complete idea of the processes in aquatic environments (Hansen et al., 2016; Kujawinski, 2011; Osterholz et al., 2016), since this interaction can be quite variable due to the close interplay between DOM and bacterioplankton. While bacterial community drives DOM cycling and changes, DOM composition also affects communities' composition and processes.

It was found that the quality of DOM can have a big effect on bacterial communities, with more bioavailable DOM selecting for specific populations within the community and modulating the bacterial production (BP) and the bacterial growth efficiency (BGE) (Dinasquet et al., 2013; Farjalla et al., 2009; Gómez-Consarnau et al., 2012; Sarmento et al., 2013). On the other hand, bacterial metabolism and dissimilarity is changed with different DOM concentrations (Eiler et al., 2003). In a recent paper, Sarmento and colleagues (2016) found that the quantity of DOM supplied to incubations can have an even greater effect than quality on specialization index of bacterial groups, at least for marine bacterial communities of a stable and oligotrophic habitat feeding on a broadly labile DOM of phytoplanktonic origin.

Given the diverse responses found in the literature and that most works related to that question did not consider the environmental trophic state where the

bacterial communities were sampled or did not work with communities from eutrophic and tropical environments, we investigated if the quantity or the quality of DOM influences more extensively the diversity and metabolism of microbial community from an eutrophic environment. Our expectation was that a community coming from an eutrophic environment would be adapted to large DOM quantities and quality variations would exert stronger influence on the community.

In laboratorial batch cultures, we tested a freshwater bacterial community growing on three quality levels across two quantity levels of phytoplanktonic DOM. The organisms used here, both bacteria and phytoplankton, were obtained from Barra Bonita Reservoir, a tropical, freshwater man-made reservoir known to be eu-to-hypertrophic (Dellamano-Oliveira et al., 2008; Vieira et al., 2013), further contributing to the understanding of microbial process in eutrophic tropical freshwater environments. Bacterial communities were characterized using cytometric diversity obtained from flow cytometry analysis and the bulk metabolic rates of production, respiration and growth efficiency. We used spectral absorbance from ultraviolet to visible light and associated indexes to characterize the DOM and its changes during incubations and aimed to correlate those changes in DOM to the changes in the bacterial community characteristics.

Material and Methods

Algal cultures and DOM preparation

Cryptomonas obovata and *Microcystis aeruginosa* (CO and MA hereafter), phytoplanktonic species present (Dellamano-Oliveira et al., 2008; Vieira et al., 2013)and isolated from Barra Bonita reservoir, and maintained in axenic conditions at Freshwater Water Microalgae Culture Collection at Federal University of São

Carlos (CCMA-UFSCar in Portuguese acronymic, in São Carlos - Brazil), were used as source of DOM. Each species was grown in three batch cultures of 1.8 L sterile WC medium (Guillard and Lorenzen, 1972) in 2 L capacity Boeco flasks, aerated with filter-sterilized (0.22 µm)compressed air, flowing at 0.05 L $^{-1}$ min^{-1}, under light intensity of200 mmol photons m^{-2}s^{-1}, at controlled temperature of 23 ±2˚C. Growth curves were prepared by monitoring optical density at wavelength 670 nm and 678 (for CO and MA, respectively the highest absorbance peak near the chlorophyll wavelength absorbance 665nm [Guillard and Lorenzen, 1972]) until late exponential/early stationary growth phase. The cultures were filtered with tangential filtration in heat sterilized (120°C) 0.6 µm pore cartridge (Xampler^{TM} CEP-6-D-4-A). The filtrate was then filtered in polycarbonate membrane with 0.22 µm pore to obtain the DOM. The 0.22 µm membranes were autoclaved and, immediately prior to filtration, washed with HCl 2M and with sterile ultrapure water in abundance. The filtered DOMs were collected in clean glass bottles, which were previously washed with HCl P.A. for at least 2 hours and kept in dark at 4°C until the dilution procedure and start of the experiment. To achieve the necessary volume to the experiment set up (described below), the DOM from the triplicate batch cultures of each algae were pooled together.

Natural bacterioplankton community

At Barra Bonita Reservoir (Tietê River, 22°29'S, 48°34'W, São Paulo, Brazil), sub-superficial sample was collected in 20 liters polycarbonate bottle, at the day before of the start of the experiment, and used for preparation of the bacterial inoculum,. The water was filtered in calcinated 0.8 µm glass filters twice to remove phytoplankton, nanoflagellates and ciliates and was kept in the dark at 4°C

until the start of experiment. Temperature and pH were measured *in situ* with multiparametric probe YSI 6600 V2 (YSI, Yellow Springs®, OH, USA) with values of 21°C and 4.8 pH, respectively, and dissolved organic carbon (DOC) was measured in the lab after filtration in 0.22 µm polycarbonate membranes prepared as mentioned above.

Experimental design

The experimental design was conceived with exudates (DOM) obtained from axenic cultures of the two microalgae MA and CO separated or in mixture (MX), resulting in the factor quality with three levels (MA, CO and MX, respectively). Through the mix of DOM from both phytoplankton species as one of the quality levels, we also aimed to test the effects of increased chemical diversity of compounds available to the development of the bacterial communities.

DOMs of the three different qualities were then diluted in terms of dissolved organic carbon per liter (mg-C L^{-1}), the factor quantity, with two concentration levels, deemed here as 'High' and 'Low', approximately 20 mg L^{-1} for High and 4 mg L^{-1} for Low. The CO, MA and MX were prepared by dilution with sterile ultrapure water to achieve the desired DOM concentrations. Therefore, we had 6 treatments as the full combination of three Quality levels *versus* two Quantity levels. We set up a degradation experiment using one unique natural bacterioplankton community and these six treatments. All treatments had excess nitrogen (2.87 mg L^{-1} NO_3) and phosphorus (0.86 mg L^{-1} PO_4) added to prevent nutrient limitation (Logue et al., 2016). A single sample from the six treatments was obtained for initial measurement of DOC concentration and spectral measurement and then each treatment was inoculated with natural bacterioplankton community in the ratio of 5% v/v and poured

into nine 250 mL glass ambar flasks with culture volume of 220 mL. In addition to the treatment flasks, we kept biological (5% v/v of bacterial inocula in sterile ultrapure water) and chemical controls (DOM without bacterial inocula of each treatment).

The culture flasks were kept in an incubator with controlled temperature, at approximately the temperature of the natural water where the bacterioplankton were obtained (21°C), with constant and mild agitation, in the dark. Since nine flasks of each treatment were prepared, we removed triplicate flasks in each experimental sampling time, which were T1 = 6h, T2 = 24h and T3 = 96h (4 days). Flow cytometry analysis (abundance, biovolume, biomass and cytometric diversity), DOC determination, absorbance of DOM on ultraviolet to visible light and associated indexes were performed for each sample.

DOC quantification and DOM analyses

Samples for DOC analysis and UV-visible absorbance spectra were filtered through sterile, acid washed (2M HCl) 0.22 µm polycarbonate membranes rinsed with copious volumes of ultrapure water. DOC determinations were performed in a total organic carbon analyzer (TOC-Vcph, Shimadzu). The instrument was calibrated with standard solutions of potassium hydrogen phthalate, and three to five readings were made, until the coefficient of variance (standard deviation/mean) of readings was smaller than 2%.

UV-visible absorbance was obtained in a Spectrofluorometer FS5® (Edinburgh Instruments, UK) in transmission scan mode, from 200 nm to 800 nm with 1 nm step. Samples were measured in a HCl acid-cleaned 1 cm quartz cuvette and ultrapure water was used as blank. Spectral slopes ($S_{275-295}$, $S_{290-350}$, $S_{350-400}$), which were calculated as a non-linear fit of an exponential function at the target

interval, were estimated. The spectral slope ratio ($S_{275-295}$/ $S_{350-400}$), DOM normalized absorbance in the UV region (specific ultraviolet absorbance, SUVA [254 nm, 280 nm,350 nm,370 nm]), and DOM normalized absorbance in the visible region (specific visible absorbance SVA [412 nm, 440 nm, 480 nm, 510 nm, 532 nm, 555 nm]) expressed as L mg C^{-1} m^{-1}, were calculated (Hansen et al., 2016; Kothawala et al., 2012).

Bacterial abundance and diversity

Samples for bacterial abundance were fixed with formaldehyde to a final concentration of 1%, flash frozen in liquid nitrogen and kept at -80°C until analysis. Analysis were performed with Syto-13 Green stain and addition of fluorescent latex beads with 0.75 µm diameter, as internal size control, in a BD FACScalibur™ cytometer and analyzed in the FlowJo®V10.2 software. The results from FlowJo were then used as input for the *flowDiv*, a newly developed R (R Core Team, 2017) package which analyze bacterial gated populations in flow cytometry for their diversity, providing measures of alpha diversity and evenness of bacterial community for each sample and a matrix of beta diversity between all samples (Wanderley et al., 2019). The diversity measures can be any of the ones available in R *vegan* package, and we used Shannon-Wiener Index as alpha diversity and Bray-Curtis dissimilarity matrix as beta diversity.

Bacterial metabolism

Bacterial production was measured by the ^{3}H-Thymidine radiolabelled method, following the protocol of Kirchman et al. (1982). Briefly, two replicates of each sample were incubated for 1 hour in the dark with 5nM (final concentration) ^{3}H-Thymidine (70 $Cimmol^{-1}$, New England Nuclear) and then fixated with cold

trichloroacetic acid (TCA) 50% (v/v). Blanks for each treatment were killed with TCA 50% before the addition of ^{3}H-Thymidine. Samples were centrifuge-cleaned twice with 1 ml ultrapure water at speed of 14000 RPM for 10 minutes, discarding the supernatant. Scintillation cocktail was added, and the radioactivity assay was carried in a Perkin Elmer Scintillator. We estimate the bacterial production as µg C l^{-1} $hour^{-1}$ following the calculations outlined in Barrera-alba et al. (2004), based in the original works of Bell et al. and Kirchman et al. (Bell et al., 1983; Kirchman et al., 1982). Briefly, this estimation starts with the conversion of disintegrations per minute (dpm, the output of the assay) to mol ^{3}H-Thymidine l^{-1} $hour^{-1}$, which is then converted to cell number produced at the same time interval. With the average biovolume of bacterial cells estimated from the flow cytometry analysis and subsequent conversion to biomass (in pg C $cell^{-1}$), we were finally able to estimate the bacterial production in µg C l^{-1} $hour^{-1}$.

Bacterial respiration (BR) was estimated by the dissolved oxygen consumption of samples in triplicates, in 24 hours incubations using an optical fiber sensor (Unisense©, Aarhus, Dinamarca) (Briand et al., 2004). This experimental estimation was only possible at the 96 hours sampling points (T3) given the low oxygen consumption at other sampling times. Therefore, for the estimation of respiration of the other time points, we followed the estimated conversion factor from bacterial production to respiration presented in del Giorgio and Cole (1998).

In T3 sampling time, it was possible to estimate the bacterial growth efficiency (BGE) directly from the relationship between the measured bacterial production and respiration by the formula BGE = BP/(BP + BR). For the sampling points where we did not had and experimentally determined BR, we also estimate the BGE from BP, following the correlation proposed in del Giorgio and Cole (1998).

Statistical analysis

To investigate the differences between treatment for each time and over the whole experiment, DOC concentration, bacterial abundance, cytometric alpha diversity, bacterial production (BP), respiration (BR), and growth efficiency (BGE) were compared through Analysis of variance (ANOVA), to a cutoff significance ($Pr(>F) < 0.05$. A distance matrix of the Euclidean distances of the chi-squared transformed DOM spectral characteristics and a Bray-Curtis distance matrix of the cytometric diversity were used as input for non-metric multidimensional scaling (NMDS) to represent and investigate sample relations from DOM and beta diversity dissimilarities matrices in a bi-dimensional space.

Permutational Multivariate Analysis of Variance (PERMANOVA) was applied both to community Bray-Curtis dissimilarities matrix and DOM Euclidean dissimilarities measures to further investigate how variations in the dissimilarities are attributed to different treatments (quantity and quality levels) and to incubation times.

Finally, the correlation between DOM similarity matrix and cytometric beta diversity matrices was computed through the Mantel correlation test, both for all data and for data separated by sampling Time. All tests were performed in R software, package *vegan* (Oksanen et al., 2017).

Results

Bacterial abundance

All six treatments showed considerable increase in bacterial density (Fig 1a), including the biological Control. The average of initial cell density for every treatment was 5.36×10^4 cells mL^{-1}. The average cell density at the end of the experiment were 2.22×10^7 cells mL^{-1} for ‘High’ and approximately three times less for ‘Low’

treatment: 7.38 x 10^6 cells mL^{-1}. The biological Control presented 1.8 x 10^6 cells mL^{-1}, over an order of magnitude lower than 'High' treatments. Three significantly different (ANOVA Pr(>F) <0.05) groups of bacterial abundance were achieved by the end of experiment (96 hours – 4 days) according to the quantity level; High, Low and Control (no addition of DOM) (Figure 1a). There were no differences in mean bacterial abundances between quality levels by the end of experiment (ANOVA (Pr(>F) > 0.05), either in specific quantity levels or in the overall data.

Bacterial density of every treatment, except for CO_High, displayed a drop in the first 6 hours of culturing followed by an exponential growth phase until 24 hours and, subsequently, a slower growth rate was maintained until the final sampling at 96 hours (4 days). Probably for this reason, the most revealing responses happened at the second sampling time (24h).

The 24h was the only sampling time that quality groups within each quantity had abundances significantly different, while quantity groups had significantly different abundances from T1 to T3 (Table 1). Treatments MX_Low had a striking increase in cell abundance that separated this treatment from the other two quality levels at the same quantity level (CO_Low and MA_Low). Also, the treatment MA_High had similar cell abundances to these two Low quantity level treatments, with significantly less cell abundance than the other two treatments with High quantity level (CO_High and MX_High). In lower DOM quantity, it seems that greater chemical diversity resulted in faster increase of bacterial abundance at short incubation time, as seen for MX Low until 24h.

Table 1: ANOVA analyses of alpha diversity (Shannon-Wiener, H), bacterial abundance and dissolved organic carbon (DOC). Results are shown, in more detail, for analyses of differences in Quality and Quantity levels for each sampling time. Analyses with whole data pooled for differences between Quantity and Quality levels are also provided.

		Alpha diversity (H)		**Bacterial abundance**		**DOC**	
		F	Pr(>F)	F	Pr(>F)	F	Pr(>F)
Quality	T0	1.512	ns	0.929	ns	0.1	ns
	T1	0.553	ns	0.862	ns	0.02	ns
	T2	5.878	**< 0.05**	4.233	**< 0.05**	5.622	**< 0.05**
	T3	0.272	ns	2.387	ns	0.361	ns
Quantity	T0	0.543	ns	1.07	ns	51.37	**< 0.05**
	T1	8.966	**< 0.05**	11.95	**< 0.05**	85.65	**< 0.05**
	T2	24.92	**< 0.05**	8.991	**< 0.05**	5.683	**< 0.05**
	T3	0.283	ns	266.2	**< 0.05**	6.992	**< 0.05**
Quality		1.564	ns	0.63	ns	0.5	ns
Quantity		0.674	ns	4.156	**< 0.05**	29.76	**< 0.05**

Significant differences are highlighted in **bold**. ns = not significant

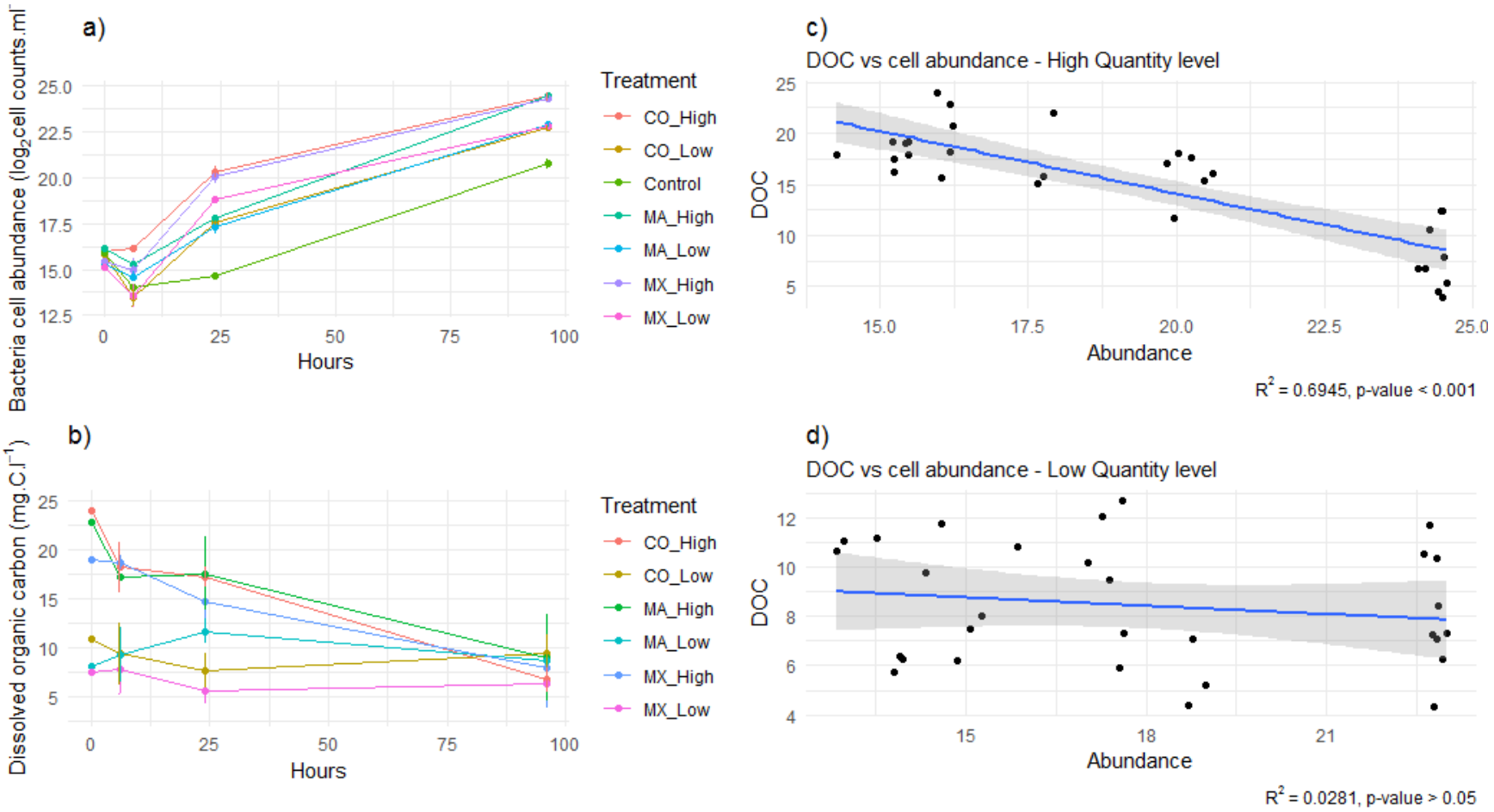

Figure 1: a) Log_2 transformed mean bacterial abundance in treatments over incubation time; b) mean dissolved organic carbon (DOC) concentration, in mg-Carbon L^{-1}, over incubation time; c) High quantity level treatment – linear regression between DOC concentrations and log_2 abundance; d) Low Quantity level treatment – linear regression between DOC concentrations and log_2 abundance. CO = *Cryptomonas obovata* DOM; MA = *Microcystis aeruginosa* DOM; MX = mixture, in equal parts, of CO and MA DOM; High = treatments with the higher DOC concentration level; Low = treatments with the lower DOC concentration level.

DOC measurements and correlation with bacterial abundance

The average of DOC concentration from quantity levels High and Low at the start of experiment was 21.94 mg-C l^{-1} and 8.77 mg-C l^{-1}, respectively, and reached significantly different averages (5.34 mg-C l^{-1} and 2.90 mg-C l^{-1}, ANOVA Pr(>F) <0.05) by the end of experiment (Fig 1b; Table 1). It is also important to mention that there was a significant carryover of environmental DOM to the treatments, which we estimate to be around 3 mg-C l^{-1}. This carryover is especially important for Low Quantity treatments, as it has a similar concentration to the intended Low Quantity level. However, this is not a problem for the comparisons between treatments, as this carryover was present in all treatments.

The percentages of DOC removal were over 60% for all “High” treatments. The DOC removal percentages in High quantity levels were bigger for CO or MX quality levels, with CO having the greatest removal (CO_High had 81.58%; MA_High had 69.24%; MX_High had 75.93%). The removal of DOC from the Low quantity treatments was marginal. DOC measurements for Low quantity level showed that the removal in MX quality level was the biggest, but there were no significant differences of DOC concentrations by the end of experiment by quality levels (Fig 1b; Table 1).

The linear regression of DOC and cell abundance for the whole data set was significant (p-value < 0.05), but with a rather low $R^2 = 0.1184$. High quantity level treatments regression was also significant, but with $R^2 = 0.6954$ (Fig 1c), while the regression was not significant for Low quantity levels (p-value > 0.05).

Community cytometric alpha diversity analysis

The cytometric alpha diversity calculated as Shannon-Wiener Index (H index) and Pielou's equitability Index (J index) is presented in Figure 2, with H index also shown for High and Low quantity levels separately (Fig 2c and 2d, respectively). All treatments, regardless of the quantity or quality level, converged to similar values of H and J indices at T3 (=96 hours), with averages of H = 108.22 and J = 0.78, without significant variation (ANOVA Pr(>F) > 0.05) (Fig 2a; Table 1, only Shannon-Wiener shown but same results for Pielou's J).

A pattern is readily apparent in Figure 2. Communities with High quantity went through an early decrease in the H diversity levels, which reached their minimal values after 24 hours of incubation, with a subsequent recovery of H diversity in all communities (Fig 2c). The lowest H values were attained by quality treatments CO and MX, both containing DOM from *C. obovata*. In the Low quantity treatments, this pattern is recalled in slightly different fashion, with this initial decrease happening either between the beginning of the experiment and 6 hours (T1, CO and Control) or at the 24h time point (MA and MX). Once more, the treatments with CO DOM included, either alone or in mixture, reached the lowest values of H index at 6 or 24 hours. Regarding the J index, all treatments had decreased values from 6 hours to 24 hours of incubation (T1 to T2), and the sharper decreases were for the treatments CO_High and MX_High, then MX_Low and MA_High. CO_Low and MA_Low displayed a less acute, steady decrease up to 96 hours of incubation (T3). The pattern of recovery of diversity was only observed for the CO_High and MX_High, and marginally for MX_Low and MA_High (Fig 2b).

Overall, the quantity level affected more strongly the communities and the response is fast, given the drop in the diversity indices until 24h (T2) (sometimes at

6h [T1]). However, this quantity effect seems to be modulated by the quality level of the DOM, as the treatments with DOM from *C. obovata*, alone or in mixture, resulted in the lowest H index values in both quantity levels.

Beta diversity

Bacterial cytometric differences between samples, the beta diversity, were represented in a non-metric multidimensional scaling (nMDS) ordination plot of Bray-Curtis dissimilarity matrix (Fig 3). The triplicates of each treatment within each sampling time grouped together and mostly without overlap after 6 hours (T1), reflecting the changes in the communities over time along axis NMDS 1, with increasing dissimilarities of samples within sampling time until 24h (T2) and less dissimilar communities at 96 hours (T3) relative to T2.

At 24 hours of experiment, communities in each treatment were more clearly separated along the NMDS 2 axis, reflecting primarily the two quantity levels, but also signaling the dissimilarities by quality levels, whose intensity (=distance) seem to depend upon the quantity level. For High quantity treatments (green symbols), tighter clusters were formed with quality CO and MX. This pattern was present, but attenuated, in Low treatments.

At 96h, the distances between samples had an overall decrease relatively to 24 hours of incubation and treatments formed a less dispersed group with samples more similar across all treatments, albeit they were still separated by quantity level and, even if not as evident as at 24 hours, by quality levels.

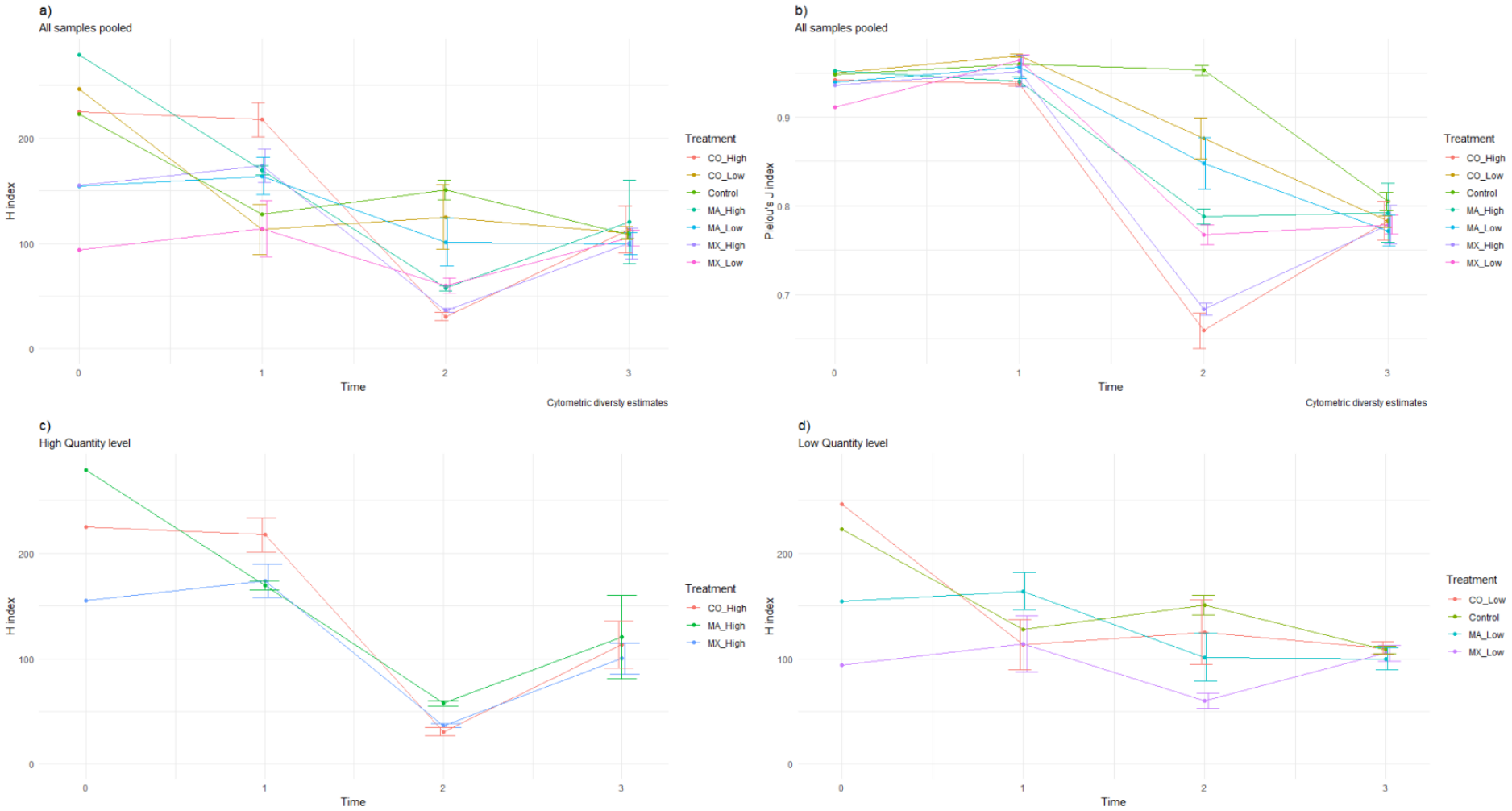

Figure 2: Shannon-Wiener and Pielou's equitability diversity index. a) Shannon-Wiener values for full data set; b) Pielou's equitability index for full data set; c) High Quantity level Shannon-Wiener index. d) Low Quantity level Shannon-Wiener index.

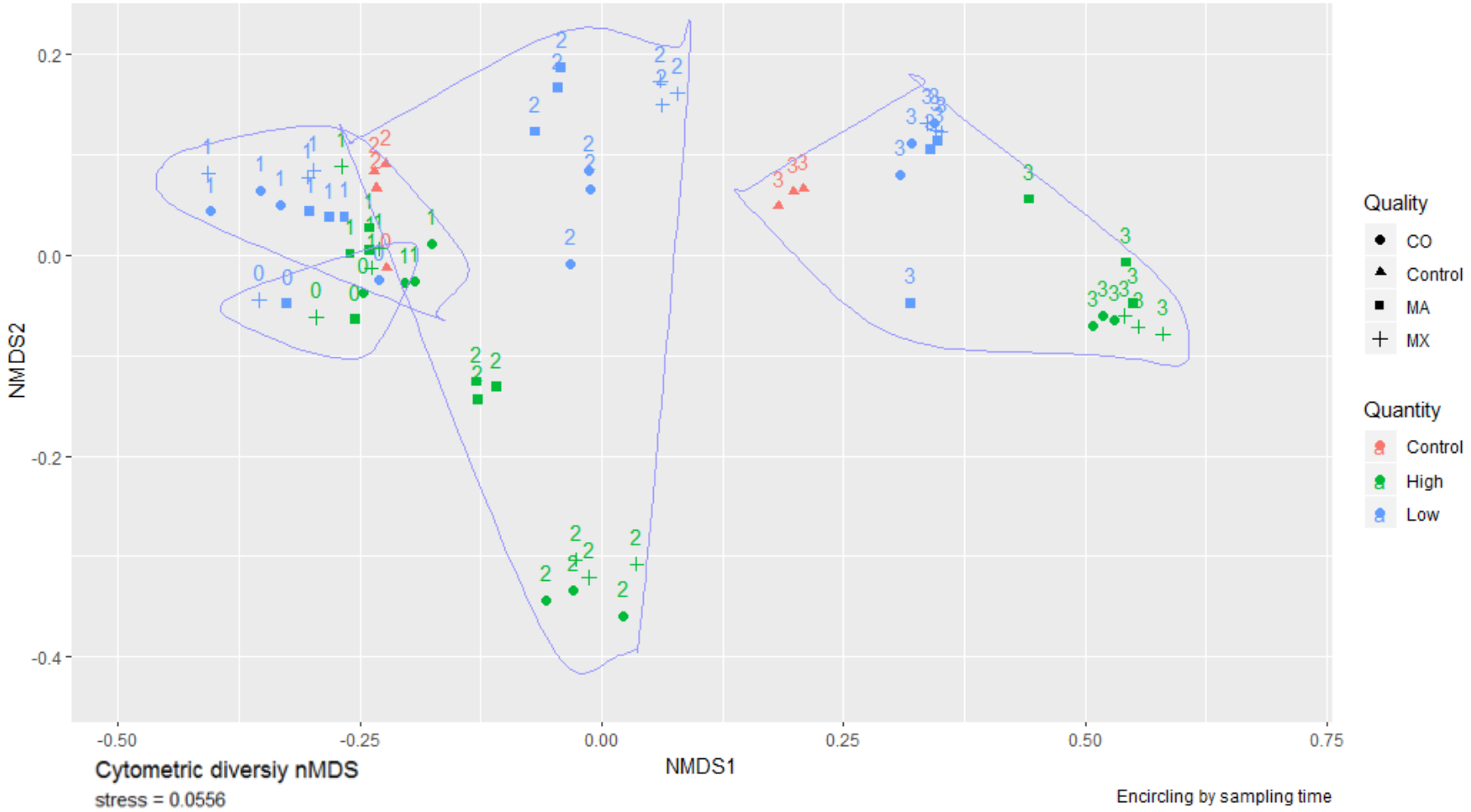

Figure 3: NMDS representation of beta diversity between samples, assessed as Bray-Curtis dissimilarity matrix. Light blue ellipses depict time samplings and are not drawn based in statistical analysis. Green symbols are High quantity treatments, blue symbols are Low quantity treatments; red triangles are biological controls. Filled circle = *C. obovata* DOM quality; Filled triangles = Control; Filled squares = *M. aeruginosa* DOM quality; Cross = Mixed DOM quality.

PERMANOVA results presented in Table 2 indicate that quantity factor significantly correlates to community variation (Pr(>F)<0.05) throughout the sampling times. Quality factor and the interaction term between factors also significantly correlated with community variation, except at last sampling time (full data set, *Data Partitioned by Time*). A more in-depth exploration of the data by quantity levels gave further evidence of the effects of the quality levels throughout the experimental time. Overall, quality levels of DOM did explain similar proportions of the variation in community composition regardless of quantity level until 24h (T2). In the last sampling time (T3, 96 hours), the quality level no longer correlated with variations in the community composition for Low quantity treatments.

Table 2: PERMANOVA analysis of the variance of beta diversity attributed to different grouping strategies of the data (all partitioned by sampling time): Full data set, High quantity treatments and Low quantity treatments.

			T1	**T2**	**T3**
		df	r^2	r^2	r^2
Full data set	Quantity	2	**0.279**	**0.486**	**0.721**
	Quality	2	**0.173**	**0.181**	0.038
	Quant:Qual	2	**0.123**	**0.172**	0.015
	Residuals	12,14,14[a]	0.422	0.160	0.192
High Quantity		df	r^2	r^2	r^2
	Quality	2	**0.4393**	**0.709**	**0.400**
	Residuals	6	0.560	0.290	0.599
Low Quantity		df	r^2	r^2	r^2
	Quality	2	**0.394**	**0.733**	0.247
	Residuals	6	0.605	0.26	0.752

Bold indicates Pr(>F) <0.05.

DOM spectral absorbance characteristics

DOM spectral absorbance characteristics separated treatments primarily due to quantity levels (Fig 4a), but a closer look within these two groups provided more information. High quantity treatments (Fig 4b) are placed in the nMDS representation according to incubation time, with T0 (initial) samples separated from the other times, while T1 (6h) and T2 (24h) are somewhat entwined. T3 (96h) samples are also separated from other sampling times. Samples were further separated by quality levels, with some superposition of samples from different quality levels between T1 and T2. In T3, although yet in a concise group, samples were more scattered.

It is also possible to discern an overall separation across all sampling times between the three quality levels. The pattern of separation consists in samples from the quality levels CO and MX closer together and samples from MA level apart from the other two. More specifically, MX samples are usually placed between samples of CO and MA.

The overall pattern for Low samples resembles this quality-driven pattern. Low treatments showed little difference by time but shared the same pattern of separation of samples according to the quality level as the High treatments. Further, CO and MX samples clustered together across the whole experimental time (with the exception of one outlier of MX at T3), while MA samples, although with some superposition with the CO and MX cluster, also stayed in a rather concise cluster throughout all sampling times.

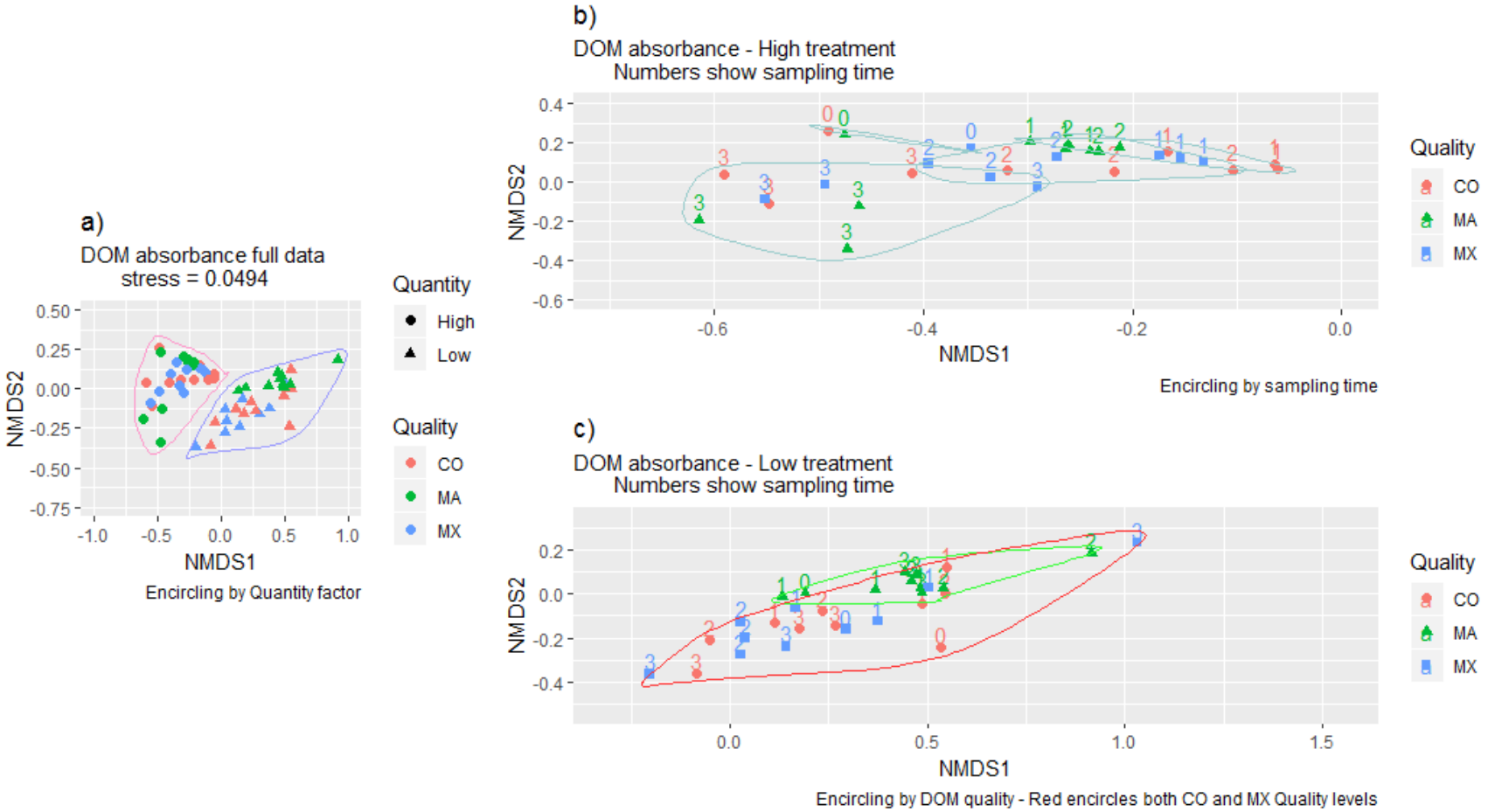

Figure 4: nMDS representation of spectral absorbance characteristics of DOM. (a) Full that set; (b) High quantity treatments, with light blue encircling depicting sampling time; (c) Low quantity treatments, with red encircling indicating the group formed with CO and MX quality levels. CO = *Cryptomonas obovata*; MA = *Microcystis aeruginosa*; MX = Mixed DOM.

PERMANOVA analysis of DOM shown in Table 3 indicates that the factor quantity significantly explained the total variance on all sampling times for the full data set, while the factor quality and the interaction term of quantity and quality were only significant for the variance of the DOM during T2. In a similar fashion to the PERMANOVA analysis of the cytometric diversity and considering the high percentage of the variance of data explained by the factor quantity, we split the data by quantity levels to further analyze it. The explained variance of the DOM characteristics by the factor quality was bigger in the treatments with Low quantity levels (High quantity, quality $r^2 = 0.3492$, Low quantity, quality $r^2 = 0.5952$.

Table 3: PERMANOVA analysis of the variance of DOM spectral characteristics attributed to different grouping strategies of the data (all partitioned by sampling time): Full data set, High quantity treatments and Low quantity treatments. Control is included only at T3.

			T1	**T2**	**T3**
		df	r^2	r^2	r^2
Full data set	Quantity	2	**0.6810**	**0.4764**	**0.4289**
	Quality	2	0,0601	**0.2099**	0.1281
	Quant:Qual	2	0,01392	**0.1215**	0.1189
	Residuals	12,14,14[a]	0.2448	0.1920	0.3239
High Quantity		df	r^2	r^2	r^2
	Quality	2	0,1643	**0.3492**	0.3819
	Residuals	6	0.8356	0.6507	0.6180
Low Quantity		df	r^2	r^2	r^2
	Quality	2	0.0677	**0.5952**	0.1027
	Residuals	6	0.9332	0.4047	0.8972

Bold indicates Pr(>F) <0.05.

Interaction between DOM spectral characteristics and cytometric bacterial diversity

The interaction between DOM spectral characteristics and bacterial diversity was evaluated from the correlation between the dissimilarity matrices of DOM and beta diversity, through the Mantel test, which varies from -1 (strongly uncorrelated) to 1 (strongly correlated) as the Pearson product-moment correlation coefficient. The Mantel test requires that the compared matrices have the same dimensions, so the biological control samples (Control) were removed from the beta diversity matrix since they were missing in the DOM matrix. Considering all samples, a positive, significant correlation was found between DOM spectral characteristics and cytometric bacterial diversity (r^2 = 0.263; p-value <0.05; Table 4). The correlation between DOM and diversity for individual sampling times were always significant and larger than for the whole data set. This correlation was bigger at T1 (T1 r^2 = 0.5255) and slightly decreased at T2 to a value that was maintained until T3 (r^2 = 0.4687 and r^2= 0.4556, respectively).

Table 4: Mantel correlation between cytometric dissimilarities matrix and DOM spectral absorbance distance matrix. r2 reported as Person's product-moment correlation.

	Full dataset	T1	T2	T3
r^2	0.263	0.5255	0.4687	0.4556
p-value	< 0.05	< 0.05	< 0.05	< 0.05

Bacterial metabolism

The bacterial metabolism is presented here with the bacterial production (BP) and bacterial growth efficiency (BGE) (Fig 5). Bacterial production was measured at T0,

24 hours (T2) and 96 hours (T3) of incubation, and there are no measurements of BP, and thus BGE, at 6 hours (T1) of incubation. The BP is minimal at T0 (Fig 5 left panel) and is greatly increased at 24 hours of incubation in those treatments that displayed greater cell growth, CO_High and MX_High, followed by the MX_Low treatment. The BGE display a similar pattern, but at 24 hours of incubation treatment MA_High has the same level as the MX_Low, and CO_Low and MA_Low have the lowest BGE of all treatments. At 96 hours of incubation, MA_High has the greatest BP, and a similar level of BGE as CO_High, while MX_High has the lowest BGE of all treatments, but still larger BP than CO_Low and MX_Low. Overall, the BP increase consistently up to the last sampling point, indication that there was still enough carbon to sustain the growth of communities.

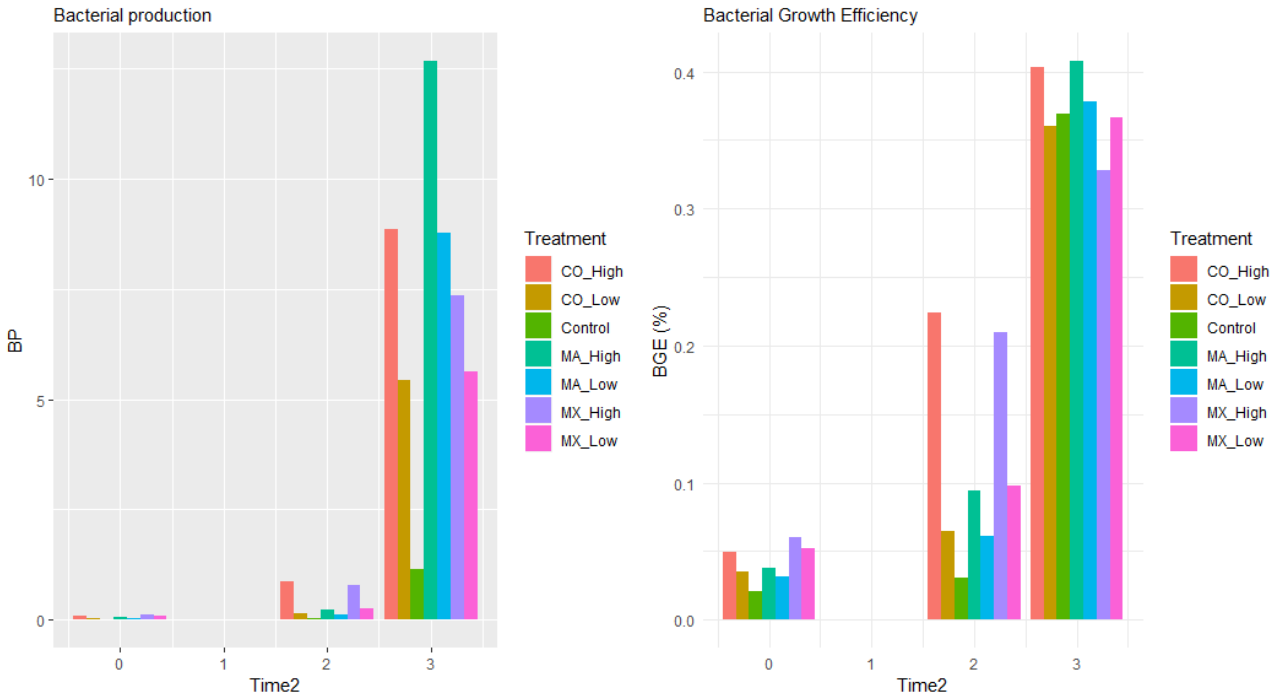

Figure 5: Bacterial metabolism measurements of treatments. Left panel: Bacterial production in µg C L^{-1} h^{-1}; Right panel: Bacterial growth efficiency, in percentage of carbon used to production (fixation of biomass).

Discussion

The assessment of bacterial diversity was performed through the cytometric diversity of the samples. In Flow cytometry (FCM), morphological and nucleic acid content - phenotypic information - are readily obtained on a single-cell basis (Props et al., 2016). Through this information, individual cells (events in FCM) are differently positioned in a scatter plot formed between two (of the many possible) FCM channels. Subsequently, the flowDiv package extracts the position (phenotypic information) and

computes ecological diversity metrics – alpha diversity, beta diversity, evenness index. Although these bins do not correlate directly to the effective number of species, they are analogous to operational taxonomic units (OTUs) and accurately captures diversity changes (Props et al., 2016; Wanderley et al., 2019).

To understand the dynamics of a community, it is necessary to understand the ecological mechanisms that control the community assembly (Goldford et al., 2018). These mechanisms can be described within four “high-level” processes: selection, dispersion, drift and speciation (Vellend, 2016). By using a closed microcosm system in a short term experiment, we simplify our approach by ensuring that the process that would have more importance to the governing microbial community assembly in our experiment is selection. Selection effects stems from the abiotic and biotic components of an environment, and here we expect it to be caused by the interplay between community members modulated by the quality and quantity of the DOM available.

We observed greater decreases in alpha diversity measurements with High quantity treatments overall, but even greater decreases within High quantity treatments containing DOM from *C. obovate* (CO and MX qualities). A previous study tested the specialization of bacteria feeding of phytoplanktonic DOM, in a combination of both quantity and quality levels, for high taxonomic bacterial ranks, and found that that specialization in the use of different DOM decreased as DOC concentration increases (120, 360 and 1200 μg l^{-1} of DOC), i.e. higher DOC concentrations favored more generalist behavior in short term experiment (5 hours) (Sarmento et al., 2016). In line with their results, quantity could be exerting a bigger influence on the community assembly, as seen with PERMANOVA results (table 2). However, we could also argue

that the effect of quantity in our experiment was only magnifying and extending in time the effects of the quality of DOM on the bacterial community, since regardless of the quantity used, qualities containing DOM from *C. obovata* had the biggest impacts: steeper decrease of alpha diversity and higher bacterial production at 6 – 24 hours and, evident in High quantity treatment but less clear in Low Quantity, more dissimilar communities compared to the Control (Fig 3). A study found that the lack of an effect of varying carbon sources could be an artifact caused by the assessment of these effects through broad taxonomical ranks. One could observe more generalist strategy for class and order levels, but when the diversity within these clades is more carefully scrutinized at genera level, specific carbon compounds may in fact stimulate the growth of specific organisms, suggesting that they are specialists (Gómez-Consarnau et al., 2012). This observation might indicate that the effects of quality could be masked, and thus quantity effects would be deemed more important in analyses with higher taxonomic rank.

Bacterial communities can also display changes in their diversity without a strong change in their composition, i.e. losing or gaining organisms, through changes in the relative abundances of their members. Thus organisms that were not selected at the beginning of the incubation could be maintained, even if without growth (Dinasquet et al., 2013), and they could start growing at a later point in the incubation, which could explain the observed recovery in the alpha diversity in a still growing, continuously productive community (Fig 1a, Fig 5).

It was also found that some bacterial taxa can be extremely efficient at consuming a given DOC pool, and even a single strain has the potential to completely consume the labile fraction of a DOC pool (Pedler et al., 2014). This finding shows how

strong selection can be if organisms that are extremely efficient in using the available DOM are present. Similarly, here it might had happened that a few fast growing bacterial taxa were selected at the beginning by the DOM of *C. obovata*, but at later stages the environment changed in a way that permitted other organisms to also grow, recovering the diversity and adding up to the community-wise metabolic rates measured. In this scenario, the whole process could be a sequence of competition, at early stages, and facilitation towards the end of the incubation, driven by the quality of the DOM, whose effects could be augmented by higher quantity. In this sense, the organisms that grow faster are outcompeting the majority of other clades by being able to use more efficiently the compounds of the fresh and more labile DOM and, by breaking these compounds into other compounds and/or excreting by-products, would enable or enhance the growth of a bigger number of taxa that are not so efficient in using this initial DOM.

Remarkably, the nMDS representation of the DOM (Fig 4), although primarily clustered by quantity level, showed that all quality levels had changes with time but still reasonably maintained their individual discernible characteristics (Fig 4b and 4c). Moreover, the DOM changes were correlated with the changes of the bacterial community (Fig. 3, Table 4), indicating a coupling of biochemical DOM diversity and biological diversity, which supports the view of a sequence of processes, with competition causing the drop in overall diversity at first and, as the biochemical characteristics of the DOM are changed, the onset of facilitation of the growth of more organisms, resulting in the partial recovery of diversity levels. Accordingly, the Mantel correlation between DOM spectral absorbance characteristics and bacterial cytometric

diversity were positive throughout the experiment. Further, the overall data had a significant correlation, and the correlation at specific times was consistently higher, peaking at 24 hours (T2), further corroborating the interactions found between DOM (quality and quantity) and bacterial cytometric diversity in our work.

Barra Bonita reservoir is dominated by *M. aeruginosa* throughout the year, with transient increases in the relative abundance of *C. obovate* (Dellamano-Oliveira et al., 2008; Vieira et al., 2013) and, thus, the community used in this study could be considered adapted to the MA DOM. Accordingly, communities growing on the MA quality DOM are more similar to Control than the communities of the other two quality levels at T2. This is another indication that quality might be a more important factor for the microbial community assembly and that it exerts an early effect on the communities.

Yet another interesting remark is that our amended DOMs were freshly produced by phytoplankton, with an overall labile characteristic (Bertilsson and Jones, 2003; Medeiros et al., 2017), while an environmental DOM would be a mixture of labile and more degraded DOM, thus being more recalcitrant (Dittmar, 2015). We observed an indication of this more recalcitrant characteristic with the non-significant linear regression between cell abundance and DOM for Low quantity treatments (Figure 1d), for which the environmental DOM carryover is more important. Therefore, the bacteria on these treatments could be using preferentially only the amended, more labile portion of the available DOM, thus preventing a general correlation between DOC concentrations and cell abundances as we observe for High quantity.

At the end of the incubation, all communities started to get closer to control again, although with High treatments still farther way. Also, communities of all qualities

start to cluster together within a given quantity level, indicating that the quality effect is transient and select organisms at early stages of the incubation, but as the DOM quality is degraded this effect is alleviated. This outcome is clear by the observation of Figure 3, where a stronger selection causes the communities to increase their dissimilarity at 6 hours and, especially, 24 hours. The effect of quality was more evident and lasting in High treatments (Fig. 3) and during exponential bacterial growth. All these observations indicate that indeed quality correlation with diversity was modulated by the quantity level of DOM and restricted mostly to early responses, at the exponential growth phase, until 24h (T2) (Table 2). After that point, the communities started to converge to a similar point, indicating that the selecting effect of the DOM is indeed decreasing, more evidently so for the treatments with Low quantity.

There is an ongoing debate about the roles of competition and facilitation on the mechanisms of community assembly, and both seem important for this assembly (Goldford et al., 2018). However, some robust support was found for a type of facilitation that does not cause a decrease in fitness level of the helper organism (Pacheco et al., 2019), meaning that facilitation could be a strong driver of community assembly, especially at later stages of community development, similarly as we observed here. Pacheco and colleagues showed that the generation of different by-products compounds by ‘first’ organisms is dependent on the identity of these first organisms, oxygen presence, and the original source of carbon. Different sets of by-products were simulated to be produced with different iterations of ‘first’ organism and original carbon source, and each set of by-products could in turn sustain specific individuals. If this concept is extended for an entire community, a competition could

happen at first, given a limited diversity of compounds available. As DOM is degraded (as any other carbon source), its diversity, in the sense of different compounds, is at first increased, although at longer time it usually is reduced, mostly to recalcitrant compounds (Dittmar, 2015). Thus naturally, the composition of this initial community depends upon the quality of the initial carbon source, as it would select for fast growing organisms at first, which would then select the next ‘generation’ of organisms that would be able to grow through the by-products that they generate, and so on (Pacheco et al., 2019).

Conclusion

Our findings show that both quantity and quality contributte to variations in bacterial community composition, metabolism and alpha diversity changes. However, quality seems to be determinant for the development of the communities and exerts its effect at the beginning of the incubation by inducing a strong competition. As the biochemical environment is modified by these organisms, a change that is correlated to the changes on the community composition, other organisms are enabled to growth. Therefore, the effect of DOM quality can be seen as sequence of competition and facilitation between communities’ members. The quantity seems to modulate the effect of quality, with larger quantity the DOM magnifying and extending the duration of the competition-dominated stage of the community assembling.

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CAPÍTULO 2 - CRYOPRESERVATION OF WHOLE HETEROTROPHIC BACTERIAL COMMUNITIES: EFFECT OF NATURAL DISSOLVED ORGANIC MATTER MEDIUM AMENDMENTS TO THE RECOVERY OF STRUCTURE AND ACTIVITY OF RESUSCITATED COMMUNITIES*

**Experiments presented in this chapter were perfomed at the Laboratoire d'Océanographie Microbienne (Lomic), Observatoire Oceanológique de Banyuls-sur-Mer, France, in a project coordinated by Dr. Sara Beier,*

Abstract

In this work, we evaluated the application of a cryopreservation method coupled with a culturing method using natural dissolved organic matter (DOM) extracts to cryopreserve and re-grow whole natural aquatic heterotrophic bacterial community, aiming to maintain its structure and diversity. Common difficulties in micro/mesocosms experimentation motivates this work, and they are related to the lack of control of the initial community, overgrown of rare taxa that significantly shifts community' composition and the limited experimental possibilities with communities from hard to access sites. Overall, we were able to successfully apply these methods, but there are some caveats. First, the initial absolute cell abundance of the community is critical; small volumes lead to greater variance in the cell abundances of the resuscitated community. Second, long time lags between sampling and cryopreservation can cause compositional changes in the communities, even if the overall performance does not show significant variations, and thus it is advisable to process the samples as fast as possible. Finally, the method did not fully recovered diversity, but recovery a significant fraction of it in a reproducible fashion. Furthermore, specialized communities from more stable, oligotrophic environments proved more difficult to re-grow in the resuscitated communities, while

communities that were tentatively identified as more generalists were remarkably similar to their original community. We then devised an experiment to showcase the potential application of the method, investigating simultaneously the effect of variation in DOM supply and coalescence of communities. Tentatively more generalist community, Canet, was more adaptable and thus was re-grown more similarly to the original community than the specialized SOLA community. Simultaneously, coalescent communities were dominated by the more generalist community. Considering the consistency of results, the method opens many possibilities to experimentation with hard to access communities and to evaluate interactions of factors in a more complete fashion.

Keywords: cryopreservation, DOM amendment, microbial ecology, bottle-effect, historical contingencies

Introduction

Ecology is the science that tries to answer the question of why different environments harbor different sets of organisms (Vellend, 2016). Microbes have enormous importance to ecosystem functioning (Fuhrman, 2009; Whitman et al., 1998) and, within their realm, heterotrophic bacteria have a key, as they are the foremost decomposers, driving the cycle of carbon and nutrients (Amado et al., 2013; Cotner and Biddanda, 2002) and, in aquatic environments, re-introducing energy and nutrients back to the food web via microbial loop (Azam et al., 1983; Pomeroy, 1971).

One effective way to expand ecological theory is with manipulation experiments, but often they are unfeasible or unethical to perform in natural environments. On the other hand, experiments with meso/microcosms allow for a constant system in which

selected variables can be manipulated at specific levels, a clear advantage for hypothesis testing. However, experimentation with microbes presents its own drawbacks, and the most prominent one is the lack of control for the start communities (Kerckhof et al., 2014). Studies aiming to test several hypotheses but starting with different communities may not be fully comparable, thus the development of a method to standardize the starting community is imperative.

The majority of cryopreservation protocols are only available for axenic cultures (Kerckhof et al., 2014) and usually are strain specific. The specificity of the protocol is related to the cryoprotective agent (CPA) of choice, the rate of freezing and thawing, storage temperature, and the medium and additives used to resuscitate the organisms (Heylen et al., 2012). Bacteria consortia and especially natural heterotrophic bacterial communities have high diversity, with hundreds to thousands different organisms, making their cryopreservation a challenge – the goal is to preserve diversity, composition and activity, but the high diversity would be exactly what prevents the development and employment of a single and efficient cryopreservation method. Therefore, it is unrealistic to expect a complete preservation of the community's composition or activity of all organisms in a natural and diverse community.

Furthermore, cultivation of natural bacterial communities in meso-microcosms result in different communities composition, both in batch or continuous systems (Vekeman and Heylen, 2017), with typical overgrow of some easily cultivated but rare taxa while naturally common and abundant species disappear (e.g. SAR11 clade members) (Giovannoni, 2016; Rappé et al., 2002). The net result is both loss of diversity in terms of richness and evenness, and an apparent turnover of species, two

aspects that prevent a readily transposition of findings of culture-based experimental approaches to natural environments. This outcome is commonly known as the “bottle-effect”, a not fully understood effect that likely is a result of excess carbon availability and/or unbalanced carbon to nutrient ratios in culture media (Agis et al., 2007; Hammes et al., 2010). Therefore, the development of both a way to cryopreserve whole starting communities and a culturing method that allows better maintenance of diversity of microbial communities in microcosm setups are necessary.

It was shown in an earlier study that typically abundant members of aquatic bacterioplankton that are hard to grow in laboratory conditions, such as members of the actinobacterial LUNA cluster, *Limnohabitans* sp. or SAR11 clade were actively growing in microcosms that were amended with DOM containing cell contents of microbes from natural aquatic ecosystems (Shen et al., 2018). Medium additives, as the signaling molecules N-acyl homoserine lactone (AHL) and cyclic adenosine monophosphate (cAMP), also might help increase the yields and efficiency of bacterial cultivation (Bruns et al., 2002), one effect that could be exploited for re-growth after resuscitation of cryopreserved communities.

To advance the possibilities of experiment inside that context, we tested a method of cryopreservation of whole natural bacterial communities and a cultivation method that would allow for the recovery of the natural diversity of the starting communities or, at the very least, the consistent recovery of a significant portion of it in the resuscitated communities. A whole community cryopreservation method was tested and the influence of highly oligotrophic natural DOM additions in the development of those microbial communities in microcosm incubations was assessed.

First it was investigated if it is possible to re-grow cryopreserved natural bacterial communities. The strategy used was to culture this communities in microcosms of artificial sea water medium amended with the DOM extracts, prepared at the laboratory, as the sole source of carbon and nutrients. Following any possible growth, the effects of variations in the absolute initial cell numbers of cultures over the reproducibility of cryopreserved and re-grown communities and the effects of addition of AHL and cAMP on the culture media were tested. The similarity of cryopreserved communities with their original community was then assessed. Later, it was investigated the effect of the time interval that the natural water sampled is kept in the sampling bottle before the preparation of the cryopreserved communities, i.e., the time lag in performing the cryopreservation protocol.

Based on the results of these initial experiments, but using new communities and DOM, it was tested if the composition of natural communities could be maintained in cryopreserved communities. Particularly, if the similarity of the re-grown community to the original community was related to the trophic level and variability of the original community's environment, as communities from different environments could have variations in their performance when frozen and/or grown in laboratory microcosms. Then the effects of both a coalescence event and differing DOM sources in the individual and coalescent communities were tested. Regarding this last experiment, we hypothesize that 1) community cultures amended with DOM extract prepared from samples of the same site as the community would facilitate the growth of the 'resident' community; 2) more diverse DOM, expressed as a mixture of two natural DOM extracts from different places, would permit a higher diversity in the re-grown communities; and

3) coalescent communities will have the highest diversity levels among the experimental communities.

Material and methods

Sampling sites

The water samples in all sampling sites were collected using polycarbonate carboys of 25 liters. These carboys were clean at the laboratory and previously rinsed with sample water from the site. The sample was transported back to the laboratory for processing.

Samples of water from the Mediterranean coast of Banyuls-sur-Mer bay (42°28' 52.1" N, 3°08' 14.6" E; hereafter called Mediterranean II) were obtained in two separated sampling events at the exact same site, on 03/22/2018 and on 04/12/2018. The water sample from the first sampling was used to obtain both the community used in five of the six treatments of experiment 1 and the DOM used in all treatments of this experiment. The sample from the second sampling was used only to prepare newly cryopreserved communities used as the sixth treatment of this experiment. This site was chosen for the first experiment because it is easily accessible, although no time series environmental data is available.

For experiments 2 to 4, two sites with distinct stability and trophic levels were sampled. Furthermore, a geographically distant site was sampled to obtain different DOM. The first sampling site was SOLA (Station d'Observation Laboratoire Arago), sampled in July 3rd of 2018 and located in the Mediterranean Sea, approximately one

nautical mile off the coast of Banyuls-sur-Mer bay, France (North Mediterranean Sea, 42° 31′N, 03° 11E) (Obernosterer et al. 2010). This is a stable and oligotrophic site (salinity mean = 37.75 ± 0.51 PSU; chlorophyll-a mean = 0.807 ± 0.58 µg. L^{-1}, n=111 – time series from 2012 to 2017 [https://wwz.ifremer.fr/surval/]). Both the communities and the DOM amendments used in the experiments were obtained from this site. Furthermore, a filter-sterilized volume of this site was used as medium in one treatment, to re-grow a community originating from this same site.

The second sampling site was Canet, a coastal lagoon highly variable and with hypertrophic events, with direct opening to the sea and input from riverine waters, located in the region of Languedoc – Roussillon, France (Canet lagoon, 42° 40'N, 3° 01'E, salinity mean = 22 PSU [max = 32.6, min = 4.9]); chlorophyll a mean = 19.5 µg. L^{-1} [max = 204 µg. L^{-1}, min = 3.00 µg. L^{-1}], time series from 2012 to 2017 [https://wwz.ifremer.fr/surval/]). From this site, only the bacterial community was used in the experiments.

The last sampling site, used to obtain a completely different DOM to be used in mixture with the DOM from SOLA in part of the treatments, was at the Baltic Sea.

Cryopreservation of communities

The water samples were pre-filtered through 0.8 µm polycarbonate membranes in order to remove primary producers, ciliates and nanoflagelates. This pre-filtered sample was used for the cryopreservation of the communities, which followed the protocol of Vekeman and Heylen (2017), with some modifications. This protocol, describing the cryopreservation of single strains of harvested bacteria cells growing in a chosen artificial liquid medium, was adapted for the cryopreservation of whole natural

communities, i.e. 0.8 µm the pre-filtered samples, that were concentrated in 0.22 µm polycarbonate membranes together with a small volume of the filtered natural water.

Briefly, a sterile stock solution of dimethylsulfoxide (DMSO), prepared by filtration through a 0.22 µm Teflon PTFE syringe filter, was used as the cryoprotective agent (CPA). This stock solution was diluted to a 10% (v/v) concentration in fresh artificial sea water (ASW) medium, which had no addition of carbon and nutrients sources, and 0.5 mL of the diluted DMSO solution was distributed in 2 mL Eppendorf tubes and kept at 4°C until use.

The 0.8 µm pre-filtered natural water samples were subsequently filtered through 0.22 µm polycarbonate membranes (25 mm of diameter) in order to accumulate bacterial cells on the filter. The volume used was variable on the first experiment and fixed for each sampling site in remaining experiments (see below). Care was taken to not completely dry the membranes by the end of the filtration procedure. A 0.5 mL aliquot of the water remaining over the membrane was used to resuspend the cells from the membrane and then pipetted into the previously prepared Eppendorf tubes, to achieve the final DMSO concentration of 5%. The membranes were then placed completely immersed into the tubes, which were maintained at 4°C for 15 minutes for acclimatization, flash frozen with liquid nitrogen and transferred to -80°C freezer for storage.

To inoculate the cultures in microcosms, cryopreserved communities were thawed at room temperature and, as soon as it could not be seen any ice in the thawed volume, the 1 mL liquid plus the filter were transferred to culture microcosms.

Preparation of natural dissolved organic matter (DOM) extracts

For the preparation of DOM extract solutions, a volume of the natural water samples was filtered directly through 0.22 µm polycarbonate membranes (150 mm of diameter) to retain all particulate matter. This volume varied and was taken into consideration for both the preparation of the solutions and the volume of these solutions used as amendment (i.e., carbon and nutrients source) in the culture media, which are described below for each experiment. These membranes were then stored frozen until the protocol for DOM extract solution preparation was carried on.

This protocol, an intracellular organic matter extraction of the natural community, performed on the stored 150 mm diameter 0.22 µm polycarbonate membranes, consisted in milling the membranes immersed in liquid nitrogen, resuspend the fragments in a known amount of ultrapure water and autoclave at 120°C for 20 minutes. Finally, the sterile suspensions containing the polycarbonate membranes pieces were filtered through burnt GF/F membranes (0.7 µm of pore size) to remove particles. The DOM extracts were stored frozen in -20°C to -30°C.

Culture parameters

Artificial seawater medium (ASW, Eguchi *et al.*, 1996) without the addition of carbon, nitrogen, phosphorus, and vitamin sources and with reduced concentration of the trace metals solution (10 µL per liter of medium, one tenth of the original recipe) was used as standard medium for all experiments. The final salinity was 35 PSU and the pH was adjusted to 8.

The source of carbon, nutrients and vitamins in the media were the DOM extracts, except in one treatment (further described below) where SOLA community was re-grown in sterile filtered natural sea water (filtration through 0.22 µm membranes) from the same site, which acted both as the culture medium and DOM source.

Three parameters were considered to determine the volume of DOM extract added to each microcosms: the volume of water sample filtered through the 0.22 µm membranes used in the DOM extraction; the final volume of the extract, i.e., the volume of ultrapure water used to resuspend the membranes after milling; and the ASW volume used in a particular microcosm. The reasoning is to provide to the communities in the microcosms an amount of carbon that is roughly correlated to the total organic carbon that the original water sample had per volume (dissolved in the extract, but particulate in the original water sample).

The DOM extract amendment volume (*y*) that relates to this approximation was calculated by the following formula:

$$y = \frac{v \,.\, \alpha}{V}$$

where *v* represents the total (final) volume of the DOM extract, *α* represents the volume of the microcosm culture and *V* the volume of the original water sample filtered through 0.22 µm polycarbonate membranes for the extraction procedure. This volume of DOM amendment extract was deemed 'complete', and in order to adapt the DOM supply to provide sufficient carbon and nutrients that were also not in excess, a volume of the DOM extract ranging from 0.1 to 0.33 times *y* was used (determined in preliminary tests, in reference to bacterial cell abundances commonly found in SOLA station, between 2-16 x 10^5 cells mL^{-1} (Obernosterer et al., 2010)).

For Experiment 1, only one DOM amendment, extracted from the same site as the communities, was used at the proportion of 0.33**y*. For Experiment 2 and 3, SOLA DOM extract, hereafter denoted as capital "S" following the identification letter of community used in each treatment, was used as the DOM amendment at the proportion of 0.1**y.* For experiment 2, a filter-sterilized (0.22 µm) sea water sample from SOLA site, without addition of DOM extracts, was used as medium and carbon and nutrients source in one treatment. Experiment 4 also had DOM amendment at the proportion of 0.1**y*, with treatments using SOLA DOM individually and in a mixture with Baltic Sea DOM in 1:1 (v/v) proportion, denoted as capital "M" following the community identification.

Table 1: Overview of experiments. Origin of the cryopreserved Community (Community), use of the additives AHL and cAMP (Add) to enhance growth, proportion of DOM amendment relative to 'complete' DOM from the environment, DOM extract used and final concentrations of Carbon [C], Nitrogen [N] and Phosphorus [P] in µM, as well as the C:N:P ratios.

	Community	**Add**	**DOM amendment proportion**	**DOM extract (volume added)**	**[C]**	**[N]**	**[P]**	**C:N:P**
Exp1	Mediterranean II	Yes[a]	0.33	Mediterranean II (100)	1.623	NA	NA	NA
Exp2	SOLA; Canet	No	0.1	SOLA (284)	0.499	0.07	0.006	83:12:1
Exp3	SOLA	No	0.1	SOLA (284)	0.499	0.07	0.006	83:12:1
Exp4	SOLA; Canet	No	0.1	SOLA* (284)	0.499	0.07	0.006	83:12:1
Exp 4	SOLA; Canet	No	0.1	Mixed* (284)	1.29	0.27	0.037	34:7:1
Exp4	SOLA; Canet	No	0.1	Baltic Sea* (284)	2.08	0.477	0.069	30:7:1

a: All treatments except one with 200 mL of volume filtered had addition of AHL and cAMP. *: Experiment 4 used only the mixed DOM extract, prepared from SOLA and Baltic Sea extracts.

Experimental design

The culture volume used in experiment 1 was different from the standardized culture volume of experiments 2, 3 and 4, all described below. Experiment 1 used 100 mL Boeco flasks with 80 mL of culture medium, while all other experiments used 1L Boeco flasks with 900 mL of culture medium. All treatments had triplicates cultures. An overview of experimental set ups of each experiment is given at Table 1.

Experiment 1 - Assessment of the growth and the effects of the initial cell numbers and culture additives on cryopreserved communities

Our first goal was to verify if we could re-grow cryopreserved natural communities in the artificial sea water medium (ASW) amended with DOM. Concomitantly, we assessed if the addition of AHL and cAMP and variable absolute initial cell numbers would increase the efficiency of the cryopreserved communities' growth. We experimented with community from Mediterrenean II site, as a representative of an oligotrophic and stable site, from which we cryopreserved communities with varying, increasing volumes filtered (=absolute accumulated cells) of 8 mL, 40 mL, 200 mL and 1500 mL. The 1500 ml volume culturing was performed at a later time than the other volumes, and two different persons performed the protocol independently. All treatments, except one of the 200 ml treatments, had AHL and cAMP added (Bruns et al., 2002). We evaluated the cell abundances that could be attained and the variability in the growth curves of replicate communities of each of the filtered volumes. These cultures had one third of the 'complete' DOM concentration used as DOM amendment, which was higher than we later established as adequate.

Experiment 2 - Similarity of resuscitated with the originally filtered communities

Subsequently, we tested how similarly re-grown cryopreserved communities from SOLA and Canet would be to their original communities. We cultured both in the ASW medium amended with DOM from SOLA with the one tenth proportion of the 'complete' concentration, without addition of additives. For SOLA community we also in used filter-sterilized (0.22 µm) sea water from SOLA site without addition of prepared DOM and additives. We evaluated cell abundances, growth curves and community composition from 16S rRNA gene tags.

Experiment 3 - Effect of time lag between sampling and freezing on the diversity of the re-grown cryopreserved communities

We evaluated the effect of time lag between sampling and freezing bacterial community from SOLA site. The bacterial community was filtered and cryopreserved immediately after sampling ("First" treatment) and 7.5 hours after sampling ("Last" treatment). We evaluated this effect assessing their cell abundances, growth curves and compositional shift using the ARISA fingerprinting technique.

Experiment 4 - Investigating the influence of different DOM sources on the cryopreserved communities, both individually and in a controlled community coalescence event

Finally, we applied the developed method to investigate the effect of different DOM sources (SOLA or Mixed DOM) to the cryopreserved communities of an oligotrophic, stable site (SOLA) and from an hypertrophic and highly variable lagoon

(Canet), both communities isolated or in a controlled community coalescence event, where cryopreserved communities from SOLA and Canet were mixed and re-grown together.

For this experiment we had 6 treatments analyzed in triplicates: treatments with Canet community re-grown in DOM from SOLA site (C_S), or in mixed DOM (C_M) (for details of mixed DOM see table 1); coalescent community of SOLA and Canet with DOM from SOLA site (SC_S), or with Mixed DOM (SC_M); and SOLA community re-grown in SOLA DOM (S_S), or Mixed DOM (S_M).

We evaluated the results based on cell abundances and growth and with the overall composition dissimilarities of communities through 16S rRNA gene tags.

Analyses

Flow cytometry

Cell enumeration was carried out by flow cytometry. Briefly, samples for bacterial abundance were fixed with formaldehyde to a final concentration of 1%, were stored in the dark for 10 minutes and placed at -80°C until analysis. Sybr green was used for staining of the cells in the samples, using 1.0 µm green fluorescent polymer microspheres (DukeScientific Corporation) as internal standard. Analyses were carried out in a BD FACSCantoII™ cytometer (Becton Dickinson). Fluorescent beads (True counts, Becton Dickinson) were used to determine the flow rate. Results were analyzed in the FlowJo®V7software. Gated populations in the SSC-H and FL1-H channels, after

exclusion of the region relative to autoflorescence of chlorophyll-a, were used to enumerate heterotrophic bacteria cell abundance per mL.

Molecular analyses

We filtered 400 to 500 mL of each sample for bacterial DNA extraction and analysis through 0.22 µm polycarbonate membrane and stored at -30 °C until extraction. Filters were cut in small pieces and DNA extraction was performed following the manufacturer protocol of QIAmpMiniKit (Qiagen) adding a bead beating with the buffer ATL as the first step of the protocol. For ARISA (Automated Ribosomal Intergenic Spacer Analysis), extracted DNA was amplified with Kappa2G reaction kit in 20 µL reactions using 10 µM of the primers 1406F and 23SRY (Frossard et al., 2012) and 1 µL DNA template. Reactions mixtures were held at 94 °C for 3 min followed by 35 cycles of amplification at 94 °C for 15s, 55 °C for 15s, 72 °C for 30s and a final extension of 72 °C for 1 minute. Samples were then purified through Sephadex G-50 resin, quantified by Pico green assay and 0.2 µL of MapMarker1000 was added to the 5 µL standardized amount of PCR products, and denaturated with formamide at 95 °C for 3 minutes. The sequencing was performed at the Bio2mar platform at the Laboratoire Arago, Banyuls-sur-mer.

Analysis of fragment data was performed using the T-Rex software (Culman et al., 2009) and was carried out as follows: alignment of fragments by bin size of 5 for fragments < 600 bp and bin size of 7 for fragments ≥ 600 bp. Noise filtering by 1.2 times the standard deviation of the appropriated dye (Brown et al., 2005; Cardinale et al., 2004).

High throughput sequencing (HTS) used 16S rRNA gene primers pairs 515yf-926r (Parada et al., 2016) for amplification of gene tag, with 300bp Paired-end sequencing in Illumina Miseq V3, with subsequent demultiplexing, adapter and primer clipping performed by LGC Genomics GmbH (Germany). Reads were processed in the DADA2 pipeline (Callahan et al., 2016) to obtain the exact sequence of organisms, the amplicon sequence variants (ASV). The sequences were taxonomically assigned in comparison with Silva database. A total of 861 ASVs were identified across all samples.

Data processing was carried out in phyloseq package (McMurdie and Holmes, 2013). For alpha diversity estimates, the only filtering done was the removal of sequences of Cyanobacterias, resulting in 851 ASVs. For all other analysis and representations of this data set, as PERMANOVA of composition, Principal Component Analysis representation and bargraphs of the composition, the data was first agglomerated by a cophenetic distance (H = 0.01), with a total of 567 ASVs. From this data, their relative abundance was calculated as the abundance of a specific ASV divided by the total sum of ASVs across all samples.

Chemical analyses

For determination of dissolved organic carbon in our DOM extracts, triplicates of 10-times diluted extracts of the DOM were transferred to pre combusted glass tubes, acidified (85% H_3PO_4, final pH = 2), closed with Teflon caps and stored in the dark until analyzed on a Shimadzu TOC-V (Shimadzu Europa GmbH). Between three to five injections were made for each sample to ensure a coefficient of variation lower than 2%. Twenty milliliter samples for nitrate, nitrite, and phosphate analyses were stored at -20 °C and analyzed on a Skalar autoanalyzer according to Tréguer and Le Corre (1975).

Results and discussion

Experiment 1 - Assessment of the growth and the effects of the initial cell numbers and culture additives on cryopreserved communities

The resuscitated cryopreserved cultures with volumes up to 200 mL filtered ($\leq$1.48 x10^8 initial cells) were characterized by high variance among replicate samples for all sampling times (Fig. 1a, Table 2).The variance were normally smaller if larger volumes (1500 mL, 2.05 x10^8 initial cells) were filtered (Fig. 1b, P1). P1 and P2 in Figure 1b refer to two different people performing the same protocol, and P2 had two of three membranes dried at the end of the filtration, which resulted in an increased variance between replicates after day 5, although the growth curves are similar and coefficient of variation results are still lower than the other low volume cultures until day 5. Thus, it is important to highlight that care and standardization is necessary to obtain reproducible communities, especially if more than one person will perform the protocol, and drying of the membranes at the final point of the filtration must be avoided.

Overall, the volume filtered through the membranes influenced the growth of the resuscitated communities, causing more variation with smaller volumes, as it translates into less cell numbers at the beginning of the cultures. Treatments with less initial cell numbers also displayed a longer lag phase, and this was more critical for the treatments with the smallest volumes and, thus, smallest starting cell numbers (Fig 1a, 8 mL).The use of additives AHL and cAMP did not resulted in any difference between the 200 mL filtered volume treatments (Fig 1a), and although we did use it in the treatment with 1500 mL filtered, we deemed these additions unnecessary and did not add any additive in the subsequent tests. Finally, the final cell abundance attained in all treatments was

approximately 5 to 10 times higher than we would expect from SOLA site (Obernosterer et al., 2010).

The observed higher variance between replicates of the cultures with smaller absolute initial cells could be caused by a higher variability of the community composition and respective population sizes that were either captured by the filtration step or successfully frozen and resuscitated with our cryopreservation protocol, both scenarios that could cause historical contingencies. In this situation, even small differences in the starting community could cause a high deviation leading to different communities as these differences are amplified over time via the growth of the community (Fukami, 2015). The consequence would be increased chance of divergent structure or function of the community, which would translate in the results of our experiment in different growth rates and increased variability among replicates. Higher cell numbers result in better efficiency of the freezing protocol (Vekeman and Heylen, 2017), which could alleviate these historical contingencies. Therefore, increasing the volume filtered resulted larger initial cell numbers, maximizing the chance to capture an adequate subset of representatives of the original community in the various frozen communities prepared and minimizing the variance between replicates.

Finally, although suggested elsewhere (Bruns et al., 2002), we did not observed a more consistent culturing of the frozen communities with the addition of the AHL and cAMP. Furthermore, we also observed that communities could grow on these compounds, even without any addition of DOM (data not shown), so we precluded these additions for the subsequent experiments.

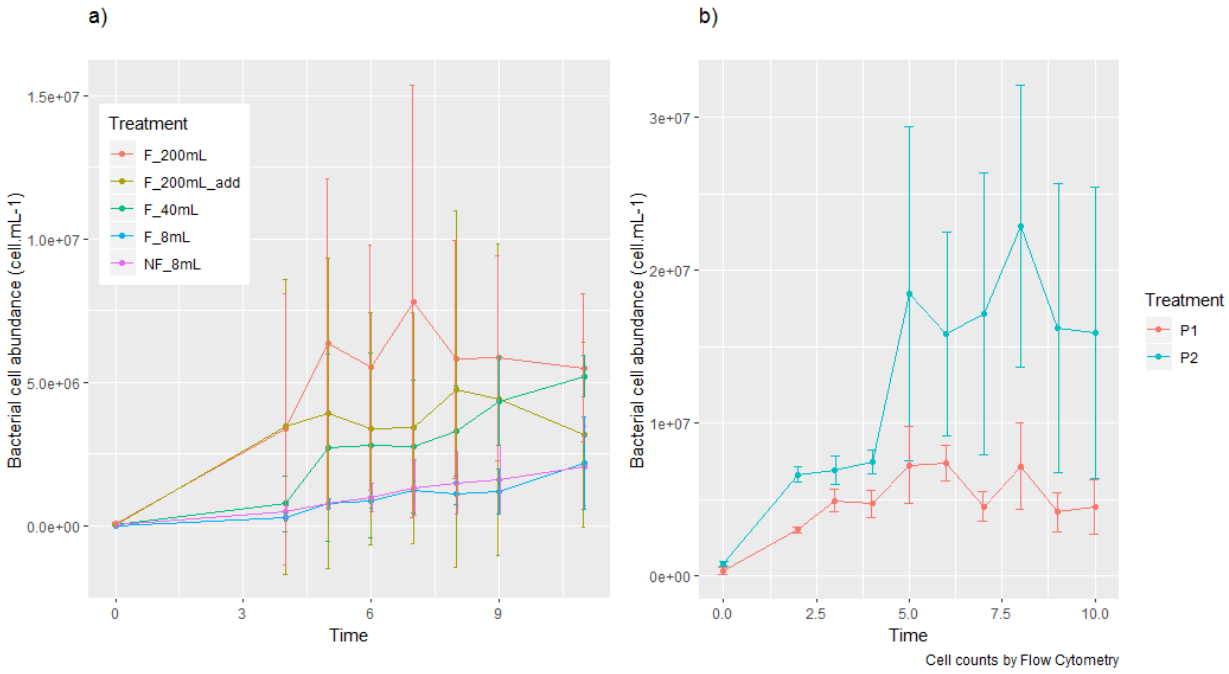

Figure 1: Growth curves of experiment 1. Cell abundances in cell mL^{-1} and error bars with the standard variation between triplicates. a) frozen absolute initial cell abundance of ≤1,48 $x10^8$. b) frozen absolute initial cell abundances of 2,05 $x10^8$.

Table 2 : Cell abundance, absolute cell abundance and coefficient of variation of the cultures with varying initial absolute cell abundance

			Coefficient of variation (CV)		
	Inoculum (cell. mL^{-1})	Absolute initial cell abundance	Day 0	Day 4	Day 6
8 ml[a]	737,848	5,9 $x10^6$	0.168253	0.338481	0.506347
8 ml[b]	737,848	5,9 $x10^6$	0.04534	0.46191	0.318832
40 ml	737,848	2,95 $x10^7$	0.289747	1.256558	1.139967
200 ml[c]	737,848	1,48 $x10^8$	0.597929	1.490081	1.19545
200 ml[d]	737,848	1,48 $x10^8$	0.161685	1.405416	0.773292
P1 - 1500 ml	136,546	2,05 $x10^8$	0.719261	0.191125	0.157583
P2 - 1500 ml	136,546	2,05 $x10^8$	0.180545	0.102856	0.42137

[a]: Non-frozen filter, addition of AHL and cAMP; [b]: Frozen filter, addition of AHL and cAMP; [c]: Frozen filter, addition of AHL and cAMP; [d]: Frozen filter, without AHL and cAMP

Experiment 2 - Similarity of resuscitated with the originally filtered communities

Overall, we were able to recover the original diversities of the communities of Canet and SOLA, but SOLA presented a more challenging community to do so, especially when grown with artificial medium amended with the DOM preparation from that same site (Figure 2, ‘S’ community, ‘S’ DOM). SOLA (S_initial) and Canet (C_initial) original communities are clearly differentiated by the dissimilarity analysis of their ASVs compositions along the first axis of the Principal Coordinates Analysis depicted in Figure 2. Also, Canet cryopreserved communities grown in ASW medium are very similar to the original Canet community, even considering that they were grown on medium amended with SOLA DOM (CS). On the other hand, SOLA cryopreserved communities grown in this same medium (SS), contrary to our expectations, were strikingly dissimilar to the original SOLA community. Our expectation was that communities grown with DOM amendments from the same site would have a better recovery of the diversity and would resemble the original community more closely, as they are expectedly more adapted to this DOM than other, different community, but this was not the case for SOLA cryopreserved communities. Conversely, the SOLA community that was grown with the sterile filtered sea water was fairly similar to the original SOLA community (SW).

Regarding the similarity between SOLA original community and cryopreserved SOLA community grown in filtered sea water (SW), it is noteworthy that in SW treatment we were able to actively grow ubiquitous pelagic organisms of natural marine environments, like some members of the SAR11 and Rickettsiales, which are difficult to maintain in microcosms cultivations (Figure 3) and are considered specialists for

oligotrophic environments, had undergone DNA streamlining and reducing their metabolic plasticity (Giovannoni et al., 2014). This difficulty in re-growing these oligotrophic ubiquitous organisms is clear in the treatment with SOLA DOM (SS), which although was successful in re-growing some of those ubiquitous organisms, did so in a much lower abundance than the treatment with sea water (Figure 3).

SOLA community had more overall changes, with overgrow of the Alteromonadales clade in both treatments, more strongly so in SS, which had a larger apparent turnover of species with selection of this clade over naturally dominant clades (SAR 11, Rickettsiales, Rhodospirillales). SW treatment maintained a more similar composition compared to the original community, but still with a significant overgrow of organisms from the Alteromonadales clade. Alteromonadales is a clade with a wide range of life styles that harbors both generalists and specialists (Gómez-Consarnau et al., 2012). Canet community composition was recovered more thoroughly, as seen in Figure 3, but also with changes in the relative abundance of its members, especially with the relative increase of the Flavobacteriales and Campylobacterales clade. However, these changes seemed to add diversity instead of cause only substitution and decrease in alpha diversity (Figure 4).

We believe that our culturing set up, with artificial media and amended DOM, even maintaining a good proportion of the original communities' diversity, can be perceived by the bacteria as a changing environment, one that can select an even more restricted subset of organisms from those that were successfully cryopreserved and resuscitated. This more restricted selection could be due to the bottle-effect (Hammes et al., 2010), or because of the changing environment itself. This second hypothesis is

partially supported when the biodiversity-insurance relationship is considered (Matias et al., 2012). Within this idea, bacterial communities with more generalists would perform better in a changing environment, being more productive and by maintaining their composition over time. Considering that Canet is a more variable and hypertrophic environment, it likely harbors a community that is consisted of more generalist organisms than SOLA environment, which is an oligotrophic and stable environment that admittedly has more specialists, like the streamlined SAR11 (Giovannoni, 2016). Following the biodiversity-insurance hypothesis, we can propose that the reason for the better recovery of cryopreserved and re-grown communities from Canet is exactly this difference in life strategy of the majority of its members, which in Canet can be considered generalists - or at least more generalists than the organisms present in SOLA. That could be the reason for cryopreserved SOLA communities grown in filtered sea water, a media more similar to the original environment, were accordingly more similar to the original community. We cannot rule out that comparable results could be achieved if we used lower DOC concentration with the DOM amendment, i.e. that the medium used in the treatments had already more carbon and nutrients than what would be optimal for the oligotrophic community from SOLA. However, we emphasize that the DOC concentrations on the culture media, as showed in Table 1, are already quite low.

These results have a potential in devising strategies for the culturing of whole communities in microcosms. For communities originating from more variable and higher trophic state environments, our approach of amendment of the media with natural DOM extracts is efficient, even not using DOM from the same environment, since in this case mostly more generalist organisms would be growing. For communities originating from

more oligotrophic and stable sites, might be more efficient to use filtered water from the site, considering how specialized, and thus sensitive to changes, the resident community is.

The net result of these compositional changes can also be observed through the alpha diversity levels of each of these samples in Figure 4. Figure 4a depicts the observed diversity and, interestingly, all treatments with cryopreserved communities had higher observed richness than the original communities, except for one triplicate of the SW treatment. This is showing that undetected organisms in the original communities increased in abundance in all treatments, resulting in the apparent turnover of species observed.

Overall, Canet re-grown communities were more reproducible than SOLA re-grown communities and consistently more diverse than the original Canet community, both in the observable richness and in the Reciprocal Simpson index. Conversely, SOLA cryopreserved communities display a decrease in the Reciprocal Simpson index, reflecting the overgrown and dominance of naturally rare clades over naturally common organisms in both treatments, but especially in SS. Another key difference between Canet and SOLA communities is that SOLA cryopreserved communities resulted in triplicates less reproducible. All these outcomes can be related, and expected, when considering both the biodiversity-insurance hypothesis of a community from an oligotrophic environment with more specialists, and historical contingencies (Fukami, 2015; Matias et al., 2012).

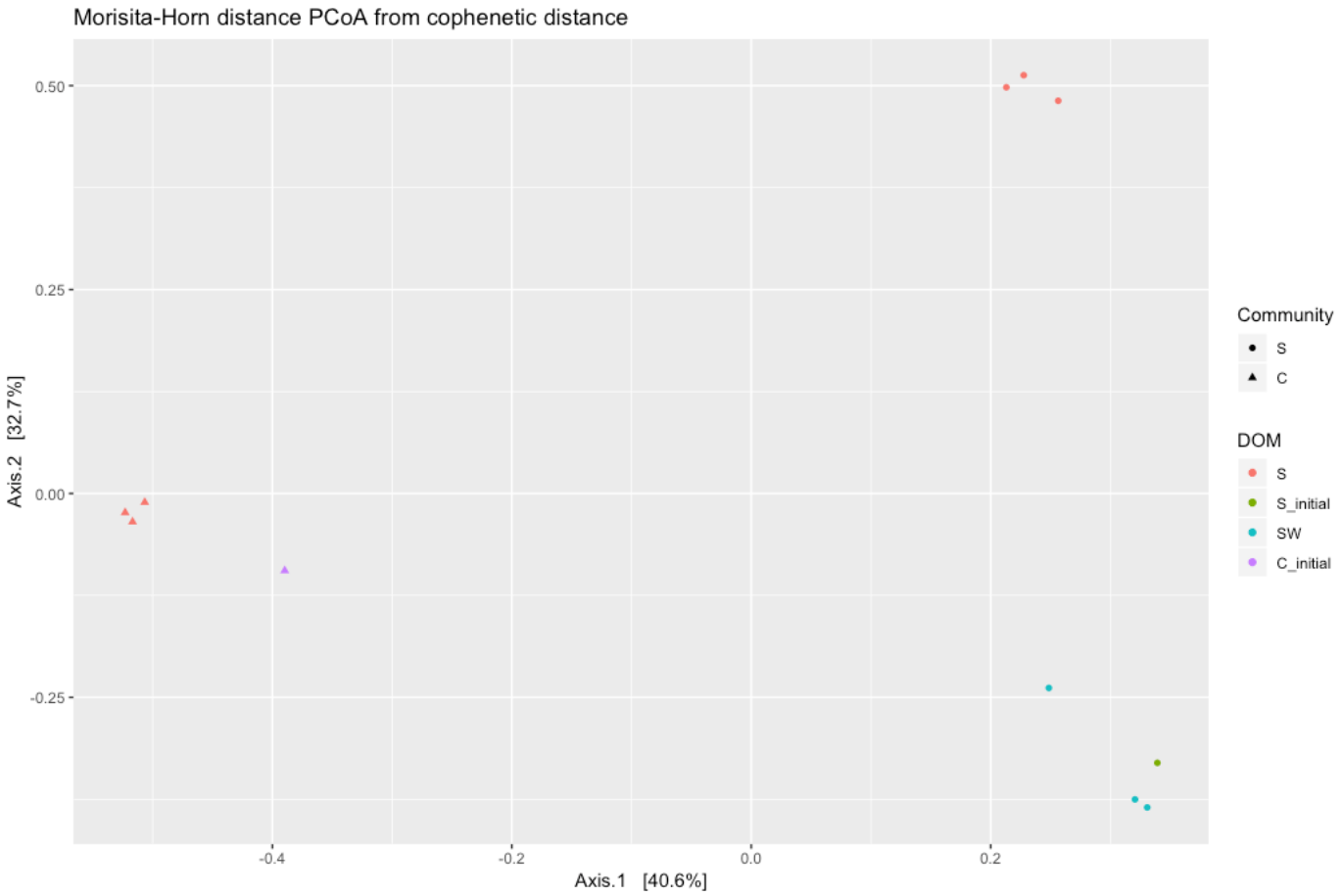

Figure 2: Principal Coordinates Analysis representation of the similarities between original and cryopreserved and re-grown samples from Canet and SOLA. S = SOLA communities and C = Canet communities, resuscitated and cultured in ASW media with S = SOLA DOM extract at 0.1 of 'complete' amount. SW = SOLA cryopreserved community resuscitated and cultured in filtered seawater from SOLA. S_initial = original SOLA community; C_Initial = original Canet community.

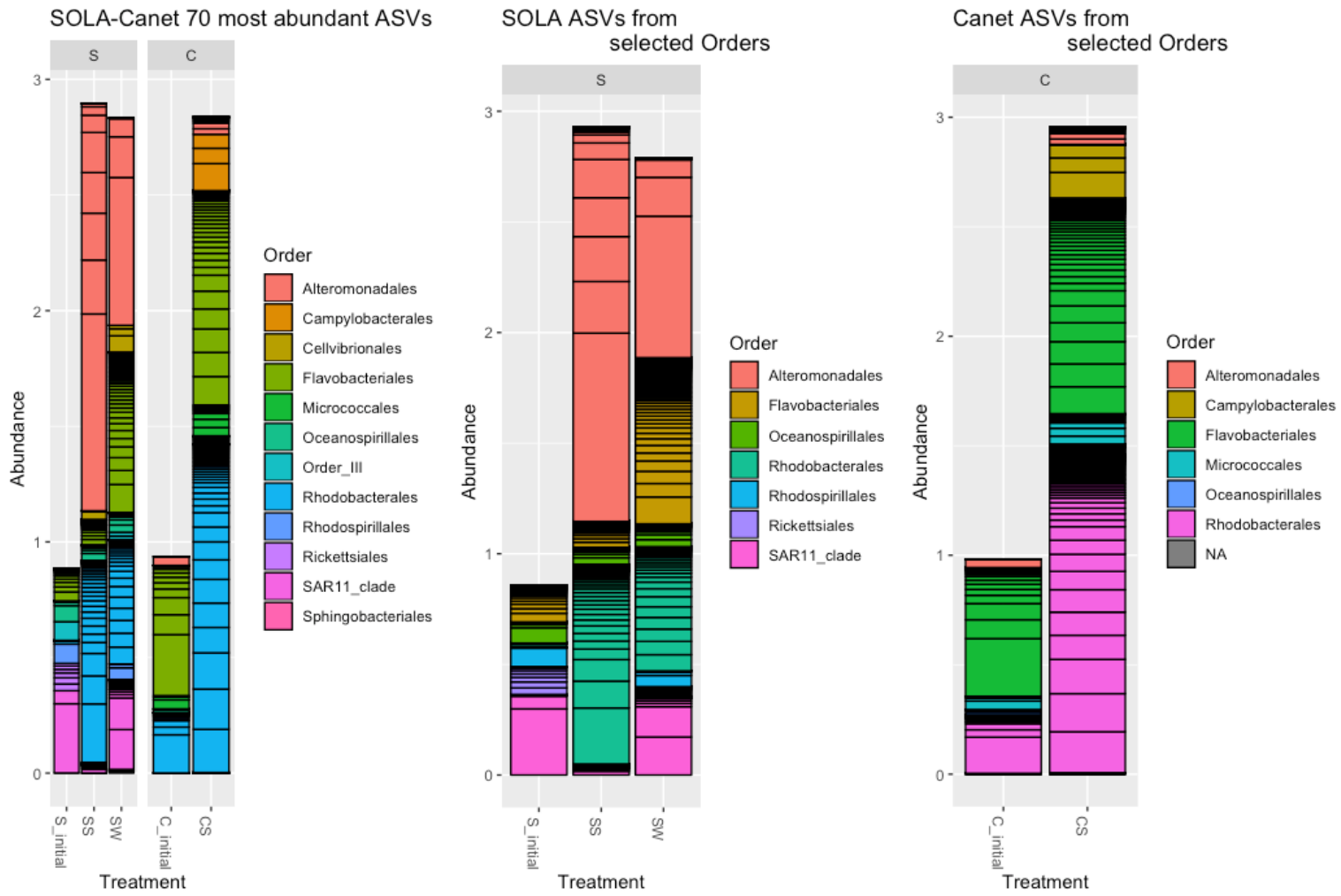

Figure 3: Bargraphs depicting relative abundances of organisms pooled by orders. S_initial = original SOLA community; SS = SOLA community cultured in ASW medium with SOLA DOM amendment; SW = SOLA community cultured in filtered sea water from SOLA site; C_Initial = original Canet community; CS = Canet community cultured in ASW medium amended with SOLA DOM.

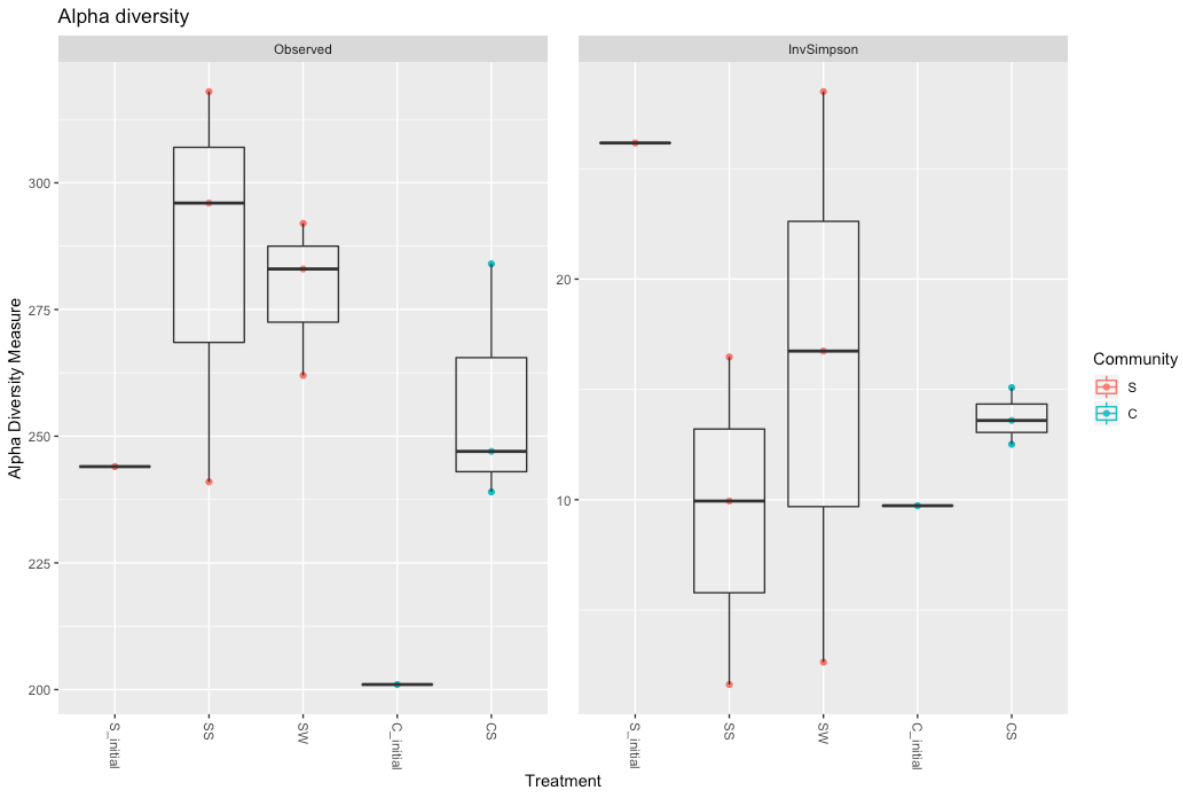

Figure 4: Alpha diversity of resuscitated communities in comparison to their original communities. Left panel depicts the observed richness. Right panel depicts the Reciprocal Simpson index for these same communities. S_initial = original SOLA community; SS = SOLA community cultured in ASW medium with SOLA DOM amendment; SW = SOLA community cultured in filtered sea water from SOLA site; C_Initial = original Canet community; CS = Canet community cultured in ASW medium amended with SOLA DOM.

Experiment 3 - Effect of time lag between sampling and freezing on the diversity of the re-grown cryopreserved communities

Diversity levels of the genetic fingerprinting technique ARISA were evaluated by Richness (S), Shannon-Wiener index and Pielou's evenness, as well as by visualization with hierarchical cluster of the Bray-Curtis dissimilarities of samples (Figure 5). The results show no significant difference between 'First' and 'Last' sets of membranes (ANOVA Pf(>F) > 0.05), but the diversity levels of both sets are lower than the original community (SOLA_1), similar to what we observed in the previous Results section. Furthermore, growth curves and cell abundances are equivalent for 'First' and 'Last' set of samples (data not shown).

The aim of a cryopreservation method for whole community is to preserve the diversity of the communities, and if several replicates of a community are intended to be preserved, replicates may not be prepared at the same time. In our specific case, the time between the first and last cryopreserved community replicates to be processed was approximately 7.5 hours (we prepared a total of 36 cryopreserved communities, 6 at a time). Thus, it was necessary to evaluate if the time that one takes to process all replicates, i.e., the time that the samples wait inside the sampling carboys, could affect the resuscitated and re-grown cryopreserved communities' diversity.

Our data clearly demonstrate this effect, with 'First' samples resembleing more the original community than 'Last' samples (Figure 5). PERMANOVA test, which takes into account the composition of the communities (organisms [i.e., fragments] presence and their relative abundance in each sample) confirmed that communities were significantly different from each other, with the different treatments (Initial community,

‘First’ and ‘Last’ set of membranes) explaining 80% of the variation in the data set (R^2 = 0.800, Pr(F) < 0.05). We believe that this influence of the time lag in processing the samples on the resuscitated community composition is likely due to specific taxa that start proliferating faster, while others decrease their activity. Thus, the community composition of the starting community could have been already changed when the last filters were prepared, or otherwise some cells meanwhile could have gone to an inactivated status and were harder to re-grow. In any case, this is a manifestation of the bottle-effect which subsequent selection of the remaining organisms (Stewart et al., 2012), possibly generating historical contingencies that propagates eventual differences in the starting communities even further (Fukami, 2015).

In summary, the results present here entices to a greater care for the samples processing regarding the elapsed time after sampling, performing the cryopreservation protocol as fast as possible and using as many parallel filtration units as possible to minimize the effect of the time lag in sample processing.

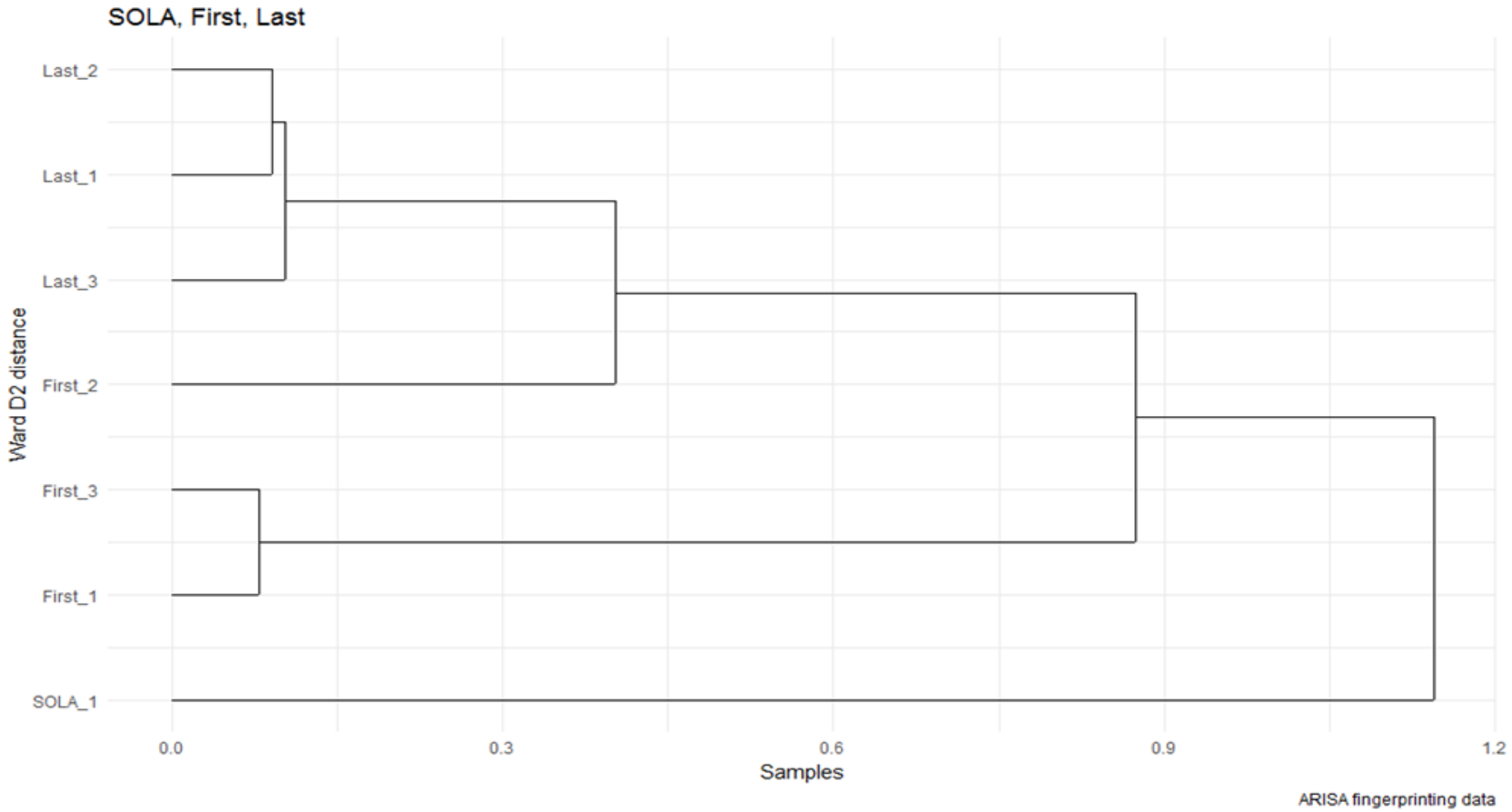

Figure 5: Dendrogram of samples using data from the ARISA fingerprinting technique. SOLA_1 = SOLA original community; First = first membranes to undergo the cryopreservation protocol as soon as the sample arrived. Last = last membranes to undergo cryopreservation protocol, approximately 7.5 hour after the First membranes.

Experiment 4 - Investigating the influence of different DOM sources on the cryopreserved communities, both individually and in a controlled community coalescence event

The core idea behind the tests of the cryopreservation and DOM amended cultivation is that this method would enable the conservation and more complete experimentation of microbial communities, e.g., from sample sites hard to access that can be sampled only during rare occasion or with big experimental setups that would render unfeasible complete experimentation at once. For these communities, a cryopreservation method makes more viable that experimental approaches could be employed in their study.

As we showed, we can achieve a good reproducibility of the complex initial microbial communities with cryopreserved communities, even if losing part of the original diversity, allowing the required control for the composition of the starting community (Fig 2), and averting the historical contingencies that might shift the community development in unpredictable ways (Fukami, 2015). This control allows the comparison of findings between different microcosms studies and a multiplicity of manipulations to address and study ecological theory under controlled conditions.

We devised an ecological experiment that would profit from the unconstrained set ups that become possible with the cryopreservation of diverse communities. We investigate the effect of changing DOM complexity and quantity, i.e. SOLA site DOM extracts (simpler, poorer in nutrients, lower quantity [“S”]) and Mixed DOM extracts from SOLA and Baltic sea (more complex, richer in nutrients higher quantity [“M”]), on two communities that originated from diverse environments, one stable and oligotrophic

(SOLA – “S”), the other variable and hypertrophic (Canet – “C”). Simultaneously, the outcome of a community coalescence event was also investigated.

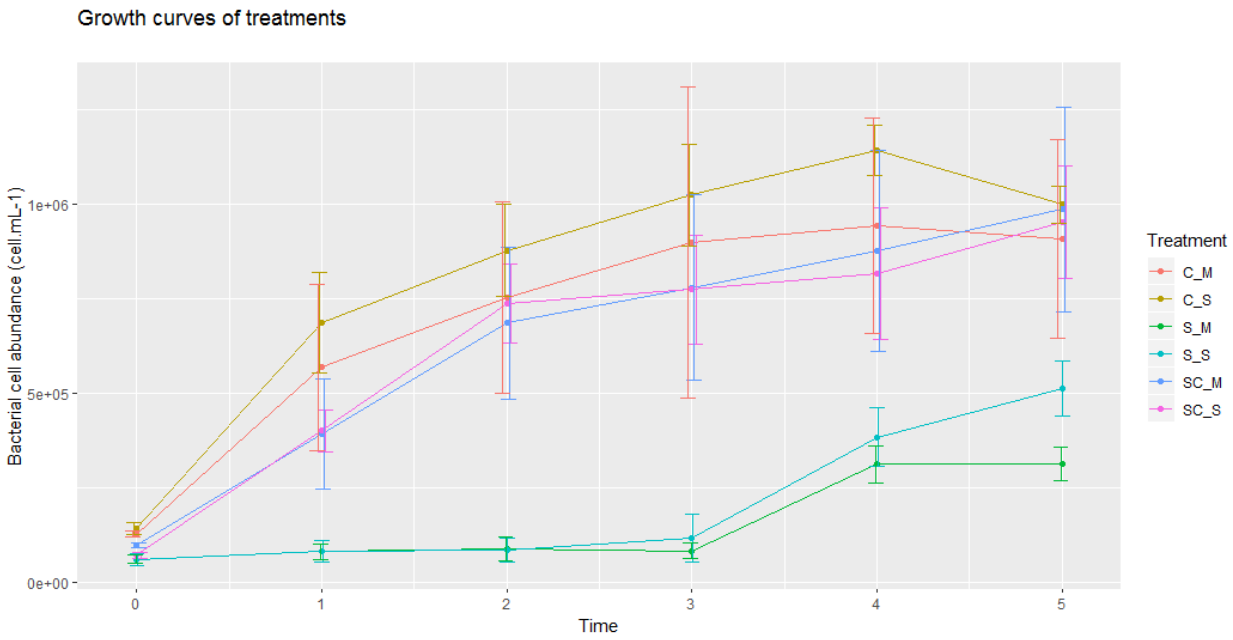

Figure 6: Growth curves of cryopreserved and resuscitated communities. Before the underline on the legend, C = Canet, S = SOLA and SC = SOLA + Canet, for communities. After underline on the legend, M = Mixed DOM (SOLA + BalticSea) and S = SOLA DOM, for DOM amendment. All treatments were cultured in ASW medium with DOM amendment.

Treatments that included cryopreserved Canet communities, both by its own or in coalescence with SOLA cryopreserved communities, had shorter lag phase than treatments exclusively with SOLA communities (Figure 6). While these Canet-included treatments grew faster, they also had higher variance between their replicates than the SOLA cryopreserved communities throughout the incubation time. By the end of the experiment, SOLA cryopreserved communities had a significant different mean

abundance than Canet and Coalescent communities (ANOVA Pr(>F) < 0.05), which had similar abundance (ANOVA Pr(>F) > 0.05). Furthermore, all treatments that included Canet cryopreserved community did not had differences in the mean cell abundance due to the DOM used in the culture medium at the end of the experiment (Canet, F = 0.342, Pr(>F) > 0.05; SC, F = 0.035, Pr(>F) > 0.05). However, SOLA communities were significantly different from each other at the end of the experiment due to the DOM amendment used (F = 16.37, Pr(>F) < 0.05).

This result is mirrored in the PCoA representation (Figure 7), which differentiates each original community and its re-grown communities in the first axis, with coalescent communities resembling more Canet, similarly as the pattern of the growth curves. SM and SS treatments are farther apart from each other compared to Canet (C_S and C_M) and coalescent community treatments (CS_S and CS_M), but still fairly close to each other. SC and C communities are superposed, evidence of a strong dominance of the Canet community over SOLA community. This would make sense if Canet community is indeed more generalist, making them more fit to adapt to a different environment (which the artificial media with DOM amended certainly is). Furthermore, it seems that samples are slightly separated by DOM amendment, along the second axis, more clearly for SOLA community.

Docherty et al. (2006) showed that bacterial community development reflects the carbon source quality and quantity it is using, but different communities tend to converge to a uniform community capable of using that source, except when a community coming from low carbon source was transferred to highest carbon source. Also, high concentrations of carbon source could magnify the effects of dissimilarity on

the community, although it could also possibly mask more fine effects if the quantity is much larger than the usual for that community (Chapter 1 of this thesis), especially so for specialized communities from oligotrophic sites (Docherty et al., 2006). This possibility is reinforced by the biodiversity-insurance hypothesis (Matias et al., 2012), which states that more specialist communities would perform worse, and more likely lose diversity and function, in a changing environment. A possible reflection of this process can be seen in the way SOLA communities present loss of alpha diversity (Figure 4), longer lag phases (Figure 6) and ultimately has a more dissimilar re-grown community comparatively to the original. Accordingly, Canet communities, obtained from a more variable and eutrophic environment, converged to overall more similar compositions that resembled the original community, without loss of alpha diversity.

The variance of the growth curves between replicates of the Canet community communities grown with mixed DOM (C_M, SC_M) was higher than with these same communities grown with SOLA DOM (C_S, SC_S). Thus, the growth curves also indicate some effect of the DOM on those treatments. A repeated measures ANOVA (rmANOVA) analysis identified that DOM do have an effect in Canet and SC (coalescent) communities, but not in SOLA (data not shown), further evidence of the differential effect of DOM quality/quantity depending on the origin of the community.

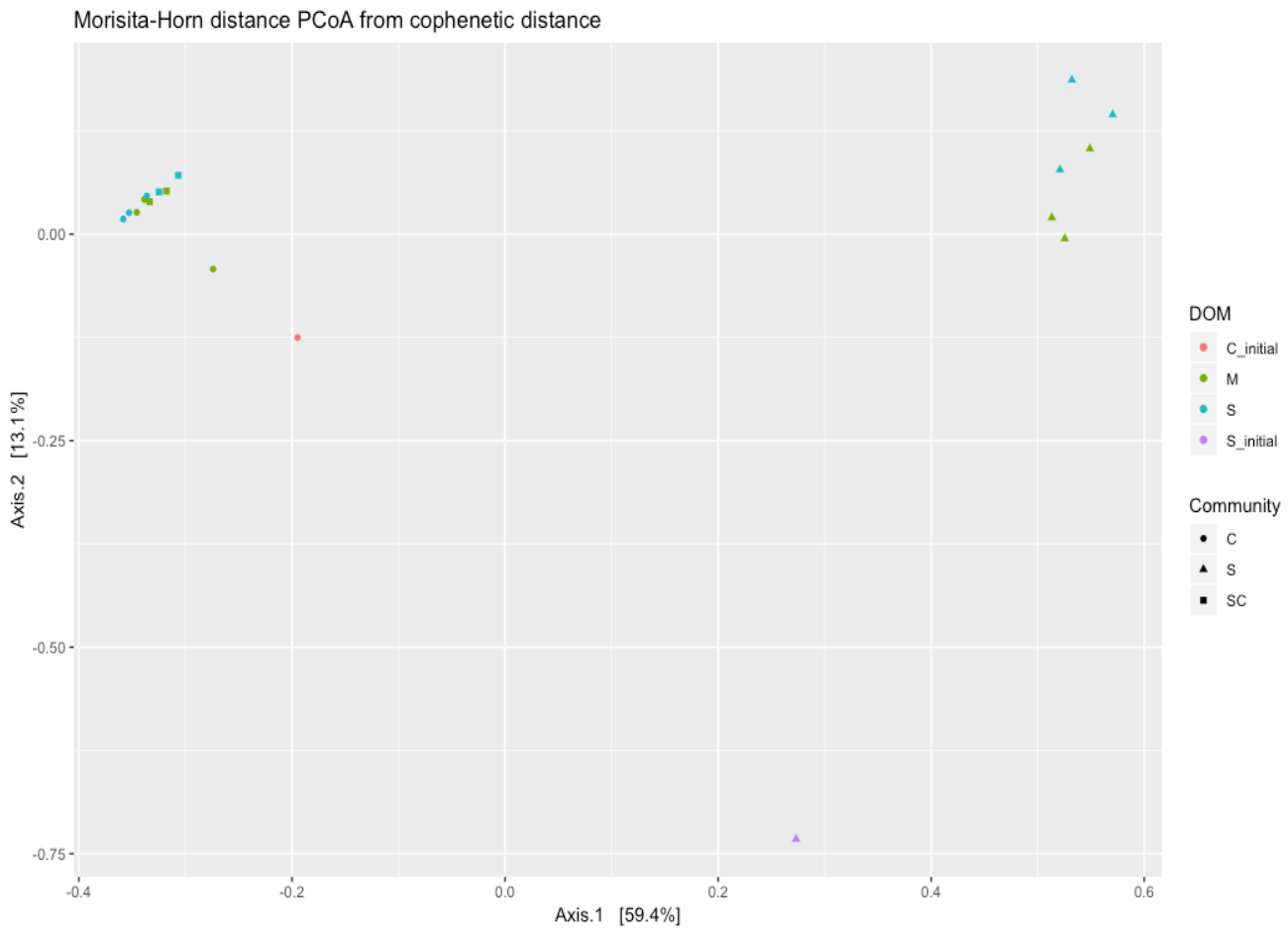

Figure 7: Principal Coordinates Analysis using Morisita-Horn distance of abundance of ASV (amplicon sequence variant).

Regarding the diversity level of each treatment, we hypothesized that increased diversity of DOM would result in more diversity in the treatments, but there is no evidence of difference in the Shannon-Wiener index of communities growing on media with amendments of different DOM complexity (ANOVA PR(>F) > 0.05; data not shown). We also hypothesized that the coalescent communities would have more diversity, but it in fact had a similar Shannon-Wiener index than Canet samples, which was higher than SOLA, indicating that the more generalist community dominated the coalescent community and the coalescent event did not resulted in higher diversity.

Conclusion

The objective of this work was to evaluate a cryopreservation method for whole bacterial communities and a culturing method with DOM amendments that would allow the recovery of original diversity and hard-to-culture organisms. In that regard, the objective was met but it is necessary to take some precautions: larger filtered volumes and caution to not dry the filter during the filtration step of the cryopreservation method, and as fast as possible processing of the samples. A fraction of the diversity was lost in the cryopreserved community, but the cryopreservation and cultivation methods allowed reproducible re-grow of the communities, both related to cell abundances and to final community composition. An experiment was devised to illustrate one of the potential uses of the method, investigating the effects of DOM variations on different communities, which were cultured either individually or in a coalescent event. The results show that a community with more generalist organisms converged to similar diversity after resuscitation and culturing, regardless of the quality of the DOM extract amendment, reaching a community composition similar to the original community. Conversely, the methods were not as successful for a community with more specialist organisms, although it was still possible to re-grow some of the ubiquitous clades of the original community. Therefore, the outcome of the community composition and diversity in microcosms cultures are not only related to the DOM quality (and possible quantity) but to the characteristics of the community being cultured and of the environment where this community came from, with clear differences between generalists and specialists communities. Considering these results, it was demonstrated that the methods ensure reproducibility and control of the initial community and allow more possibilities to

experimentation with hard to access communities and to scale up in the number of possible manipulations that could be applied to experimental communities.

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Conclusões gerais

O tema geral dessa tese é a interação de comunidades bacterianas naturais com a matéria orgânica dissolvida (MOD) em experimentos em microcosmos. Em meu primeiro capítulo, nos queríamos entender a interação e efeito de quantidade e qualidade de MOD na estruturação de comunidade microbiana natural. O que encontramos foi que ambos os fatores se correlacionam com a estrutura da comunidade bacteriana, causando o que parece ser uma alternância de processos de seleção. No início, por competição, ocorre a seleção daqueles organismos que podem crescer mais rápido utilizando aquela fonte de carbono específica. No entanto, no decorrer da incubação, demonstramos que a MOD vai sendo alterada, e sua variação se correlaciona com a variação da diversidade de organismos, o que parece ser a causa da recuperação parcial em 96 horas de incubação da diversidade perdida nas primeiras 24 horas. Interpretamos isso com base no trabalho de Pacheco et al. (2019), que mostra que a degradação de um único composto simples no começo de uma incubação pode gerar diversos outros compostos que têm a capacidade de sustentar o crescimento de outros e mais diversos organismos. Transpondo esta idéia para uma comunidade, ela poderia ficar sob pressão de competição no começo por limitação nos tipos de compostos (=fontes de carbono) disponíveis, e sua diversidade cairia a princípio. Mas se outros e novos compostos que são gerados durante a degradação da MOD inicial podem sustentar mais organismos, a diversidade perdida pode ser parcialmente recuperada, exatamente como observamos. Nesse sentido, consideramos que a qualidade exerce uma pressão predominante, sendo modulada pela quantidade, que pode aumentar a intensidade da competição inicial ou a duração do seu efeito.

Em nosso segundo capítulo, discutimos a causa dos principais problemas de experimentos em microcosmos, e como poderíamos, pelo menos, minimizá-los. Experimentos em microcosmos muitas vezes são a única forma de testar hipóteses específicas e experimentalmente modificar o ambiente das comunidades bacterianas, constituindo uma poderosa, e uma das poucas disponíveis, ferramentas dos ecólogos microbianos para este fim. Embora uma poderosa ferramenta, esse tipo de experimento tem problemas, especialmente relacionados ao chamado “efeito garrafa” e às contingências históricas (Fukami, 2015; Hammes et al., 2010), que, respectivamente, introduz uma variabilidade inicial na comunidade, que pode ser uma variação estocástica, que é então amplificada com o passar das gerações, tornando, e.g., réplicas experimentais cada vez mais diferentes e impedindo uma adequada padronização das comunidades iniciais (Kerckhof et al., 2014), requerimento mínimo para a comparação de resultados e a formulação de teorias que podem ser expandidas para outros sistemas (Vellend, 2016). Considerando este contexto, nos utilizamos duas estratégias, que preferencialmente devem ser usadas em conjunto, para minimizar essas variações indesejadas nos experimentos da ecologia microbiana. A primeira é a aplicação de um método de cultivo que previna essa seleção de organismos raros no ambiente nos cultivos em microcosmos. Como recentemente reportado, a utilização de DOM natural, com conteúdo intracelular total da comunidade, parece ser eficaz em manter uma maior diversidade, inclusive permitindo do crescimento de organismos reconhecidamente difíceis de cultivar em laboratório (Shen et al., 2018). A segunda estratégia é a aplicação de um método que permita a padronização das comunidades iniciais. Assim, testamos um método de criopreservação de comunidade natural

inteiras. Estas comunidades foram ressuscitadas e cultivadas em meio com adição de MOD extraídos do ambiente natural. O método teve boa recuperação da diversidade original, embora não a recuperou completamente, e ainda permitiu o crescimento daqueles organismos difíceis de cultivar. No entanto, o sucesso desse método foi talvez mais relacionado ao status trófico do ambiente de onde as comunidades congeladas vieram e se o estilo de vida característico da comunidade era de generalistas ou especialistas. No geral, comunidades com característica mais generalista, que vieram de ambientes mais variáveis e eutróficos, foram mais fáceis de cultivar e manter a diversidade similar à natural, obtendo comunidades muito semelhantes à comunidade natural original. Comunidades originárias de ambientes oligotróficos e estáveis, de característica mais especialista, foram mais difíceis de cultivar e manter uma diversidade similar à natural, embora o sucesso tenha sido muito maior quando utilizamos água deste mesmo local esterilizada por filtração. Fizemos entaão um experimento para ilustrar a aplicabilidade dessas técnicas, onde testamos simultaneamente o efeito de diferentes MOD e de coalescência de comunidade. Em resumo, comunidade de característica mais especialista foi de fato mais diferente em relação à comunidade original e apresentou alguma variação na diversidade final à diferença de MODs, enquanto comunidades de características mais generalista foram muito similares a comunidade original, mas quase não apresentaram diferenças com relação às diferentes MODs.

Por fim, de modo geral tanto a quantidade quanto a qualidade de MOD influenciam a composição e desenvolvimento de uma comunidade bacteriana, o que não é absolutamente algo novo. No entanto, pensamos que a qualidade exerce uma

seleção mais específica sobre a comunidade no começo do contato da comunidade com uma determinada MOD, e a intensidade e duração dessa seleção é modulada pela quantidade de MOD, até que a interação entre os membros da comunidade passe de primariamente negativa, competição, para uma interação de facilitação. No entanto, é preciso considerar como talvez ainda mais importante as características do ambiente de origem da comunidade a ser testada, que pode influir na resposta da comunidade ainda mais fortemente que a qualidade e quantidade de MOD.