

ORIGINAL ARTICLE

The allometry of the smallest: superlinear scaling of microbial metabolic rates in the Atlantic Ocean

Francisca C García¹, Enma Elena García-Martín^{2,5}, Fernando González Taboada³, Sofía Sal^{1,6}, Pablo Serret^{2,4} and Ángel López-Urrutia¹

¹Centro Oceanográfico de Gijón, Instituto Español de Oceanografía, Gijón, Spain; ²Universidad de Vigo, Departamento de Ecología y Biología Animal, Carretera Colegio Universitario, Vigo, Spain; ³Departamento de Biología de Organismos y Sistemas, Universidad de Oviedo, Oviedo, Vigo, Spain and ⁴Estación de Ciencias Marinas de Toralla, Universidad de Vigo, Toralla Island, Vigo, Spain

Prokaryotic planktonic organisms are small in size but largely relevant in marine biogeochemical cycles. Due to their reduced size range (0.2 to 1 µm in diameter), the effects of cell size on their metabolism have been hardly considered and are usually not examined in field studies. Here, we show the results of size-fractionated experiments of marine microbial respiration rate along a latitudinal transect in the Atlantic Ocean. The scaling exponents obtained from the power relationship between respiration rate and size were significantly higher than one. This superlinearity was ubiquitous across the latitudinal transect but its value was not universal revealing a strong albeit heterogeneous effect of cell size on microbial metabolism. Our results suggest that the latitudinal differences observed are the combined result of changes in cell size and composition between functional groups within prokaryotes. Communities where the largest size fraction was dominated by prokaryotic cyanobacteria, especially *Prochlorococcus*, have lower allometric exponents. We hypothesize that these larger, more complex prokaryotes fall close to the evolutionary transition between prokaryotes and protists, in a range where surface area starts to constrain metabolism and, hence, are expected to follow a scaling closer to linearity.

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Introduction

Heterotrophic bacterioplankton (bacteria and archaea) are considered responsible for a large fraction of the respiration in marine ecosystems, controlling the carbon cycle mainly in the vast oligotrophic areas (Williams, 1981; del Giorgio and Duarte, 2002; Robinson and Williams, 2005). Marine bacteria and archaea form an extremely diverse and specialized assemblage (Giovannoni and Stingl, 2005). They hold high capability to acquire genetic diversity mainly by lateral gene transfer and other mechanisms for gene expansion favoring their functional diversity and, therefore, a widespread diversification of metabolic pathways (Whitman *et al.*, 1998; Doolittle and Papke, 2006). This implies a competitive advantage allowing them to be adapted to the surrounding conditions (Giovannoni and

Stingl, 2005; Massana and Logares, 2013), which largely explains the changes in heterotrophic bacterioplankton composition recorded along different spatio-temporal gradients (Giovannoni *et al.*, 1996; Morris *et al.*, 2005; Pommier *et al.*, 2007; Gilbert *et al.*, 2009). Therefore, bacterial taxonomic and functional versatility have been the main variables used to explain changes in bacterioplankton metabolism.

Size and functional diversity are considered as key factors controlling species metabolism (Litchman *et al.*, 2007; Finkel *et al.*, 2010; Edwards *et al.*, 2012; Marañón *et al.*, 2013). Although much progress has been made in allometric studies of other small organisms such as phytoplankton, the effects of cell size on marine heterotrophic bacterioplankton respiration have been hardly considered and continue essentially unanswered (Makarieva *et al.*, 2005; Massana and Logares, 2013). The effects of cell size on metabolism are usually characterized using a power law (West *et al.*, 1997; Brown *et al.*, 2004; DeLong *et al.*, 2010),

$$Y = Y_0 M^b \quad (1)$$

where Y is the metabolic rate, Y_0 is a normalized constant, M is the body mass of an individual and b is the scaling exponent.

Correspondence: FC García or Á López-Urrutia, Centro Oceanográfico de Gijón, Instituto Español de Oceanografía, Avda Príncipe de Asturias 70 bis, Gijón E-33212, Asturias, Spain.
 E-mail: paquigrgrc@gmail.com or alop@gi.ieo.es

⁵Present address: School of Environmental Science, University of East Anglia, Norwich Research Park, Norwich NR4 7TJ, UK.

⁶Present address: Department of Life Sciences, Imperial College London, Silwood Park, Ascot, Berkshire SL5 7PY, UK.

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The debate on the exact value of the allometric exponent b is extensive (Kleiber, 1932; Hemmingsen, 1960; West *et al.*, 1997; Enquist *et al.*, 2003; Brown *et al.*, 2004; Huete-Ortega *et al.*, 2012). Recent empirical studies have suggested values for the scaling exponent in small-size organisms significantly different from the traditional $\frac{3}{4}$ scaling suggesting the need to further review the value of this exponent (Dodds *et al.*, 2001; Makarieva *et al.*, 2005; Zubkov, 2014). In this sense, DeLong *et al.* (2010) have found that the scaling of metabolism is not universal for all forms of life and is sublinear ($b < 1$) for metazoan, linear ($b = 1$) for protists and superlinear ($b > 1$) for prokaryotes. Following the DeLong *et al.* (2010) hypothesis, the superlinear scaling for prokaryotes is the result of the correlation between genome size and cell size within this functional group. Larger cells contain larger genomes that allows for an increasing diversity of substrates that can be metabolized leading to higher metabolic power.

The allometric studies of prokaryotes have generally used data compilations mostly from laboratory cultures (Makarieva *et al.*, 2005; DeLong *et al.*, 2010; Mara  n *et al.*, 2013). Most of the information on the allometry of microorganisms in the ocean is for phytoplankton groups (L  pez-Urrutia *et al.*, 2006; Mara  n *et al.*, 2007; Finkel *et al.*, 2010; Zubkov, 2014), while the allometric scaling of bacterial communities from natural environments remains mostly unexplored. Therefore, our main objective is to test whether the metabolic rates of prokaryotic picoplanktonic groups in marine environments follow the superlinear allometric scaling. We carried out respiration measurements by *in vivo* 2-para (iodo- phenyl)-3(nitrophenyl)- 5(phenyl) tetrazolium chloride reduction approach (ivINT) of size-fractionated marine microbial plankton communities ($< 0.8 \mu\text{m}$). Our aim is to determine how individual bacterioplankton respiration rate scales with cell size and to identify the biotic and abiotic factors that could affect this relationship.

Materials and methods

Sampling strategy

Seawater from 30 stations along a latitudinal transect in the Atlantic Ocean was taken during the 2011 Atlantic Meridional cruise (AMT21) on board the RRS Discovery. The latitudinal transect covered from 50°N (Avonmouth, UK) to 50°S (Punta Arenas, Chile). One station was sampled daily between the 29 September and the 14 November of 2011. Seawater samples were collected in Niskin bottles at predawn, from one or two different depths: near surface (2–5 m depth) and at the depth of the chlorophyll maximum (DCM). Seawater was transferred from the Niskin bottle to a 3 l bottle for subsequent subsampling and analysis of size-calibration experiments, *in vivo* INT reduction and flow cytometric determination of the size

structure, as outlined below. All bottles were previously rinsed with fresh water, followed by HCl and milliQ water at the end.

In vivo INT reduction capacity method

In vivo INT reduction capacity measurements were performed using INT. This method has the advantage of estimating community respiration by measuring the reduction of INT within individual cells, allowing the filtration of cells into size classes after the incubation, which is not possible with traditional methods like dark bottle incubation (Winkler) method. The INT reduction method has been criticized by its lack of specificity (INT can be reduced by organic matter), and by assuming a linear decay of the rate of INT reduction with time (Maldonado *et al.*, 2012). The ratio between oxygen consumption and INT reduction is also variable depending on the trophic state (that is, oligotrophic or meso-eutrophic conditions; Garc  a-Mart  n, personal communication). Despite these limitations, there is a good correlation between the INT reduced by planktonic organisms and their respiration measured by dark bottle incubation (Winkler method; Mart  nez-Garc  a *et al.*, 2009). Indeed, further studies have validated the method at large spatial scales, finding a good statistical correlation between both techniques ($r^2 = 0.74$, $P < 0.0001$, $n = 398$, Garc  a-Mart  n *et al.*, in preparation). Therefore, we used INT reduction capacity as a proxy of respiration rate, although we avoided converting INT moles to O_2 units.

Four replicates of 500 ml were collected in plastic bottles. One replicate was immediately fixed by adding formaldehyde (2% w/v final concentration) and used as a killed control. After 15–20 min, all replicates were inoculated with a sterile solution of 8 mM iodonitrotetrazolium salt (INT) to give a final concentration of 0.8 mM. All replicates were incubated between 2 and 4 h. Samples were fixed by adding formaldehyde, as for the killed control. Samples were sequentially filtered after 15 min through 0.8, 0.6, 0.4 and $0.2 \mu\text{m}$ pore-size polycarbonate filters, air-dried and stored frozen in 1.5 ml cryovials at -20°C until further processing. The $0.8 \mu\text{m}$ filter was used to remove the larger planktonic cells from the analysis. So then, respiration was estimated for three different size classes: 0.8–0.6, 0.6–0.4 and $0.4\text{--}0.2 \mu\text{m}$. The reduction of the INT was determined following Mart  nez-Garc  a *et al.* (2009).

Size structure

A water sample of 500 ml was collected for the flow cytometric determination of the size structure and filtered through the same sequence of 0.8, 0.6, 0.4 and $0.2 \mu\text{m}$ pore-size Nucleopore filters. A 500 μl aliquot from each filtrate was fixed with paraformaldehyde 2% final concentration and used for flow cytometry analyses of heterotrophic bacteria. The samples were stained with SYBR Green I (1:10 000 final dilution of

initial stock) and potassium citrate (0.1% w/v) and kept 1 h in the dark before analysis. Then, to calibrate the flow cytometric signal, 25 μl of a mixture of yellow-green 0.5 and 1.0 μm beads (Fluoresbrite Microparticles, Polysciences, Eppelheim, Germany) in pre-filtered seawater was added to each sample. Samples were analyzed for 60 s at a low flow rate (15 $\mu\text{l min}^{-1}$ approximately) with a FACSCalibur flow cytometer (Becton Dickinson, Oxford, UK).

Bacterioplankton abundance, respiration per cell and cytometric properties estimates

Flow cytometry analyses during the size-fractionated respiration experiments were used to estimate abundance and bio-volume of the community. The ivINT method estimates the respiration of the plankton fraction retained between the two consecutive filters (0.8–0.6, 0.6–0.4 and 0.4–0.2, respectively). However, flow cytometry measurements were performed on the filtrates of each filter. The cell abundance retained by one filter was calculated by subtracting the number of cells in the filtrate from the number of cells in the suspension before passing through that filter.

To estimate the mean size of the cells retained on each filter, we used a flow cytometer parameter related to cell size, the side scatter (Burkill *et al.*, 1993; Zubkov *et al.*, 1998; Calvo-Díaz and Morán, 2006). Side scatter units were transformed into size units by using a bacterioplankton size calibration performed during the AMT21 cruise (Supplementary information). The flow cytometer returns, for each sample, a flow cytometry standard file with the recorded flow cytometric signals for each cell. Each flow cytometry standard file was processed using the Bioconductor package flowCore (Hahne *et al.*, 2009), hence, we were able to access to the cell size distribution of the community. To estimate the size distribution of the cells retained on each filter, we subtracted the size distribution of the cells in the filtrate from the size distribution in the suspension before passing through that filter. Knowledge on the abundance and mean size of the cells retained on each filter allowed us to estimate the average respiration per cell and the size scaling of respiration. The respiration per cell was calculated as the respiration of the different fractions divided by the cell abundance at that fraction.

Statistical analysis

The theoretical relationship between bacterioplankton respiration per cell and cell bio-volume (Equation 1) can be linearized by logarithmic transformation. The equation has two unknowns: the normalizing constant α and the scaling exponent β that were estimated from *in situ* measurements using a mixed effects model due to the hierarchical structuring of our experimental design (Gelman and Hill, 2007). This allowed us to account for unbalanced

observations and to quantify variability associated to changes between replicates within the same experiment, and among different experiments along the transect;

$$\ln R_i = \alpha_{jk[i]} + \beta_{jk[i]} \ln V_i + \varepsilon_i \quad (2)$$

where R and V represent the respiration and the bio-volume measurements. The subscript $jk[i]$ indicate that respiration was measured for the replicate k within experiment j . Intercepts and slopes were modeled as normal random deviates with mean μ_α and μ_β , respectively. Deviations from this mean vector for each replicate were characterized by a variance-covariance matrix partitioning total variance among changes between replicates and between experiments (that is, σ_k^2 and σ_j^2). Remaining errors were assumed independent and normally distributed ($\varepsilon \sim N(0, \sigma_{\ln R_i})$). The model was fitted using restricted maximum likelihood (R Core Team, 2014) with the package lme4 (Bates *et al.*, 2014). Data were centered before the analysis to avoid spurious inflation of parameter correlations. Model selection based on Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) discarded simplified versions of the model presented above (that is, constant intercepts or slopes) and models considering differences between replicates within experiments (that is, the best model assumed $\sigma_k = 0$ for both α and β , highlighting the robustness of our measurements). We assumed that the magnitude of errors in respiration per cell was much larger than the magnitude of errors in cell size measurements, and thus that the later can be safely ignored. Although this assumption is common in allometric studies (Gillooly *et al.*, 2002; Makarieva *et al.*, 2005), it might lead to the underestimation of scaling exponents (Warton *et al.*, 2006; DeLong *et al.*, 2010). Therefore, estimates performed using ordinary least squares method were compared with estimates derived from reduced major axis regression in order to explore the effect of the fitting method in the superlinearity found, the scaling exponent and the intercept for each station (Supplementary information). A comparison with estimates derived using reduced major axis regression for each experiment resulted in around a 20% increase of scaling exponents, so we consider our approach conservative to reject the hypothesis of linear or sublinear scaling.

Results

The size-fractionation experiments along the 30 stations sampled revealed a strong correlation between cell size and metabolic activity along a latitudinal transect across the Atlantic Ocean (Figure 1). Mixed effects models provided a reasonable fit of the relationship between bacterioplankton cell size and respiration per cell along the Atlantic Ocean. The best model was a linear mixed effects model with random intercept and slope (Equation 2,

AIC = 549.98, BIC = 571.23, $P < 0.0001$). This implies significant differences in both slope and intercept along the Atlantic latitudinal transect experiments.

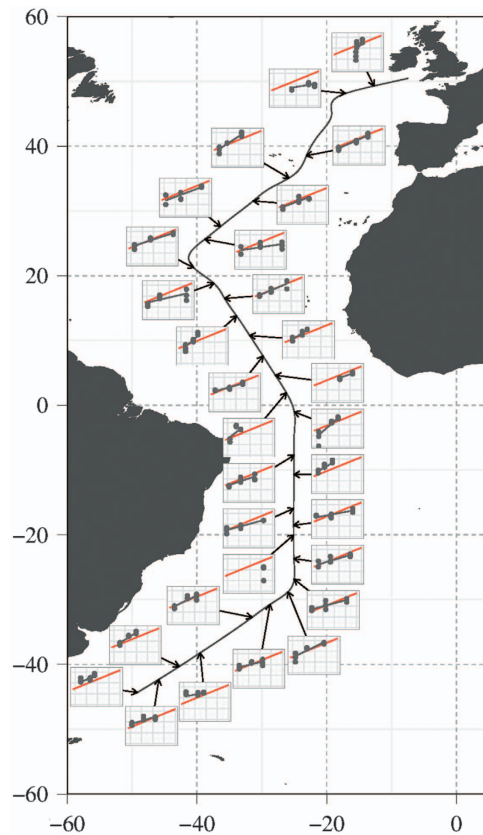


Figure 1 The relationship between respiration per cell (y axis) and bio-volume (x axis) for each experiment along the Atlantic latitudinal transect and the general fit provided by the model. Gray dots represent the values for each size fraction and gray dashed line the linear fit provided by the mixed model for each experiment. The red line corresponds to the population level and mean scaling relationship for all experiments.

The overall slope in our model suggests that respiration rate scales with bio-volume superlinearly, with a scaling exponent significantly larger than one (Figures 2, $\beta_1 = 1.67 \pm 0.19$, $P < 0.0001$). For 80% of the experiments performed along the latitudinal transect the slope was significantly higher than unity. Although the model concluded that there are significant differences between stations, the comparison of the fitted slope for each experiment with the general slope revealed that the superlinearly detected for the overall slope was also followed by the majority of the experiments (Figure 1). The comparison between the scaling exponents obtained by ordinary least square and reduced major axis regressions (Supplementary information, Supplementary Table S1) further demonstrates the superlinear scaling recorded by the mixed-effect models used in our analyses.

A caterpillar plot revealed that estimates of the allometric exponent deviated only slightly from the overall mean in most of the experiments (Figure 3, that is, 95% confidence intervals for individual estimates overlapped the general slope in more than three quarters of the experiments). However, none of the factors tested could be clearly associated to these deviations. Nevertheless, some experiments deviated significantly from the overall trend (red lines in Figure 3), highlighting the heterogeneity in slopes along the transect. In an effort to understand the differences found for the slope, we tested the relationship between the scaling exponents provided by the lineal mixed-effects model and different external factors: latitude, temperature, depth and chlorophyll.

Those bacterioplankton communities composed of larger cells evidence a decrease in the scaling exponent (Figures 2a and b). Indeed, there is a strong inverse correlation between the scaling exponent and the mean size of the cells that integrated the community in each experiment ($r = -0.57$, $P < 0.01$, Figure 4a).

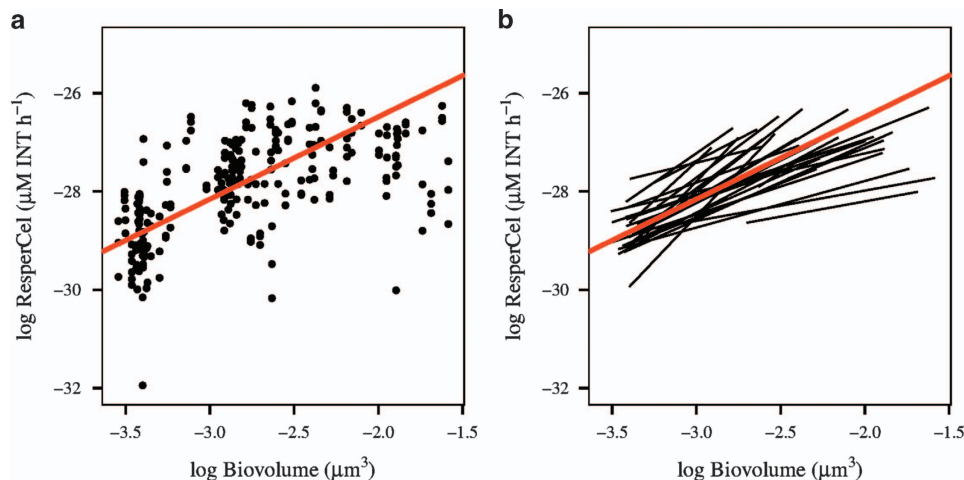


Figure 2 Relationship between respiration per cell (y axis) and bio-volume (x axis) for each experiment size fraction (a) and the linear fit for each experiment (b). The red line in each panel represent the mean scaling relationship derived from the mixed effects model.

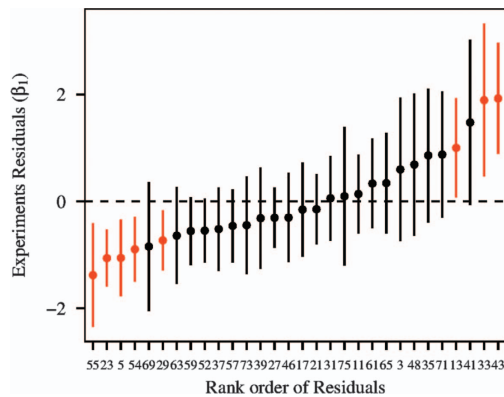


Figure 3 Caterpillar plot showing the random effects (mean and 95% confidence intervals) on the allometric exponent (β_1) for each of the 30 size fractionated experiments, which are ordered by the relative rank of the deviations. Experiments where the population level mean exponent does not fall within the confidence interval are labeled in red. These large deviations reflect the heterogeneity in slopes among experiments.

This decrease in the scaling exponent when the community is composed of larger cells implies that changes in community composition may be causing the differences in the slope term. We explored this hypothesis through the use of the average bio-volume of the cells retained by each of the different pore-size filters in an experiment. Although no remarkable changes were found in the mean bio-volume for the two smallest size fractions, there was a considerable range of average cell sizes of the largest size fraction along the experiments. A significant relationship was found between the slope term and the mean bio-volume of the cells retained on the $0.6\ \mu\text{m}$ filter ($r = -0.68$, $P < 0.001$, Figure 4b). Nonetheless, no remarkable changes were found in the mean bio-volume for the other size fractions along the experiments. This suggests that changes in the larger size populations of the microbial community may be responsible for the drop-off observed in the scaling exponent.

In addition to a change in slope, Figure 2 also denoted a decrease in the height of the scaling relationship with increasing mean bio-volume of the community. Because the slopes differ, comparison of the intercepts provided by the model is meaningless. To study these changes in the height of the scaling relationship, we selected an intermediate cell volume ($0.068\ \mu\text{m}^3$) and we calculated a size-normalized respiration rate per cell at this fixed size for all the experiments. To minimize the variability, only experiments performed at the DCM were taken into account. Similarly to the scaling exponent, the size-normalized respiration was highly correlated with average bio-volume, especially for $0.6\ \mu\text{m}$ filter size, indicating that communities composed of larger, more complex cells have lower metabolic rates per cell for a given cell size (Figures 4c and d). These effects of community composition are also evidenced by a decreasing size-normalized metabolism with an increasing percentage of

Prochlorococcus abundance in relation to heterotrophic bacteria ($r = -0.60$, $P < 0.01$) and a strong, positive relation with heterotrophic bacteria abundance ($r = 0.71$, $P < 0.001$).

These compositional changes were paralleled by changes in several ancillary environmental parameters. When the DCM was located deeper, size-normalized metabolism decreased ($r = 0.68$, $P < 0.001$). Although the DCM was deeper mainly in oligotrophic areas, a nutrient limitation index (calculated as the difference between the mixed layer depth and nitracline depth (Behrenfeld *et al.*, 2002)) showed no clear relationship with size-normalized respiration ($r = 0.35$, $P = 0.12$). This implies that albeit significant correlations were found, no environmental parameter could explain alone the differences found in the heights along the study.

Discussion

The effect of cell size on prokaryotic metabolism has been hardly considered to be important. However, our experiments using oceanic communities from natural environments demonstrate a strong effect of cell bio-volume on metabolic processes for tiny organisms like bacteria. Our results reveal a super-linear scaling (> 1) for the smallest ($< 0.8\ \mu\text{m}$) planktonic organisms along a latitudinal transect in the Atlantic Ocean. The model providing a best fit to our data estimated an overall, station-average scaling exponent value larger than one ($b = 1.67$) and in the range of the value of 1.7 – 2.0 reported by DeLong *et al.*, (2010) from a compilation of data for terrestrial and aquatic prokaryotes mostly from cultures.

DeLong *et al.*, (2010) hypothesized that the super-linear scaling is due to the correlation between cell size and genome length in prokaryotes (Shuter *et al.*, 1983; Doolittle, 2002; Tanaka *et al.*, 2003). Larger cells have longer genomes resulting in an increase in the number of metabolic pathways and, hence, in their functional diversity (Whitman *et al.*, 1998; Stepkowski and Legocki, 2001; Islas *et al.*, 2004; Price *et al.*, 2004). Accordingly, the metabolism of prokaryotes would not be constrained by surface area as in protists but by the number of metabolic pathways that they can use to acquire energy. When the size of prokaryotes is large enough so that they have a complete complement of most metabolic pathways, surface area constraints their metabolism (DeLong *et al.*, 2010).

Our model also concludes that this general scaling exponent is not universal, with significant deviations along the transect (Figure 3). This means that the overall value estimated by our model ($b = 1.67$) is not universal. Shifts in the metabolic size-scaling exponent has been also reported at evolutionary and species level (DeLong *et al.*, 2010; Kempes *et al.*, 2012).

Our analyses revealed that the heterogeneity around the universal scaling relationship may be explained in terms of changes in community

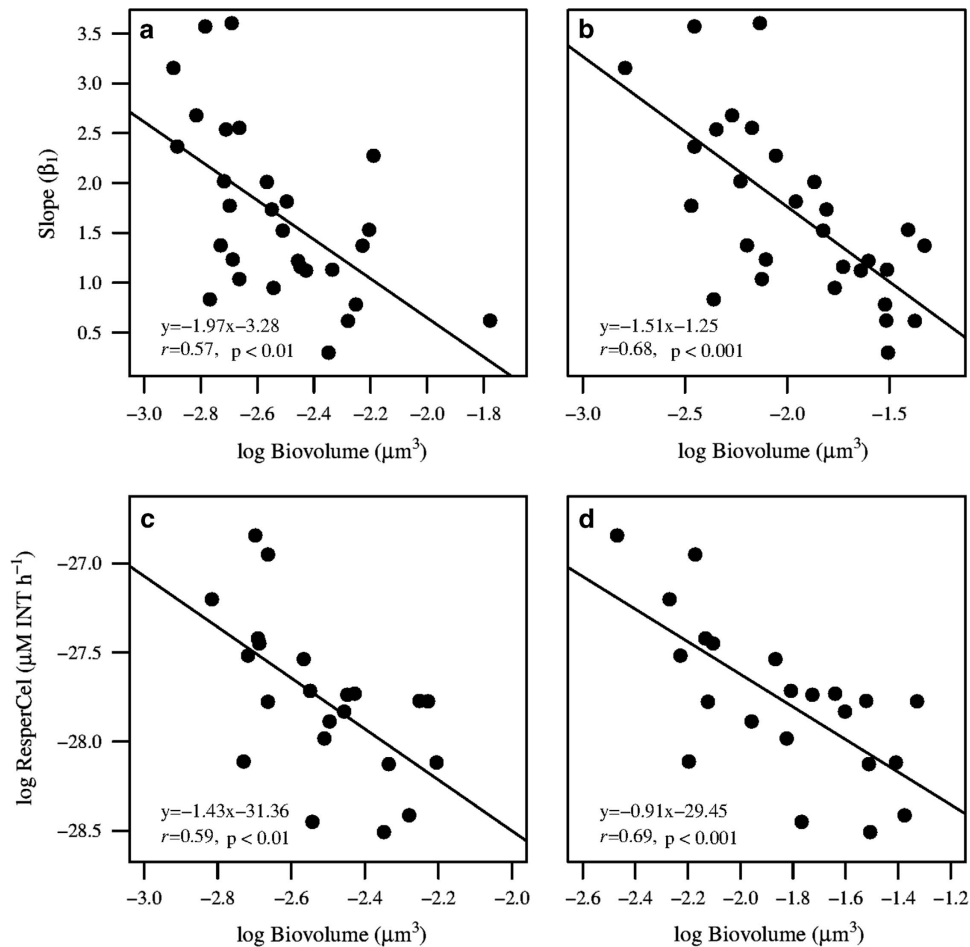


Figure 4 Relationship between the allometric slope β_1 (y axis) and the mean bio-volume (x axis) of the whole community (a) and of the cells retained onto the $0.6 \mu\text{m}$ filter (b). Relationship between the size-normalized respiration per cell (normalized for a cell with an average volume of $0.068 \mu\text{m}^3$; y axis) for experiments performed at the DCM and the mean bio-volume (x axis) of the whole community (c) and of the cells retained onto the $0.6 \mu\text{m}$ filter (d).

composition and size distribution. Communities composed of smaller cells have higher scaling exponents than those composed of larger cells (Figures 4a and b). Along the Atlantic transect there are compositional changes in the relative importance of the phylogenetic groups within prokaryotic picoplankton (bacteria, archaea and cyanobacteria; Fuhrman *et al.*, 1993; Giovannoni and Stingl, 2005) and in the cell sizes within each group. Although our experiments separated the community into three size fractions smaller than $0.8 \mu\text{m}$, these compositional changes resulted in shifts in the size range covered in our experiments and in the mean cell sizes of the whole community. We were able to detect these changes, thanks to the measurement of the size-frequency distributions within each size fraction using flow cytometry. If we use the average size of the community as an indicator of cell complexity, taking into account the relationship between cell size and the number of genes of a prokaryotic cell reported by DeLong *et al.* (2010), a similar pattern can be observed in our experiments with communities composed of large and, thus, more complex

cells having an scaling exponent closer to the unity (Figures 4a and b).

We hypothesize that these changes are the result of shifts in the allometric scaling between functional groups within prokaryotes. Experiments where heterotrophic prokaryotic groups (bacteria and archaea) predominate have a metabolism that is not constrained by surface area and scale superlinearly as suggested by DeLong *et al.* (2010). There is a drop-off in the scaling exponent (Figures 4a and b) when larger, more complex cyanobacteria, especially *Prochlorococcus*, dominate the largest size fractions of the community as in the Atlantic oligotrophic areas (Zubkov *et al.*, 1998, 2000). We suggest that these larger, more complex prokaryotes fall close to the evolutionary transition between prokaryotes and protists, in a range where surface area starts to constraint metabolism (DeLong *et al.*, 2010) and, hence, are expected to follow a scaling closer to linearity.

The change in the size-scaling slope was accompanied by a marked decreasing in the heights of the scaling relationship for communities dominated by larger, more complex cells (Figure 2).

The size-normalized respiration at the DCM was inversely correlated with mean cell size of each community, especially for the largest size fraction (Figures 4c and d). *Prochlorococcus* predominates at the DCM depth in both Atlantic oligotrophic gyres (Zubkov *et al.*, 1998), a pattern that we also found in the 0.6 µm filter in our experiments. The resulting increasing ratio of *Prochlorococcus* abundance to heterotrophic bacteria abundance in oligotrophic areas is reflected in a decrease in the size-normalized respiration (Figure 2).

The relevance of cell size in the metabolism of phytoplankton groups has been fully recognized (López-Urrutia *et al.*, 2006; Litchman *et al.*, 2007; Finkel *et al.*, 2010; Edwards *et al.*, 2012; Zubkov, 2014). The importance of these groups on the productivity of the oceans together with their wide size range, spanning several orders of magnitude, has favored that phytoplankton has been largely studied compared with other groups much smaller as bacteria, whose allometry has remained essentially unknown. Here, despite the narrow size range covered (0.2–0.8 µm), we have demonstrated that cell size is a key factor controlling the respiration of marine bacterioplankton. The strong compositional changes along the transect and its correlation with the size-scaling parameters highlights the influence of cell complexity on the allometry of the microbial community. We have shown that cell size is a key functional trait in bacterioplankton communities. Understanding the deviations from the general allometric scaling from knowledge of bacterial taxonomic and functional diversity might allow a better comprehension of their contribution to the biogeochemical cycles, especially the marine carbon cycle.

Conflict of Interest

The authors declare no conflict of interest.

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