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Exudation of organic carbon by marine phytoplankton: dependence on taxon and cell size

Daffne C. López-Sandoval[1,*], Tamara Rodríguez-Ramos[1], Pedro Cermeño[1,2], Emilio Marañón[1]

[1]Departamento de Ecología y Biología Animal, Universidad de Vigo, 36210 Vigo, Spain

[2]*Present address:* Instituto de Ciencias del Mar, Consejo Superior de Investigaciones Cientificas, Passeig Maritim de la Barceloneta 37–49, 08003 Barcelona, Spain

ABSTRACT: We determined the relationship between photosynthetic production of dissolved organic carbon (OC) and phytoplankton cell size and taxonomic composition in cultures of marine phytoplankton at 3 different growth stages. We measured OC exudation in 22 species belonging to 5 phyla and spanning >7 orders of magnitude in cell volume. The extracellular release of OC in our cultures represented on average ~2% of total carbon fixation, was not statistically different between growth stages, and was not correlated to cell size. The cell-specific OC exudation rate held an isometric relationship with cell size during the different growth phases (average slope: 0.95), which implies that general allometric models cannot be used to predict exudation in marine phytoplankton.

KEY WORDS: Phytoplankton · Exudation · Cell size · Taxonomic affiliation · Organic carbon

INTRODUCTION

The extracellular release in dissolved form of newly synthesized metabolites is a normal process (Mague et al. 1980) that takes place during all growth phases in phytoplankton (Hellebust 1965, Obernosterer & Herndl 1995). The percentage of extracellular release (PER, the fraction of total primary production [PP] released in dissolved form) in natural phytoplankton assemblages ranges between 10 and >35% of total PP (Anderson & Zeutschel 1970, Teira et al. 2001b, Morán et al. 2002, Marañón et al. 2005, López-Sandoval et al. 2010), being more important in oligotrophic areas (Fogg 1983, Karl et al. 1998, Teira et al. 2001a, López-Sandoval et al. 2011). In phytoplankton cultures, PER values tend to be smaller, ranging from <1 to 20%, with few species reaching values >25%, depending on the growth phase and culture conditions (Hellebust 1965, Mague et al. 1980, Malinsky-Rushansky & Legrand 1996, Finkel 1998).

Exudation can be the result of 2 non-mutually exclusive processes: passive diffusion of small molecules (<900 Da) through the cell membrane (Bjørnsen 1988), or a self-induced mechanism that occurs when phytoplankton organisms experience stressful conditions such as low nutrient concentration or high irradiance (Fogg 1983, Wood & Van Valen 1990). Large molecules (>1500 Da) with low diffusion rate are frequently detected as part of the excreted material (Hellebust 1965, Lancelot 1984), suggesting that other mechanisms in addition to passive diffusion must be involved. Some phytoplankton species respond to a specific stimulus (e.g. exposure to certain wavelengths) by storing polymers in secretory vessels and then releasing them via autoregulated exocytosis (Chin et al. 2004, Orellana et al. 2011), but the mechanisms involved in exudation of other kinds of macromolecules still remain largely unknown.

*Email: daffne@uvigo.es

While there are several sources of labile dissolved organic carbon (OC) in the microbial plankton community (including cell leakage due to viral attack, sloppy feeding by zooplankton, breakage of faecal pellets, etc.) (Nagata 2000), in the present study we address only the production of dissolved OC that was fixed by photosynthesis and then released during a 2 h incubation period (Mague et al. 1980, Fogg 1983).

Some studies support the hypothesis that exudation should be more important in smaller cells, due to their high surface to volume quotient (Malinsky-Rushansky & Legrand 1996, Teira et al. 2001a), but this view is not always shared (Finkel 1998, Marañón et al. 2004, López-Sandoval et al. 2010). The differences in the percentage of exudation among contrasting systems suggest a possible link between exudation and phytoplankton cell structure; however, it is difficult to test if cell size by itself has a direct effect on exudation, mainly because, in the field, other controlling variables tend to covary with phytoplankton cell size.

General allometric theory predicts that a metabolic rate (M) is related to body size (W) through the relationship: $M = W^b$, where b is the size-scaling exponent, which usually takes a value of ¾ ('Kleiber's law'). Several models based on resource distribution networks have been proposed to explain why size-scaling relationships tend to have exponents that are multiples of ¼ (West et al. 1997, 1999, Banavar et al. 2002, 2010). The validity of Kleiber's ¾ power rule has been shown in animals (Savage et al. 2004), plants (Niklas & Enquist 2001) and marine phytoplankton (Blasco et al. 1982, Finkel 2001, Finkel et al. 2004). A limitation of the latter studies is that they covered only a small number of phytoplankton species and a modest range in terms of cell size and taxonomic variability. Field studies and data meta-analyses have shown that this 'universal law' — which implies that the metabolic demand of an organism, per unit of mass, decreases as body size increases — does not hold true in the case of phytoplankton (Tang & Peters 1995, Marañón et al. 2007, Marañón 2008, Huete-Ortega et al. 2011). To our knowledge, there are no published studies of the size-scaling relationship of exudation in phytoplankton over a wide range of cell sizes and taxonomic affiliations.

Here we present data of exudation rates in 22 different species of phytoplankton in monospecific cultures, grown under the same controlled conditions and measured at 3 different growth phases with the same protocol. We provide data on the size scaling of dissolved OC production over a wide phylogenetic (5 phyla) and cell size (7 orders of magnitude in cell volume) range.

MATERIALS AND METHODS

Phytoplankton cultures

Species used for this study covered a cell size range from 0.12 to 2 500 000 μm^3 (Table 1). Cultures were obtained from Provasoli-Guillard National Center for Marine Algae (USA), Roscoff Culture Collection (France), Culture Collection of Algae and Protozoa (UK), Instituto Español de Oceanografía (Spain) and Estación de Ciencias Mariñas de Toralla (Spain). Cultures were grown in a 4 l round-bottom flask on filtered (0.2 µm), autoclaved and enriched seawater medium (details in Table 1), with silicate excluded in the case of non-diatoms and additional trace metals (L1 trace element solution) added in the case of dinoflagellates. The concentration of dissolved inorganic nitrogen was reduced 4-fold, so that the N/P molar ratio was ~6 and nitrogen limitation was ensured. Culture flasks were set up in a culture chamber at a constant temperature (18 ± 0.5°C), with continuous aeration (except for dinoflagellates), and were exposed to a photon flux density of ~250 µE m^{-2} s^{-1} and a 12:12 h light:dark cycle. Cells were kept in semi-continuous growth for 3 complete acclimation cycles in 1 l aerated flasks before conducting measurements of exudation. An acclimation cycle was defined from the time when the inoculum was added to fresh medium to the time when the population reached the exponential phase and an aliquot was transferred to fresh medium again, thus starting a new cycle. Our cultures were not axenic. However, we regularly collected samples for bacterial abundance and found that the contribution of bacterial biomass to total OC was always <0.4%.

Growth rates, cell density and cell size

Growth was monitored daily by *in vivo* fluorescence measured with an Aquafluor Turner Design fluorometer, cell counts under the microscope and measurements of chl *a* concentration. Depending on the species' cell size, a Neubauer haemacytometer (1 mm^2) or a 1 ml Sedwick-Rafter chamber were used to determine cell abundance. For *Coscinodiscus radiatus* and *C. wailesii*, cells were counted by sedimenting 5 or 10 ml aliquots for 24 h in Utermöhl chambers. Enough cells were counted to keep the coefficient of variation of the mean population abundance estimate <20%. The abundances of *Synechococcus* sp., *Prochlorococcus* sp., *Micromonas pusilla* and *Ostreococcus tauri* were determined by flow

Table 1. List of phytoplankton species studied

Phylum	Class	Species	Clone/origin	Size (μm^3)	Culture medium, NO^{3-}/NH^{4+}
Ochrophyta	Bacillariophyceae	*Skeletonema costatum*	CCAP 1077/1C	242	f/4, f/16[a]
		Thalassiosira rotula	CCAP 1085/20	2597	f/8, f/32[a]
		Phaeodactylum tricornutum	ECIMAT	93	f/4, f/16[a]
		Thalassiosira weissflogii	CCMP 1336	1163	f/4, f/16[a]
		Melosira nummoloides	ECIMAT	2285	f/4, f/16[a]
		Coscinodiscus radiatus	CCMP 312	81 955	f/4, f/16[a]
		Coscinodiscus wailesii	CCMP 2513	2 498 458	f/4, f/16[a]
		Ditylum brightwellii	CCMP 361	75 827	f/4, f/16[a]
Myzozoa	Peridinea	*Protoceratium reticulatum*	IEO-Vigo	23 823	L/2, L/8[b]
		Akashiwo sanguinea	IEO -Vigo	47 349	L/2, L/8[b]
		Alexandrium minutum	CCMP 113	5575	L/2, L/8[b]
		Alexandrium tamarense	EF04	88 836	L/2, L/8[b]
Haptophyta	Prymnesiophyceae	*Emiliania huxleyi*	CCMP 371	158	f/4, f/16[a]
		Gephyrocapsa oceanica	CCMP 2051	82	f/4, f/16[a]
		Calcidiscus leptoporus	RCC1169	51	f/4, f/16[a]
		Isochrysis galbana	ECIMAT	64	f/8, f/32[a]
	Pavlovophyceae	*Pavlova lutheri*	CCMP 1325	45	f/4, f/16[a]
Ochrophyta	Eustigmatophyceae	*Nannochloropsis gaditana*	ECIMAT	8.6	f/4, f/16[a]
Chlorophyta	Mamiellophyceae	*Micromonas pusilla*	RCC 496	10.7	K/2, K/8[c]
		Ostreococcus tauri	RCC 116	2.4	K/2, K/8[c]
Cyanophyta	Cyanophyceae	*Synechococcus* sp.	RCC 33	0.41	f/4, f/16[a]
		Prochlorococcus sp.	RCC 267	0.12	PCR-S11, PCR-S11/4[d]

[a]Guillard (1975); [b]Guillard & Hargraves (1993); [c]Keller & Guillard (1985); [d]Roscoff Culture Collection's recipe

cytometry in 2.5 ml samples, fixed with 0.250 µl paraformaldehyde (1% final concentration) and glutaraldehyde (0.05% final concentration), using a FACScan flow cytometer (Becton Dickinson). Chl *a* concentration was measured fluorometrically on a TD-700 fluorometer after filtration of duplicate 5 ml samples on GF/F filters, freezing of the filters at −20°C and extraction with 90% acetone. Biovolume was measured with a Leica DLMB microscope using the NIS-Elements BR 3.0 image analysis software. Critical cell dimensions were obtained in at least 100 cells by assigning different geometric shapes that were most similar to the real shape of each phytoplankton species Sun & Liu (2003).

Particulate organic carbon determination

Duplicate, 10 ml aliquots of culture were filtered onto pre-combusted (450°C for 8 h) GF/F filters, which were then stored at −20°C. Before analysis, filters were placed in a desiccator for 48 h at room temperature. Samples were analysed with a Carlo Erba Instruments EA1108 elemental analyser using an acetanilide standard as reference. In the case of coccolithophorids, 2 extra samples were taken and acidified with HCl fumes prior to analysis, to remove the carbon present as calcium carbonate. The elemental analysis procedure measures all carbon present in the sample, both organic and inorganic (e.g. $CaCO_3$). Hence, OC in coccolithophores was determined from the difference between the carbon measured in non-acidified (all carbon is measured) and acidified (only OC is measured) samples. Carbon biomass of each species was calculated by dividing the concentration of particulate OC by cell abundance.

Dissolved and particulate primary production

Sampling was conducted during the exponential growth phase, an intermediate stage and during the stationary phase. Exudation rates were measured as described in detail by Marañón et al. (2004). For each phase, 100 ml aliquots from each culture were taken and placed in an acid-washed Pyrex glass bottle; 5 subsamples were taken: 3 light and 2 glass vials (20 ml) were filled with the sample, spiked with 1 µCi (37 KBq) of $NaH^{14}CO_3$, and incubated for 2 h. At the end of the incubation period, 2 aliquots of 5 ml from each incubation bottle were filtered through 0.2 µm polycarbonate filters (25 mm in dia-

meter) using low vacuum pressure (<50 mm Hg). After being acidified to a pH of ~2 with 100 µl of 50% HCl, filtrates were maintained overnight in open scintillation vials (20 ml) placed on an orbital shaker. After inorganic ^{14}C removal, 15 ml of high sample capacity scintillation cocktail was added to each filtrate. Filtrates were stored in the dark until counting. The inorganic ^{14}C present in the filters was removed by exposing them to concentrated HCl fumes for 12 h. The filters were then placed in 5 ml scintillation vials to which 4 ml of scintillation cocktail were added. The radioactivity of each sample was determined using a Tri-Carb 3100TR scintillation counter. To calculate the rates of dissolved and particulate carbon production, the black bottle DPMs (disintegrations per minute) were subtracted from the light bottle DPMs for correction of any non-photosynthetic ^{14}C incorporation; possible errors were due to organic contamination of ^{14}C stocks, or incomplete removal of inorganic ^{14}C from the filtrates. As shown by Markager (1998), most of the ^{14}C-signal in black bottle samples during short (<3 h) incubations arises from incomplete removal of inorganic ^{14}C during acidification as well as ^{14}C adsorption onto particles. Previous experiments using the same protocol indicated that Time 0 samples have similar DPM counts to those obtained from dark bottle incubations (Maranón et al. 2004), which indicates that the ^{14}C signal in dark bottle samples does not represent a biological process of CO_2 fixation. Thus, failing to subtract the black bottle DPM counts may result in severe overestimation of the real rates of PP, as concluded also by Banse (1993). The DPM count in the filtrates from the light bottle was always in the order of several hundreds, and the light to dark bottle DPM count ratio was always >2. We used a constant value of 25700 mg C m^{-3} for the concentration of dissolved inorganic carbon. To the extent that different species had different growth rates, it is conceivable that equilibration of the labelled carbon inside the cells may have proceeded at different paces in different cultures. However, both small (e.g. <10 µm^3 in cell volume) and large (>10000 µm^3) species had similar growth rates, which means that the overall size-scaling slope for the rates of dissolved and particulate PP should not have been affected by differences in the time required for isotopic equilibration.

RESULTS

The percentage of extracellular release was on average ~2% of total carbon fixation (Table 2), ranged from 0.3 to 10% (among species and growth phases) and was not correlated to cell size (Fig. 1). The carbon-specific exudation rate, which took a mean value of 0.001 h^{-1} (±0.001 SD), and the percentage of extracellular release did not show significant differences among growth phases (Kruskal-Wallis *H*-test: p = 0.91 and p = 0.51, respectively).

To assess how the percentage of extracellular release varied with cell size and among species, all data were grouped in different size classes (<2, 2 to 20 and >20 µm in equivalent spherical diameter), and also according to taxonomic affiliation (diatoms, dinoflagellates, coccolithophorids, cyanobacteria and others). The lowest percentage of exudation was

Table 2. Percentage of extracellular release (PER) of dissolved organic carbon measured for each phytoplankton species during the 3 growth phases. Further taxonomic details, see Table 1

Species	N	PER (%)			95% conf. int.	
		Mean	SD	Median	Lower	Upper
S. costatum	3	0.30	0.17	0.30	−0.13	0.72
T. rotula	3	0.39	0.11	0.39	0.10	0.67
P. tricornutum	3	0.31	0.03	0.32	0.23	0.38
T. weissflogii	3	0.91	0.90	0.52	−1.32	3.13
M. nunmuloides[a]	1	1.25				
C. radiatus	3	1.30	0.19	1.22	0.82	1.77
C. wailesii	3	3.53	1.68	3.38	−0.65	7.71
D. brightwelii	3	2.26	0.82	2.13	0.21	4.30
P. reticulatum	3	1.68	1.07	1.08	−0.98	4.34
A. sanguinea	3	10.37	2.62	10.36	3.86	16.88
A. minutum	3	4.23	2.70	3.54	−2.48	10.95
A. tamarense	3	3.13	2.32	3.54	−2.62	8.88
E. huxleyi	3	0.41	0.12	0.47	0.12	0.70
G. oceanica	3	1.83	0.66	1.75	0.19	3.47
C. leptoporus	3	0.95	0.47	0.83	−0.21	2.12
I. galbana	3	0.92	0.78	0.63	−1.03	2.87
P. lutheri	3	1.03	0.33	0.89	0.21	1.86
N. gaditana	3	1.14	0.27	1.16	0.46	1.82
M. pusilla	3	9.14	1.67	8.39	4.99	13.28
O. tauri	3	1.69	0.87	1.84	−0.46	3.84
Synechococcus sp.	3	1.41	0.61	1.39	−0.11	2.93
Prochlorococcus sp.	3	1.92	0.76	1.78	0.03	3.80
All data	64	2.31	2.81	1.32	1.61	3.01

[a]Data available only for the stationary phase

observed in nanophytoplankton (PER < 2% in the 3 growth phases) and also in coccolithophorids, during exponential and intermediate phases (mean PER = 0.8 and 0.9%, respectively) (Fig. 2); the highest percentages were calculated for micro- and picophytoplankton (<2.0 µm) (Fig. 2).

Although the percentage of exudation varied markedly among phylogenetic groups and size classes, the differences were not significant (Kruskal-Wallis *H*-test: $p > 0.05$ for each growth phase in both cases). The higher percentages of exudation within the dinoflagellates (average PER > 4%) and the >20 µm size class (average PER > 2%) (Fig. 2) was due to the high values of *Akashiwo sanguinea* (mean PER = 10.4%), whereas those found in the <2.0 µm size class were due to the flagellate *Micromonas pusilla* (mean PER = 9.1%) (Table 2, Fig. 2). Even though there were no significant differences in PER among phylogenetic groups, highly significant differences existed between species (Kruskal-Wallis *H*-test: $p < 0.001$).

Both cell volume and cell biomass were very good predictors of the cell-specific exudation rate (Table 3, Fig. 3). The slope of the log-log relationship between the exudation rate and cell volume ($b = 0.95$) and cell biomass ($b = 1.08$) was significantly higher than 0.75 in both cases (*t*-test: $p < 0.01$) and not significantly different from 1.0 (*t*-test: $p > 0.05$) (Table 3, Fig. 3); this pattern remained during the 3 different growth phases (Table 3). Hence, our results indicate that OC exudation does not scale allometrically either with cell biovolume or cell biomass, but scales isometrically. Cell carbon scaled with cell biovolume with an exponent of 0.88 (i.e. $C \propto V^{0.88}$; data not shown), and, given that exudation rate scaled with V with an exponent of 0.95 ($M \propto V^{0.95}$), the relationship between exudation rate and biomass presented an even higher exponent. The 95% confidence intervals revealed no significant differences in the size-scaling exponent of exudation rate between growth phases (Table 3), which highlights the robustness of these patterns.

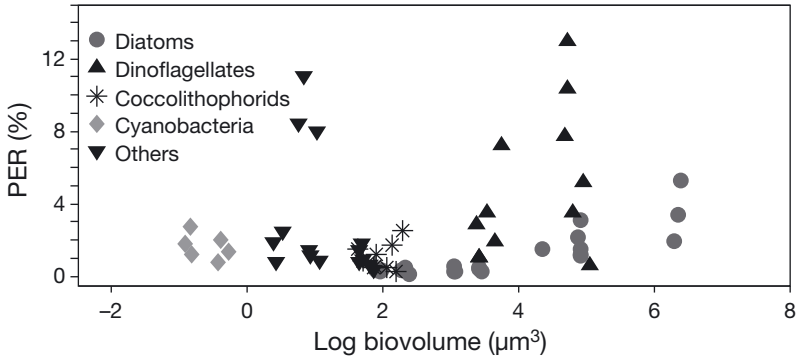

Fig. 1. Relationships between the percentage of extracellular release (PER) and cell size measured as cell biovolume (µm³) for 3 different growth phases (exponential, intermediate and stationary)

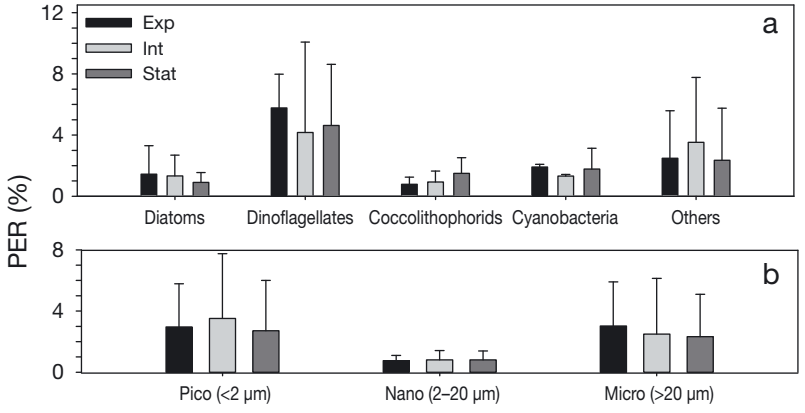

Fig. 2. Mean (±SD) percentage of extracellular release (PER) of (a) different phytoplankton groups and (b) different size classes measured throughout 3 growth phases (exponential [Exp], intermediate [Int] and stationary [Stat])

DISCUSSION

When microalgae experience uncoupling between carbon fixation and growth rate (due to nutrient deficiency), strategies such as exudation of dissolved organic compounds might help cells to cope with the 'excess carbon' obtained during photosynthesis (Wood & Van Valen 1990, Berman-Frank & Dubinsky 1999). Previous studies on exudation in phytoplankton cultures suggesed that as growth rates decline (at the stationary stage, when nutrients become limiting), exudation of dissolved OC increases (Myklestad 1977, Zlotnik & Dubinsky 1989, Obernosterer & Herndl 1995). However, in these studies only a small number of phytoplankton species were analysed, and only a few of them included >1 measurement during the phytoplankton growth cycle. In this study we obtained data from 3 different growth phases and 22 phytoplankton species, providing evidence that exudation remains constant between growth phases.

All data for the stationary phase were obtained when nitrate concen-

Table 3. Percentage of extracellular release (PER; mean ± SD) and parameters of the size-scaling relationships for exudation rate at 3 growth phases. Reduced major axis regression was used to determine the relationship between log cell volume (μm^3 $cell^{-1}$) or cell biomass (pgC $cell^{-1}$) (independent variables) and log exudation rate (pgC $cell^{-1}$ h^{-1}) (dependent variable). Bootstrap confidence limits (95%) for the intercept and slope in parentheses. p-values refer to the comparison between the size-scaling slope of the exudation rate with expected values of 1.0; at an expected slope of 0.75, $p < 0.001$ in all cases

Growth phase	Volume (μm^3)	Biomass (pgC)
Exponential		
(PER = 2.5 ± 2.6%; n = 21)		
Intercept	−3.9 (−4.3, −3.4)	−3.2 (−3.4, −2.9)
Slope	0.94 (0.8, 1.1)	1.06 (0.9, 1.1)
r^2	0.90	0.95
p	0.36	0.24
Intermediate		
(PER = 2.3 ± 3.4%; n = 21)		
Intercept	−4.0 (−4.4, −3.7)	−3.3 (−3.5, −3.1)
Slope	0.92 (0.8, 1.0)	1.04 (0.9, 1.1)
r^2	0.94	0.96
P	0.13	0.39
Stationary		
(PER = 2.1 ± 2.6%; n = 22)		
Intercept	−4.4 (−4.8, −4.0)	−3.6 (−3.9, −3.3)
Slope	1.0 (0.9, 1.1)	1.13 (1.0, 1.2)
r^2	0.93	0.95
P	0.90	0.03
All data		
(PER = 2.3 ± 2.8%; n = 64)		
Intercept	−4.1 (−4.3, −3.9)	−3.4 (−3.5, −3.2)
Slope	0.96 (0.9, 1.0)	1.08 (1.0, 1.1)
r^2	0.91	0.95
P	0.20	0.01

tration in the bulk medium was near or below the detection limit and the carbon-specific carbon fixation rate had the lowest values. However, it is possible that the cells' ability to store nitrogen intracellularly may prevent a strong nutrient deficiency, which tends to be associated with high PER values (Obernosterer & Herndl 1995).

Differences in exudation among species (Hellebust 1965, Beardall & Raven 2001) might be important even within one single class (diatoms) (Finkel 1998, Beardall & Raven 2001). Our results agree with previous reports indicating that not only the quality but also the quantity of dissolved organic matter exuded by algae may change depending on the species (Wolter 1982, Romera-Castillo et al. 2010). However, at the same time, the percentage of exudation for a given species will vary according to the conditions that the population has previously experienced (Bertilsson et al. 2005, Borchard & Engel 2012).

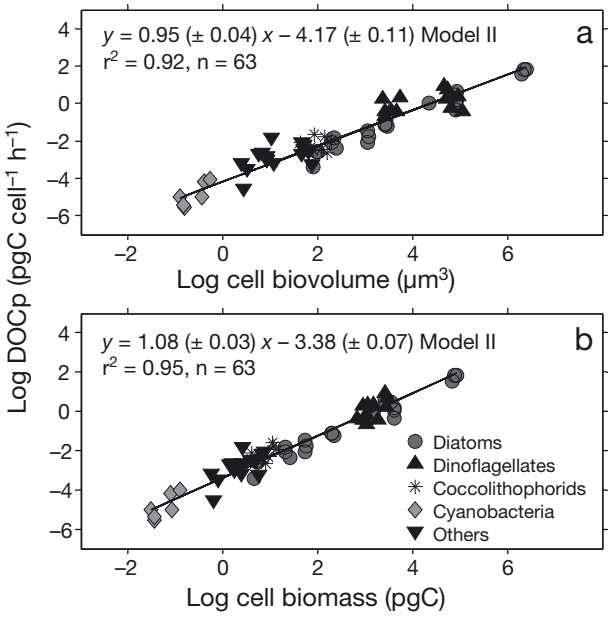

Fig. 3. Relationship between log cell-specific exudation rates (DOCp) and log cell size measured as (a) biovolume (μm^3) and (b) biomass (pg C $cell^{-1}$) for different phytoplankton groups throughout the 3 growth phases

The larger surface to volume quotient and the intrinsically thinner diffusion boundary layer which facilitates nutrient uptake in smaller cells (Chisholm 1992, Raven 1998) could also favour a higher diffusion (exudation) of low-molecular weight compounds through the cell membrane. Malinsky-Rushansky & Legrand (1996), using cultures of 3 different phytoplankton species (*Navicula filata*, *Pavlova lutheri* and a *Chlorella*-like picoeukaryote), with a size range from 1.5 to 8 µm in diameter, showed that the percentage of exudation was higher in the picoeukaryote-like cells (12.6%). In contrast, our results obtained from a much wider range in size (0.1 to 10^6 μm^3 in cell volume) demonstrate that the percentage of exudation has no relationship with cell size. This counterintuitive result indicates that exudation of recently synthesised metabolites, as measured with the ^{14}C-uptake technique over short time scales, depends on the rate of total carbon fixation and not on diffusion processes. As we discuss below, the rate of mass-specific photosynthetic carbon fixation, as well as other metabolic rates, seem to be largely independent of cell size in phytoplankton.

Recently, the applicability of Kleiber's ¾ power rule has been assessed for unicellular organisms (Johnson et al. 2009, DeLong et al. 2010, Finkel et al. 2010) and specifically for phytoplankton (Marañón et al. 2007, Marañón 2008, Huete-Ortega et al. 2011).

These studies all suggest a departure from the ¾ power relationships toward isometry in the metabolic rate of photosynthetic unicells. Here we provide robust data indicating that this departure also holds true for a metabolic loss process such as exudation, which scales isometrically with cell size. Thus, on a biomass- or biovolume-specific basis, the relative importance of dissolved PP is largely size-independent in marine phytoplankton.

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