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Tissue and skeletal changes in the scleractinian coral *Stylophora pistillata* Esper 1797 under phosphate enrichment

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ABSTRACT

Long-term phosphate enrichments (0, 0.5, and 2.5 μmol L^{-1}; 4 to 11 weeks) were used to assess a possible limitation in phosphorus of zooxanthellae and to complement data on the effect of phosphate enrichment on calcification and elemental composition of the tissue in the scleractinian coral *Stylophora pistillata*. Phosphate addition mainly affected the coral symbionts. Indeed, at 2.5 μmol L^{-1} P-enriched, zooxanthellae had a greater photosynthetic efficiency, their intracellular carbon and nitrogen contents increased by 70% and their phosphorus content by 190%, while their specific growth rate increased by 18%. C:P and N:P ratios in zooxanthellae were much higher than the Redfield ratios advocated for nutrient-repleted phytoplankton, and decreased with phosphate enrichment. Collectively, these results suggest a phosphorus limitation of the zooxanthellae growth *in hospite*. However, the increase in zooxanthellae specific growth rate did not lead to the building of a higher symbiont density, as zooxanthellae growth just matched the tissue and skeletal growth of the enriched corals. Benefits of phosphate supplementation were thus not substantial enough to lead to the building of higher zooxanthellae density and to their balanced growth, which suggests that symbiont growth was likely limited by another nutrient as well, probably nitrogen. At the host level, there were no changes in the elemental composition or in the protein levels, while skeletal growth rate increased by 31% between unenriched and 2.5 μmol L^{-1} P-enriched corals. Phosphate-enriched corals also incorporated 1.7 times more phosphorus into their skeleton than did unenriched corals. These results evidenced that zooxanthellae and the skeleton are the two accumulation sites of inorganic phosphorus within the symbiotic association.

1. Introduction

Coral reefs generally develop in clear, oligotrophic waters, where nutrient, and especially phosphorus levels are among the lowest in the world (Furnas, 1991; Szmant, 2002). Phosphorus is however an essential nutrient because it enters into the composition of many biological molecules (deoxyribonucleic and ribonucleic acids, phospholipids), including adenosine triphosphate (ATP), which has a major role as energy supplier. Hence, around most atolls and barrier reefs, its low availability may limit coral calcification and photosynthesis (Annis and Cook, 2002; D'Elia, 1977; Jackson and Yellowlees, 1990), and phosphorus is therefore taken up by corals at a relatively fast rate (D'Elia, 1977; Godinot et al., 2009; Sorokin, 1992). Conversely, many coastal reefs are subject to increased phosphorus levels, due to continuous nutrient release from sewage discharges, rainfall, rivers and ground waters. Seawater eutrophication was shown to strongly affect the community equilibrium, usually to the detriment of corals (Bell and Tomascik, 1993; McCook, 1999; Tomascik and Sander, 1985), which are often replaced by benthic algae (Bellwood et al., 2004; McCook, 1999; Smith et al., 2001).

Due to the important role of phosphorus in reef ecosystems, both under oligotrophic and eutrophic conditions, it is essential to understand the effect of phosphate enrichment on the coral-zooxanthellae symbiosis, as well as the fate of phosphorus in this association. Previous work on the subject has mainly focused on the effect of nitrogen enrichment, alone or in combination with phosphorus, on coral physiology (more than twenty studies, from Kinsey and Davies, 1979; Koop et al., 2001; Kumarsingh et al., 1998; LaVigne et al., 2008, 2010; Mason et al., 2007; McCook, 1999; Miller and Yellowlees, 1989; Murphy and Riley, 1962; Muscatine et al., 1989; Rasmussen, 1988; Rasmussen and Cuff, 1990; Redfield, 1934; Rees, 1991; Renegar and Riegl, 2005; Reynaud et al., 2007; Rodolfo-Metalpa et al., 2010; Smith et al., 2001; Snidvongs and Kinzie, 1994; Sorokin, 1992; Stambler et al., 1991; Steven and Broadbent, 1997; Stimson and Kinzie, 1991; Szmant, 2002; Tambutté et al., 1995; Tanaka et al.,

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2007). However, the effect of phosphorus enrichment alone on coral physiology has not been extensively investigated (Table 1), mainly because physiological parameters studied are scattered among different works, coral species and conditions. Moreover, studies have reported conflicting results, difficult to interpret because each parameter has been considered separately from the others. Concerning calcification for example, phosphate enrichment was found to decrease it (Ferrier-Pagès et al., 2000; Koop et al., 2001; Rasmussen and Cuff, 1990; Renegar and Riegl, 2005), increase it (Koop et al., 2001; Rasmussen and Cuff, 1990; Steven and Broadbent, 1997), or have no effect (Koop et al., 2001; Stambler et al., 1991) depending on the enrichment, the species, and the technique used for calcification measurement. The same is true for the C:P ratios of the tissue, although results come from only three studies (Table 2): while Belda et al. (1993) reported a decrease in C:P ratios for the giant clam *Tridacna gigas* after a 3-month phosphate enrichment of 2–10 μmol L^{-1}, two other studies (Snidvongs and Kinzie, 1994; Stambler et al., 1991) found an increase in C:P ratios in the coral *Pocillopora damicornis* with phosphate enrichments of 0.5–2 μmol L^{-1}.

Consequently, the scleractinian coral *Stylophora pistillata* was maintained 4 to 11 weeks under phosphate enrichment to i) assess a possible limitation in phosphorus of zooxanthellae growth and/or coral primary productivity, ii) complement data on the effect of phosphate enrichment on calcification and elemental composition of the tissue; and to iii) determine the accumulation sites of phosphorus within the symbiosis, among animal tissue, zooxanthellae, and skeleton. To our knowledge, the extent of phosphorus accumulation in the skeleton versus external phosphorus enrichment has not yet been experimentally assessed for tropical corals, although they have been reported to concentrate phosphorus relative to calcium in phosphate-rich environments (Dodge et al., 1984; Kumarsingh et al., 1998; LaVigne et al., 2008, 2010). From all the above data, we calculated the percentage of phosphorus accumulation within each compartment (animal, zooxanthellae, skeleton) of phosphate-enriched corals relative to unenriched corals.

2. Materials and methods

2.1. Organisms and culture conditions

Colonies of the zooxanthellate coral *S. pistillata* were obtained from the Red Sea, and maintained in natural seawater under controlled conditions (26 °C, salinity of 38) and under an irradiance (photosynthetic active radiation, PAR) of 150 μmol m^{-2} s^{-1},with a 12 h:12 h dark:light cycle. Light was supplied by 400 W Hydrargyrum Quartz Iodide lights (HQI), and measured using a spherical quantum sensor (LiCor LI-193). 1.5 months before the experiment, 120 nubbins (i.e. branch tips) were prepared by cutting 12 branches (2.5 ± 1.0 cm long and 0.6 ± 0.3 cm in diameter) of ten parent colonies with pliers (Tambutté et al., 1995). Nubbins were attached to nylon filaments and suspended in aquaria until tissues fully covered the skeleton. They were then randomly assigned to six 30-L aquaria, and allowed to acclimate for a week. Temperature was regulated in each aquarium at 26.0 ± 0.1 °C. After the first week of acclimation, two aquaria were randomly assigned to each of the 3 following treatments: 0 (unenriched control), 0.5, and 2.5 μmol L^{-1} phosphate enriched, considering that P concentration in the natural seawater was almost undetectable during the whole experiment (<0.05 μmol L^{-1}). The 0.5 μmol L^{-1} enrichment represented a phosphate concentration which has been reported on some reefs under eutrophication (Kinsey and Davies, 1979), whereas the 2.5 μmol L^{-1} enrichment was used to highlight the effect of phosphate on coral physiology. Concentrated stock solutions of phosphate (as KH_2PO_4, 2.0 and 10.0 μmol L^{-1} respectively) were maintained in 2 separate 50-L tanks kept in the dark and were continuously pumped with a peristaltic pump to the enriched tanks. Pre-heated (24 ± 0.2 °C) natural seawater with trace phosphate (<0.05 μmol L^{-1}), nitrate (<0.4 μmol L^{-1}), and ammonium (<0.5 μmol L^{-1}) concentrations was also continuously pumped into the aquaria, in order to obtain final phosphate concentrations of ca. 0, 0.5, or 2.5 μmol L^{-1}. The seawater flow rate was ca. 1.2 L h^{-1}. Phosphate concentrations were regularly checked in the experimental tanks according to Murphy and Riley

Table 1
Summary of coral responses to phosphate in the literature. "+" indicates a positive effect, "−" a negative effect, and "0" an absence of effect.

Species	Experimental design		Parameters measured and effect of P								References
	In situ or laboratory	Phosphate enrichment (in μmol L^{-1})	Prot	Zoox	Chl	Growth	Photo-synthesis	Respiration	Elemental ratios of tissues	Others	
Acropora longicyathus	ENCORE reef experiment	4			+	+/0					Bucher and Harrison (2000)
Stylophora pistillata	Laboratory enrichments	2.8				−	+				Ferrier-Pagès et al. (2000)
Acropora longicyathus, *Acropora palifera*, *Acropora aspera*, *Pocillopora damicornis*, *Stylophora pistillata*	ENCORE reef experiment	2.5–5.1		+/0	0	+/0/−	+	+		+ (lipids), + / 0 / − (reproduction), + (NH_4 uptake)	Koop et al. (2001)
Stylophora pistillata	Laboratory enrichments	2	0	0	0				0 (C:N)	− (lipids), 0 (mitotic index)	Muscatine et al. (1989)
Acropora formosa	Laboratory enrichments	1,2 and 4								− (Sr in skeleton, thinning of skeletal walls, void enlargement)	Rasmussen (1988)
Acropora formosa	Laboratory enrichments	2, 4, and 8				+/−				− (Sr in skeleton)	Rasmussen and Cuff (1990)
Acropora cervicornis	Laboratory enrichments	2 and 4				−					Renegar and Riegl (2005)
Pocillopora damicornis	Laboratory enrichments	1 and 2			0				+ (C:P)		Snidvongs and Kinzie (1994)
Pocillopora damicornis	Laboratory enrichments	0.5 and 2	0	0	0	0			+ (C:P)		Stambler et al. (1991)
Acropora palifera	ENCORE reef experiment	4		+	0	+					Steven and Broadbent (1997)
Stylophora pistillata	Laboratory enrichments	0.5 and 2.5	0	0	−	+	+	+	− (N:P)	+ (P/Ca), + (Mg/Ca), − (Sr/Ca) in skeleton	This study

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Table 2
Elemental ratios of C, N, and P in the coral *Stylophora pistillata* exposed to 0, 0.5, and 2.5 μmol L^{-1} phosphate-enriched seawater for 11 weeks; and summary of data in the literature on C:P and N:P ratios from unenriched and phosphate-enriched corals and clams. Results from the present study are expressed as the mean ± SE of 6 nubbins randomly pooled by groups of two (3 groups per treatment).

P enrichment	Whole organism			Animal			Zooxanthellae			Reference/species
	C:P	N:P	C:N	C:P	N:P	C:N	C:P	N:P	C:N	
Unenriched				433 ± 47	66 ± 6	6.6 ± 0.2	551 ± 58	87 ± 8	6.3 ± 1.3	This study
0.5 μmol L^{-1}				417 ± 40	65 ± 6	6.4 ± 0.4	520 ± 26	73 ± 17	7.1 ± 1.7	*Stylophora pistillata*
2.5 μmol L^{-1}				389 ± 73	58 ± 10	6.8 ± 0.1	411 ± 55	56 ± 7	7.3 ± 1.2	
Unenriched							355			Snidvongs and Kinzie (1994)
1.2 μmol L^{-1}							500			*Pocillopora damicornis*
0.1 μmol L^{-1}	233									Stambler et al. (1991)
0.5 μmol L^{-1}	416									*Pocillopora damicornis*
2.0 μmol L^{-1}	375									
Unenriched				172 ± 35	26.6 ± 4.4	6.4 ± 0.4	365 ± 49	21.4 ± 3.1	19.7 ± 4.0	Muller-Parker et al. (1994)
										Pocillopora damicornis
Unenriched	130	22					279 ± 18	52 ± 1		Belda et al. (1995)
2.0 μmol L^{-1}	100	17					256 ± 15	46 ± 1		*Tridacna gigas*
5.0 μmol L^{-1}	75	13					376 ± 101	66 ± 14		
10.0 μmol L^{-1}	95	16					238 ± 20	39 ± 4		

(1962) and did not vary by more than ± 0.05 μmol L^{-1} throughout the experiment. The pH_T was also checked and did not change by more than 0.01. Corals were not fed during the 11 weeks enrichment, in order to control phosphorus levels.

2.2. Physiological measurements

Skeletal growth rate was assessed using the buoyant weight technique (Jokiel et al., 1978) on 10 nubbins per treatment (5 nubbins per aquarium) one week prior to the beginning of the experiment, the first day of incubation and after one, two, four, six and eight weeks of incubation. Since nubbins were entirely covered with tissue, no algal growth was possible on the skeleton. Moreover, due to the small tissue thickness and incubation length, increase in tissue weight per cm^2 was negligible, as demonstrated by Davies (1989), so that only skeletal weight was monitored. Specific growth rate (G) was expressed as the slope of the regression line relating the natural logarithm of coral nubbin weight (mg) versus the experimental time (days).

Net photosynthesis (P_n) and respiration (R) were measured after 4 and 7 weeks (T_4 and T_7), on 5 randomly selected nubbins per treatment (modified after Ferrier-Pagès et al., 2000) under dark or light conditions (PAR of 0 and 150 μmol m^{-2} s^{-1}). Data were normalized either to the total chlorophyll content (according to Godinot et al., 2009) or to the surface area of the nubbins (using the wax technique described in Stimson and Kinzie, 1991). Protein content was also determined on the 5 nubbins used for photosynthesis measurements, after extraction in 1 mol L^{-1} NaOH for 30 min at 90 °C (measured using the commercially available BC Assay Interchim kit, according to Hoogenboom et al., 2010). Zooxanthella density was assessed at T_4 and T_7 on 4 additional nubbins for each incubation time and condition as in Godinot et al. (2009). The mean areal zooxanthella density was used to calculate mean photosynthetic and respiration rates per zooxanthella, as well as mean chlorophyll content per zooxanthella. Change in the total number of zooxanthellae between T_4 and T_7 was used to determine the zooxanthellae specific growth rate (μ) in the three P-conditions, using the equation:

$$\mu = \frac{1}{Z}\frac{dZ}{dt}$$

where Z is the number of zooxanthellae cells and t the incubation length (Falkowski et al., 1984, 1993).

In order to examine the photosynthetic efficiency of PSII, the maximum quantum yield of photosystem II (dark F_v/F_m of PSII) was measured at T_7 on 5 randomly selected nubbins per treatment using pulse-amplitude-modulated chlorophyll fluorometry (diving PAM, Waltz; method described in Rodolfo-Metalpa et al., 2010).

2.3. Changes in tissue and zooxanthellae C:N:P

The total carbon:nitrogen:phosphorus (C:N:P) ratios were also measured, after 11 weeks, in the animal tissue and zooxanthellae of 6 nubbins per treatment. Coral tissue was separated from the skeleton in 10 mL 2 μm-filtered seawater, using an Air-Pik. Tissues from two coral nubbins were pooled together to improve measurement precision. Therefore, three replicates were obtained by using six nubbins. Tissues were homogenized using a tissue grinder. A 500-μL sample was taken for zooxanthellae counts, and bare skeletons were used for surface area measurements. For each sample, zooxanthellae and animal tissues were separated using three successive centrifugations (1300 g, 25 °C, 5 min). The success of the separation was visually checked using an optical microscope. Animal and zooxanthellae samples were each equally divided into two aliquots for the determination of total carbon and nitrogen content on one side and total phosphorus content on the other side. Carbon and nitrogen contents were measured on freeze-dried samples using a CHN elemental analyzer (Flash EA Thermoquest). 4 tubes containing freeze-dried filtered seawater (FSW) served as controls. Aliquots used for phosphorus assays were ashed (2 h, 500 °C) in Pyrex containers and subsequently acidified (with hot HCl 0.2 mol L^{-1}; modified after Snidvongs and Kinzie, 1994). Phosphorus was then measured following the technique of Murphy and Riley (1962). Three control bottles containing FSW were also run as a control for seawater phosphorus concentration. Previous testing of this technique yielded a 99.3% recovery rate for both inorganic (KH_2PO_4) and organic (methylumbelliferyl P) phosphorus.

2.4. Multiple element/calcium ratios in the skeleton

Analyses of ^{26}Mg, ^{31}P, ^{43}Ca and ^{84}Sr were determined by inductively coupled plasma mass spectrometry (ICP-MS) using the multi-collector Axiom in single collection mode at Lamont-Doherty Earth Observatory, following the standard addition method (Vandecasteele and Block, 1997) to correct for matrix effects. Approximately 50 mg of coral powder was carefully scraped off the outermost portion (2 to 5 mm-thick portion at the tip of apexes) of the coral skeletons from the three 11-week treatments. Three skeletons from each phosphate treatment were pooled together to get one measurable sample. The aragonite powder was soaked in H_2O_2 (30%, ~8.9 mol L^{-1}) for two days, then placed at 80 °C for 30 min, in order to remove any residual organic matter. The samples were subsequently dried in an oven at 50 °C for 6 h and dissolved overnight with double distilled 1 mol L^{-1}

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HNO_3. Working solutions were further diluted in 0.45 mol L^{-1} HNO_3 (~2%) resulting in a final calcium concentration of ~80 μg mL^{-1}. Procedural blank solutions were made with the same vials and acid used for the treatment of the samples. A multielemental stock standard mixture was prepared gravimetrically with 0.45 mol L^{-1} HNO_3 and High-Purity Standards (Charleston, SC), mixed in appropriate concentrations to match the typical composition of coral skeletons. A four point standard curve was prepared by adding increasing volumes of the stock solution to three pre-cleaned vials containing a constant volume of the unknown solution (sample "control") and 0.45 mol L^{-1} HNO_3. A drift correction was performed by analysing an indium-spiked mixed solution every five samples and using a linear interpolation for all the elements. Also all the working solutions were spiked with indium as an internal standard to further correct for the instrumental drift. Analytical reproducibility based on the RSD (1σ) of six analyses of the *Porites* coral standard JCp-1 (Geological Survey of Japan) (run as an unknown) are 0.7% for Mg/Ca, 5% for P/Ca and 0.3% for Sr/Ca ratios, and 6% for P concentration.

2.5. Statistical analyses

Differences among replicated tanks were tested using *t* tests on skeletal growth rate measurements. No significant differences were found at any of the sampling dates (*t* tests, all $p>0.05$), thus corals were pooled together for other statistical analyses.

Using the software GraphPad Prism (version 5.0), we compared the slopes of the regression lines calculated for growth, in order to look for possible statistically significant differences between the three phosphate enrichments. The global effect of time and treatment on photosynthesis, chlorophyll, protein, and zooxanthellae content was examined using repeated measures ANOVAs (StatView version 5.0). For significant effects, one-way ANOVAs and protected least significant difference (PLSD) Fisher post hoc tests were performed at each sampling time. Equality of variances and normality of residuals were tested using Levene and Shapiro-Wilk tests (Statgraphics Centurion version 15). Dark F_v/F_m at T_7 were compared using a one-way ANOVA followed by PLSD Fisher post hoc tests. As the normality assumption was not met for carbon, nitrogen, and phosphorus contents of coral tissues, these data were compared using non parametric Kruskal–Wallis tests, followed by Man–Whitney post hoc tests. All results are presented as mean values ± standard error except for elemental ratios of the skeleton, for which error bars represent the analytical reproducibility (1 σ, RSD).

3. Results

Repeated measures ANOVAs showed that there was no effect of time or treatment (P enrichment) on the zooxanthellae density ($F=3.02$, $p=0.12$ for time; and $F=0.42$, $p=0.96$ for treatment) and protein content ($F=0.93$, $p=0.79$ for time; and $F=1.15$, $p=0.35$ for treatment). Mean concentrations were $3.7\pm0.1\times10^6$ zooxanthellae cm^{-2} and 1.5 ± 0.1 mg protein cm^{-2} in all samples. Conversely, there was an effect of phosphate enrichment on the chlorophyll content, with a 44% and 58% decrease in the 0.5 and 2.5 μmol L^{-1} P-enriched corals as compared to the unenriched corals after 7 weeks (one-way ANOVA, PLSD Fisher tests, both $p<0.001$; Fig. 1A). Chlorophyll content per zooxanthellae therefore decreased from 4.3 ± 0.7 pg Chl $zoox^{-1}$ in unenriched corals to 2.3 ± 0.4 and 1.8 ± 0.2 pg Chl $zoox^{-1}$ in 0.5 and 2.5 μmol L^{-1} P-enriched corals. As a consequence of this decrease, rates of respiration (R) and gross photosynthesis (P_g) normalized to chlorophyll content significantly increased for enriched corals (Fig. 1B; PLSD Fisher tests, $p=0.002$ and 0.007 for differences between unenriched and 2.5 μmol L^{-1} P-enriched corals respectively for the respiration or gross photosynthesis rates at T_7). These rates however didn't significantly

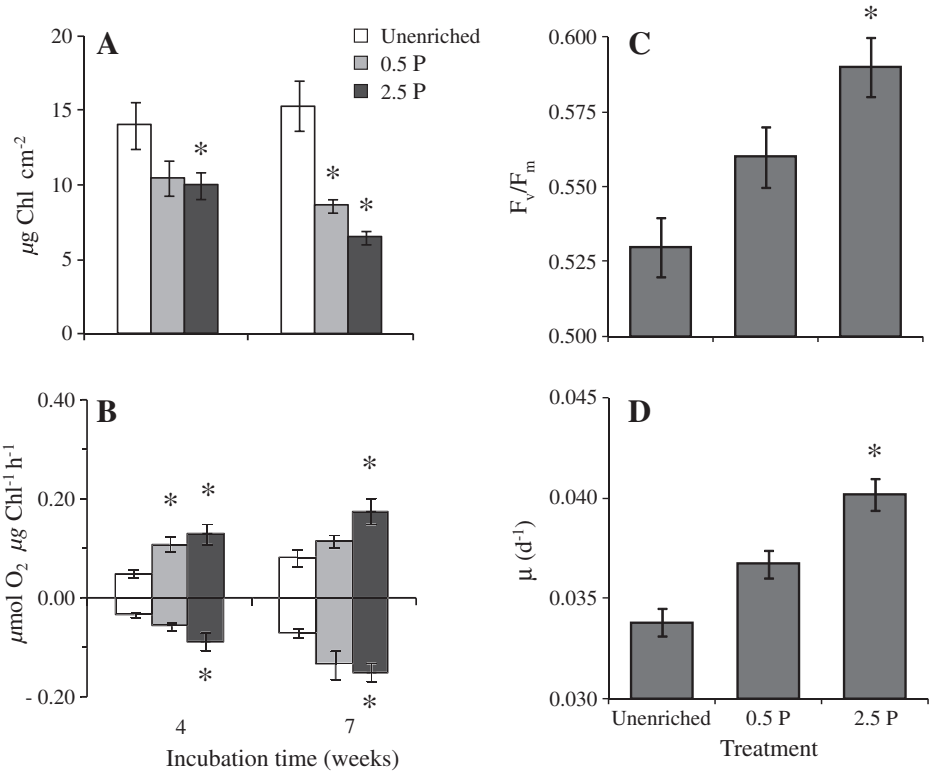

Fig. 1. Zooxanthellae parameters of *Stylophora pistillata* nubbins enriched with 0 (unenriched), 0.5, and 2.5 μmol L^{-1} phosphate. (A) Areal chlorophyll content measured after 4 and 7 weeks of enrichment. (B) Respiration (R, below the x-axis) and net photosynthesis (P_n, above the x-axis) after 4 and 7 weeks of enrichment. Data are normalized per chlorophyll content. (C) Maximum quantum yield of photosystem II (dark F_v/F_m of PSII) measured after 7 weeks of enrichment. (D) Calculated specific growth rate of the zooxanthellae population. Data are presented as the mean ± SE of 5 nubbins per treatment. Data significantly different from the unenriched treatment are indicated by an asterisk for each parameter and sampling date.

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change with other normalizations (all $p>0.05$). After 7 weeks, they were equal to a mean value of 1.1 and -1.0 μmol O_2 cm^{-2} h^{-1} or 2.9 and -2.7 μmol O_2 $zoox^{-1}$ h^{-1} for P_n and R respectively. The photosynthetic efficiency of PSII was higher for 2.5 μmol L^{-1} P-enriched corals than for unenriched corals (PLSD Fisher test, $p=0.001$; Fig. 1C).

Specific growth rate, calculated as the slope of the regression line between the natural logarithm of the skeletal weight and the time (Fig. 2A), was 31% higher in the 2.5 μmol L^{-1} P-enriched corals ($1.27\pm0.04\%$ d^{-1}) than in the unenriched ones ($0.97\pm0.11\%$ d^{-1}; $F=7.77$, $p=0.006$). Specific growth rate was $1.11\pm0.09\%$ d^{-1} in the 0.5 μmol L^{-1} P-enriched corals, and was not significantly different than in the unenriched corals ($F=0.35$, $p=0.14$).

Surface area of the coral nubbins increased over the course of the experiment, from 2.8 ± 0.3 cm^2 on average at the beginning of the experiment, to 4.8 ± 0.4 and 6.5 ± 0.8 cm^2 on average after 4 and 7 weeks of phosphate enrichment respectively (Table 3). As nubbins grew, the animal tissue and symbiotic cells also grew. Between weeks 4 and 7 of the enrichment, 8.5, 9.2, and 10.0×10^6 zooxanthellae were formed in unenriched, 0.5, and 2.5 μmol L^{-1} P-enriched corals respectively, with specific the growth rate of the zooxanthellae population significantly increasing from 0.034 ± 0.001 to 0.040 ± 0.001 d^{-1} between unenriched and 2.5 μmol L^{-1} P-enriched corals (PLSD Fisher test, $p=0.007$; Fig. 1D).

In the animal compartment, there was no change in total areal carbon (65.3 ± 1.8 μmol C cm^{-2}), nitrogen (9.9 ± 0.3 μmol N cm^{-2}), and phosphorus (0.16 ± 0.01 μmol P cm^{-2}) contents, as well as in C:N:P ratios ($427:65:1\pm28:4:1$) after 11 weeks of incubation in the different treatments (Tables 2 and 4; Kruskal–Wallis tests, all $p>0.3$). In zooxanthellae, C, N, and P content increased in phosphate-enriched corals as compared to unenriched corals, both when normalized to the surface area (Mann Whitney post hoc tests, all $p<0.05$; Table 4) and to zooxanthella (Mann–Whitney post hoc tests, all $p<0.05$; Fig. 3). The increase in P was the highest (increase of 190% when normalized per zooxanthellae, Fig. 3). As a consequence, molar N:P ratios were significantly lower in the 2.5 μmol L^{-1} P-enriched zooxanthellae (Mann Whitney post hoc test, $p=0.49$; Table 2).

In the skeletal compartment, total phosphorus content was 0.90, 1.45 and 1.55 μmol P g^{-1} for unenriched, 0.5, and 2.5 μmol L^{-1} P-enriched corals respectively, i.e. 0.10, 0.17 and 0.21 μmol P cm^{-2} respectively. P/Ca ratios were 62% and 71% higher (±9%) and Mg/Ca ratios were 8% and 10% higher (±1.6%) in the 0.5 and 2.5 μmol L^{-1} P-enriched corals, respectively. Sr/Ca ratios were 0.90% lower (±0.28%) in the 2.5 μmol L^{-1} P-enriched corals than in the other corals (Fig. 2B).

The percentage of phosphorus accumulation in each compartment of P-enriched corals relative to unenriched corals after 11 weeks of incubation is presented in Fig. 4. An increase in ambient phosphate concentration resulted in a linear accumulation of phosphorus in the zooxanthellae, while a tendency towards saturation was observed in the skeleton, and no change occurred in the tissue.

Table 3
Evolution of the average surface area in the coral *Stylophora pistillata* exposed to 0, 0.5, and 2.5 μmol L^{-1} phosphate-enriched seawater for 7 weeks, and concomitant growth of zooxanthellae over the last three weeks of the enrichment. All data are presented as the mean ± SE of 4–10 nubbins per treatment.

		Unenriched	0.5 μmol L^{-1}	2.5 μmol L^{-1}
Mean surface area (cm^2)	T_0	2.78 ± 0.32	2.78 ± 0.32	2.78 ± 0.32
	T_4	4.64 ± 0.39	4.82 ± 0.45	5.04 ± 0.42
	T_7	6.10 ± 0.74	6.43 ± 0.79	6.82 ± 0.71
Zooxanthellae formed between T_4 and T_7 (10^6 cells)		8.53 ± 0.09	9.19 ± 0.12	9.97 ± 0.08

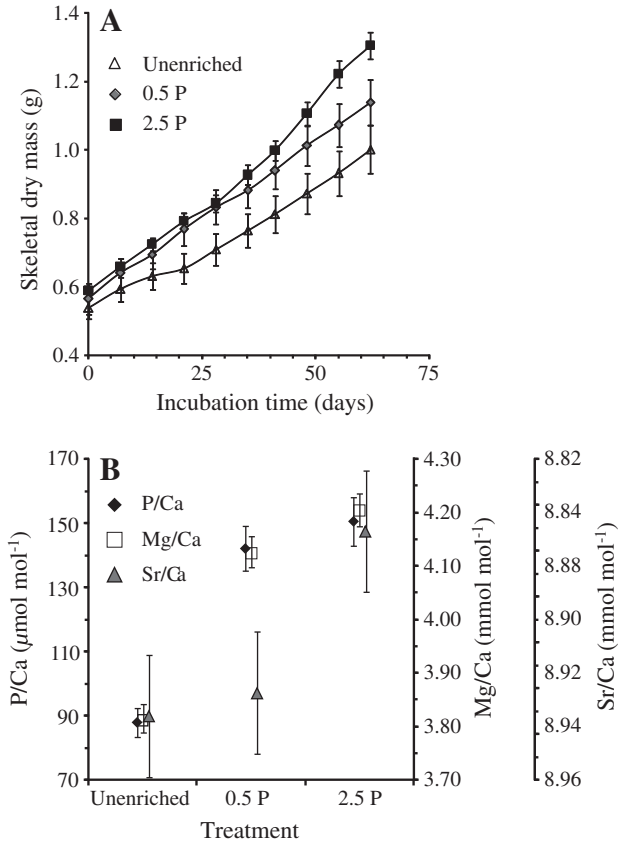

Fig. 2. Skeletal parameters of *Stylophora pistillata* nubbins enriched with 0 (unenriched), 0.5, and 2.5 μmol phosphate L^{-1}. (A) Evolution of dry mass over 8 weeks of enrichment under laboratory conditions. Each point represents the mean ± SE of 10 nubbins. (B) P/Ca, Mg/Ca and Sr/Ca ratios of nubbins enriched for 11 weeks. Data represent the ratios measured on three skeletons pooled together for each phosphate treatment. Error bars represent the analytical reproducibility based on the RSD (1σ) of six analyses of the *Porites* coral standard JCp-1 (Geological Survey of Japan). Note that the y-scale is inverted for Sr/Ca ratios.

4. Discussion

Overall, results obtained in this study have shown that phosphate enrichment mainly affected the coral symbionts, by decreasing their C:P and N:P ratios, while increasing their carbon, nitrogen, and phosphorus contents, as well as their specific growth rate, maximal photosynthetic efficiency of the PSII, and rate of photosynthesis normalized to chlorophyll content. Phosphate enrichment also affected the skeletal compartment, by increasing the skeletal growth and the P/Ca ratio. Conversely, few changes were observed in the animal host tissue.

Concerning the elemental composition of the symbionts, the C:P and N:P ratios were >400:1 and 50:1 respectively (Table 2), and were thus much higher than the Redfield ratios advocated for nutrient-repleted phytoplankton of 106:1 and 16:1 (Redfield, 1934), suggesting a nutrient limitation. The decrease in C:P and N:P with P-enrichment, together with the increase in the cellular phosphorus content, and in the specific growth rate of the symbiont population, suggest that there was a phosphorus-limitation of zooxanthellae growth *in hospite*, in agreement with the hypothesis of Belda et al. (1993), who reported a similar decrease in C:P ratios after phosphate enrichment in the giant clam *T. gigas* (Table 2). This limitation was partially alleviated by phosphate supplementation. Phosphorus-limitation of zooxanthellae growth was also hypothesized by Miller and Yellowlees (1989) based on physiological evidence related to cytoplasmic pH, as well as inorganic and organic phosphorus uptake activity of corals and their symbionts. The increase in the rates of photosynthesis per chlorophyll content and in the PSII photosynthetic efficiency after P-enrichment suggest that, in addition to the growth of the symbiont population, phosphorus might also have been a limiting factor for the many steps of the light–dark phases of zooxanthellae photosynthesis. Phosphorus is indeed needed in photophosphorylation as a component of ATP during the conversion of light

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Table 4
Total carbon (C), nitrogen (N), and phosphorus (P) content in the coral *Stylophora pistillata* exposed to 0, 0.5, and 2.5 μmol L^{-1} phosphate-enriched seawater for 11 weeks. Elemental contents are expressed in μmol cm^{-2} for carbon and nitrogen, and in nmol cm^{-2} for phosphorus. They are all presented as the mean ± SE of 6 nubbins randomly pooled in groups of two (3 groups per treatment).

P enrichment	Animal			Zooxanthellae		
	Total C (μmol cm^{-2})	Total N (μmol cm^{-2})	Total P (nmol cm^{-2})	Total C (μmol cm^{-2})	Total N (μmol cm^{-2})	Total P (nmol cm^{-2})
Unenriched	68.7 ± 2.9	10.4 ± 0.7	159 ± 21	28.6 ± 1.9	4.5 ± 0.7	52 ± 3
0.5 μmol L^{-1}	62.0 ± 2.6	9.7 ± 0.3	149 ± 12	33.2 ± 2.9	4.7 ± 1.1	64 ± 3
2.5 μmol L^{-1}	65.2 ± 3.5	9.6 ± 0.6	168 ± 27	40.9 ± 6.6	5.6 ± 0.9	100 ± 5

energy to chemical energy (Goodman et al., 1953; Hall and Rao, 1987), and is involved, during the dark phase, in many components of the Calvin cycle, such as ribulose 1,5-bisphosphate, 3-phosphoglycerate, 1,3-bisphosphoglycerate, glyceraldehydes-3-phosphate and ribulose-5-phosphate (Goodman et al., 1953; Hall and Rao, 1987). Increased photosynthetic efficiency of the zooxanthellae may explain the decrease in chlorophyll content per surface area (Fig. 2C) or per cell. A decrease in chlorophyll content was similarly observed in the giant clam *T. gigas* (Belda et al., 1993) with higher phosphate enrichments (2, 5, and 10 μmol P L^{-1}), but was attributed to an increase in the zooxanthellae density and a possible iron limitation. Underlying mechanisms for this observed decrease in chlorophyll content remain to be further investigated, especially because phosphate enrichment had also no effect on the chlorophyll content of the corals *P. damicornis* (Snidvongs and Kinzie, 1994; Stambler et al., 1991), *Acropora palifera* (Steven and Broadbent, 1997), and *S. pistillata* (Koop et al., 2001; Muscatine et al., 1989), with both similar and higher phosphate enrichments (Table 1).

As a consequence of a higher photosynthetic efficiency in phosphate-enriched zooxanthellae, and a limited enhancement of their growth rates, the cellular carbon and nitrogen contents of these zooxanthellae were enhanced by 70%. Carbon supply is not limited in symbiotic corals, and nitrogen was certainly obtained and concentrated from the host excretion and the efficient uptake of the low concentrations in seawater. Indeed, a total of 10×10^6 zooxanthellae were produced between weeks 4 and 7 in the 2.5 μmol L^{-1} P-enriched corals (Table 3), with a mean N content of $4.2 \cdot 10^{-12}$ mol N $zoox^{-1}$ (Fig. 3). A total of ca. 42 μmol N were therefore needed during this period to afford the zooxanthellae production. If we consider a daily flux of nitrogen from the host to the zooxanthellae of 0.26 μmol N cm^{-2} d^{-1} (Falkowski et al., 1993), ca. 32 μmol N (= 0.26 μmol N cm^{-2} d^{-1} × 21 d × 5.9 cm^2 on average) were available to the corals from host excretion. An additional total amount of 20 μmol N was provided via the uptake of dissolved ammonium in seawater (considering a mean uptake rate of 2.7 μmol N mg

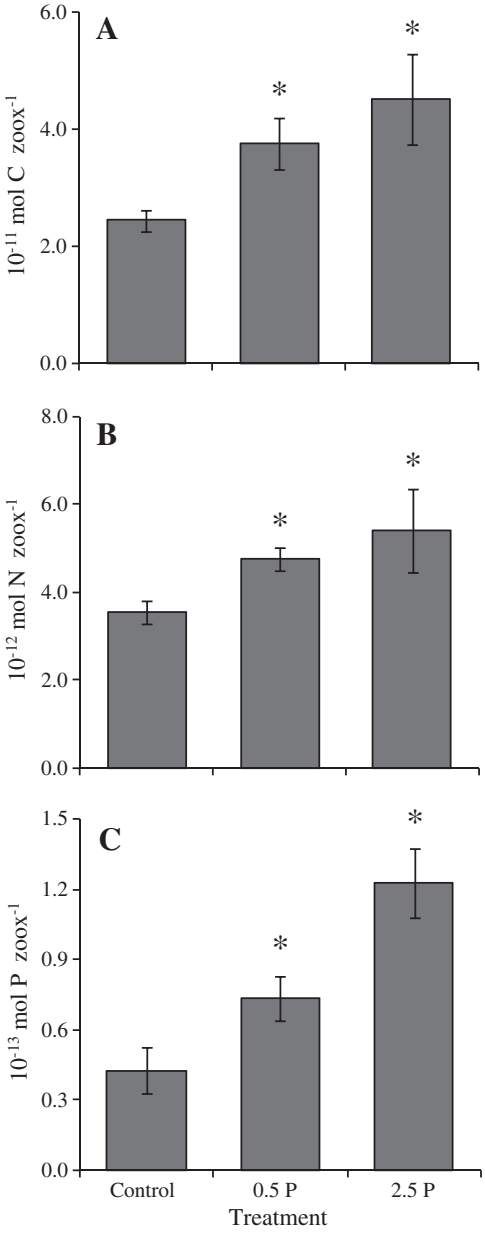

Fig. 3. Total carbon (A), nitrogen (B), and phosphorus (C) content of the zooxanthellae compartment of nubbins after 11 weeks of continuous phosphate enrichments of 0 (unenriched), 0.5, and 2.5 μmol L^{-1}. Each bar represents the mean ± SE of 6 nubbins. Data significantly different from the unenriched treatment are indicated by an asterisk for each element.

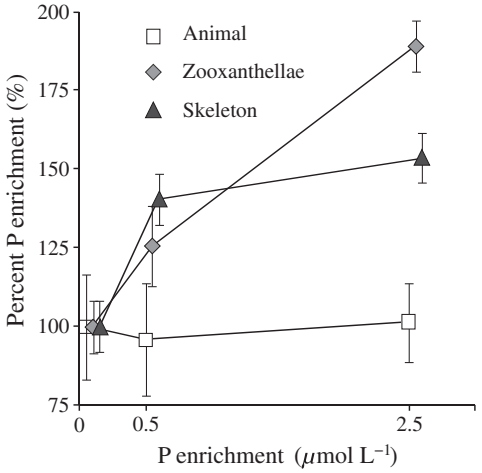

Fig. 4. Percentage of phosphorus accumulation relative to unenriched corals in each compartment (animal, zooxanthellae, skeleton) of 0.5, and 2.5 μmol L^{-1} phosphate-enriched corals after 11 weeks of continuous enrichment. Error bars represent the standard error of 6 nubbins for the animal and zooxanthellae compartment, and the analytical reproducibility for the skeleton.

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Chl^{-1} h^{-1} at an ambient concentration of <0.5 μmol NH_4^+, 12 h light, and 3×10–9 mg Chl zoox^{-1}; Godinot et al., 2011). These two nitrogen sources were therefore sufficient to cover the needs of the zooxanthellae production in P-enriched corals.

The increase in zooxanthellae specific growth rate with phosphate supplementation however did not lead to the building of a higher zooxanthellae density, which remained unchanged in P-enriched corals compared to control corals (i.e. zooxanthellae growth just matched the tissue and skeletal growth of the enriched corals). The specific growth rate calculated at T_7 (0.034–0.040 ± 0.01 d^{-1}) was also ca.10 times lower than the theoretical maximal exponential growth rate of 0.3 d^{-1}for cultured zooxanthellae (Chang et al., 1983). Benefits of phosphate to the zooxanthellae growth were therefore not substantial enough to reach balanced growth, suggesting that another nutrient was limiting, likely nitrogen. Indeed, the lack of change in zooxanthellae density tends to support the view that nitrogen was also largely limiting zooxanthellae growth, as addition of this nutrient is generally followed by a zooxanthellae burst in the coral tissue (Falkowski et al., 1993; Muscatine et al., 1989; Rees, 1991). Such a nitrogen-phosphorus co-limitation may also explain why phosphate addition alone did not lead to the negative impact on the coral calcification generally observed when phosphate and nitrogen are provided together (Ferrier-Pagès et al., 2000; Kinsey and Davies, 1979; Renegar and Riegl, 2005), in which case the overgrowth of zooxanthellae induces the disruption of the symbiotic association.

At the host level, there was no change in the elemental composition of the cells or in the protein content. This fact was already observed in the scleractinian coral *S. pistillata* and in the clam *T. gigas*, and it was hypothesized that protein synthesis was rather limited by the supply of amino acids and nitrogen (Belda et al., 1993; Muscatine et al., 1989). Nonetheless, the skeletal growth increased with phosphate enrichment, as was observed in some other studies (Table 1), maybe due to the possibility of adding sufficient zooxanthellae biomass in the newly formed tissue and skeleton. The discrepancy observed with other studies that reported a decrease in skeletal growth (Table 1) might be due to different ways in measuring calcification, i.e. total skeletal weight with the buoyant weight technique, versus linear extension rate.

Measurements of the amount of phosphorus and of the P/Ca ratio in the skeleton confirmed an enrichment of this compartment after addition of phosphate, and shows that phosphorus is not a toxic element for calcification, at least at these concentrations. However, the small difference in P/Ca ratio between the two phosphate-enriched treatments clearly demonstrated that there was a maximum limit in the amount of skeletal phosphorus accumulation. The mechanism of this incorporation is still unknown, and two pathways are possible: i) phosphorus can be deposited from the host cytoplasm, or ii) it can directly be acquired from seawater, without entering through the animal compartment (Braun and Erez, 2004; Erez and Braun, 2007). LaVigne et al. (2008, 2010) suggested the existence of two pools of skeletal phosphorus, an organic pool representing the background level, and a skeletal inorganic pool that increases with the increasing seawater phosphorus concentration. In the present study, phosphorus was measured in this latter inorganic pool, since the organic matrix was suppressed by the sample treatment. P/Ca values measured in enriched nubbins were remarkably similar to the first values reported by LaVigne et al. (2008) for *Pavona gigantea* (ca. 118 μmol mol^{-1}) from the Gulf of Panamá, characterized by a seasonal surface water phosphate increase between ca. 0.1 to ca. 0.6 μmol L^{-1}. Concerning the other trace elements analyzed, Mg/Ca and Sr/Ca ratios were respectively positively and negatively correlated with the skeletal growth rate, as previously reported (de Villiers et al., 1994; Gaetani and Cohen, 2006; Inoue et al., 2007; Mason et al., 2007; Rasmussen, 1988; Rasmussen and Cuff, 1990; Reynaud et al., 2007). The exact mechanisms responsible for this calcification-rate related kinetic effect are not entirely understood, although several hypotheses have been proposed, including a Rayleigh fractionation process and cation partitioning during aragonite crystal formation (Gaetani and Cohen, 2006; Inoue et al., 2007). However, the present study does not attempt to examine in detail the reasons behind the correlation between the growth rate and Mg/Ca and Sr/Ca ratios.

Under enrichment conditions, phosphorus concentrations did not change in the animal compartment when compared to unenriched conditions (Fig. 4), confirming that animal cells do not represent an accumulation site of inorganic phosphorus within the symbiosis. Conversely, the importance of phosphorus inside the skeleton and zooxanthellae increased (Fig. 4), confirming that these compartments are the two accumulation sites of inorganic phosphorus within the symbiotic association. In the zooxanthellar compartment, phosphorus accumulation was directly proportional to the ambient phosphate concentration (Fig. 4). Conversely, the skeletal phosphorus accumulation seemed saturable as phosphorus concentration was the same, within analytical error, for skeletons from the 0.5 and 2.5 μmol L^{-1} phosphate enrichments.

By addressing issues of nutrient supply to corals, this study provides a better understanding of the phosphorus metabolism of corals. The present results suggest that a 0.5 μmol L^{-1} P enrichment, which is usually the level encountered in eutrophicated areas, did not significantly change the physiology of the *S. pistillata* host, as measured through protein content and skeletal growth. However, the physiology of the symbionts was affected: although the zooxanthellae density remained constant, the photosynthetic efficiency increased, and phosphorus accumulated in the zooxanthellae, changing their elemental composition (after 11 weeks of enrichment). Phosphorus also accumulated in the skeleton of those corals.

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