A nanoscale secondary ion mass spectrometry study of dinoflagellate functional diversity in reef-building corals

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Summary
Nutritional interactions between corals and symbiotic dinoflagellate algae lie at the heart of the structural foundation of coral reefs. Whilst the genetic diversity of Symbiodinium has attracted particular interest because of its contribution to the sensitivity of corals to environmental changes and bleaching (i.e. disruption of coral–dinoflagellate symbiosis), very little is known about the in hospite metabolic capabilities of different Symbiodinium types. Using a combination of stable isotopic labelling and nanoscale secondary ion mass spectrometry (NanoSIMS), we investigated the ability of the intact symbiosis between the reef-building coral Isopora palifera, and Symbiodinium C or D types, to assimilate dissolved inorganic carbon (via photosynthesis) and nitrogen (as ammonium). Our results indicate that Symbiodinium types from two clades naturally associated with I. palifera possess different metabolic capabilities. The Symbiodinium C type fixed and passed significantly more carbon and nitrogen to its coral host than the D type. This study provides further insights into the metabolic plasticity among different Symbiodinium types in hospite and strengthens the evidence that the more temperature-tolerant Symbiodinium D type may be less metabolically beneficial for its coral host under non-stressful conditions.

Introduction
Coral reefs are among the most productive and biologically diverse ecosystems on Earth. The unicellular dinoflagellate algae (genus Symbiodinium), which live within coral tissue, play a fundamental role in the maintenance of healthy reefs by providing corals with important nutrients (Muscatine, 1990). Thus, corals are ‘polytrophic’, being able to acquire nutrients from sunlight through their photosynthetic symbionts (‘autotrophy’), feeding on plankton (‘heterotrophy’) and absorbing dissolved nutrients from the surrounding seawater. These multiple strategies increase the nutritional options for corals in the oligotrophic environment they commonly inhabit.

Symbiotic dinoflagellates of the genus Symbiodinium are divided into nine broad genetic clades, (clades A–I) defined by rRNA gene sequences (Rowan and Powers, 1991a; Coffroth and Santos, 2005; Pochon and Gates, 2010). These clades contain various genetically distinct types according to comparative analyses of internal transcribed spacer (ITS) sequence regions (van Oppen et al., 2001; LaJeunesse et al., 2004a, b; Pochon et al., 2007; Sampayo et al., 2007). Corals can simultaneously harbour dinoflagellates from different types in variable densities depending on coral species and environmental conditions (Baker and Romanski, 2007). Differences in physiological properties between Symbiodinium types...
can influence photosynthetic efficiency, growth and thermal tolerance of the host (Iglesias-Prieto et al., 2004; Little et al., 2004; Berkelmans and van Oppen, 2006; Sampayo et al., 2008). Although considered as a key factor that defines the health of coral reefs in a changing climate, the functional diversity of most Symbiodinium types remains under-explored.

Assimilation of carbon and nitrogen is essential for corals, and therefore, adequate supply of these two nutrients is critical to sustain optimal growth, development and response to a wide array of stresses. As yet, however, most nutritional investigations of the functional diversity of symbiotic dinoflagellates have focused on the metabolism of photosynthetically fixed carbon (Loram et al., 2007; Stat et al., 2008). Recently Baker and colleagues (2013) compared nitrate assimilation in juvenile corals infected with different Symbiodinium clades and suggested that under non-stressful conditions, Symbiodinium type C1 outcompetes an undefined D type via enhanced nitrogen acquisition. Nevertheless, assimilation of ammonium, which is the preferred source of inorganic dissolved nitrogen for coral dinoflagellates (Grover et al., 2008), has so far never been compared between Symbiodinium types from different clades. Furthermore, most bulk isotope analysis studies measuring the relative assimilation of carbon and nitrogen by symbiotic dinoflagellates suffer from potential cross-contamination of coral host and dinoflagellate fractions, as the intertwined nature of the coral–dinoflagellate endosymbiosis makes the relative quantification of dinoflagellate symbiont metabolism in hospite extremely difficult (Yellowlees et al., 2008). In this context, recent advances in nanoscale secondary ion mass spectrometry (NanoSIMS) have the potential to provide direct imaging and precise quantification at the individual cell level of up to seven different isotopes or molecular species within an intact symbiosis (Musat et al., 2008; Pernice et al., 2012; Berry et al., 2013; Kopp et al., 2013) (for review, see Orphan and House, 2009; Hoppe et al., 2013). Here, we use isotopic labelling in combination with single-cell NanoSIMS imaging of the intact coral symbiosis to offer an integrated view of the differential ability of Symbiodinium types from different clades to acquire dissolved inorganic carbon and nitrogen.

In this study, we investigated (i) whether different Symbiodinium types that naturally associate with a single coral species possess different metabolic capabilities and (ii) to what extent these genetically distinct symbiont types affect coral host metabolism. To address these questions, we compared the assimilation of dissolved inorganic carbon and nitrogen between colonies of the reef-building coral Isopora palifera (Wallace et al., 2012) (Fig. 1A), which associates with Symbiodinium types from clades C and D in its natural habitat.

Results and discussion

Sixty-eight adult I. palifera colonies growing at different sites adjacent to Heron Island Research Station (HIRS, 231330S 1511540E) were assessed regarding the diversity of Symbiodinium before November 2011, when corals were sampled for the labelling experiment. For each colony, Symbiodinium clades were identified by extracting genomic DNA from a single branch and analysing the pattern of PCR-restriction fragment length polymorphism (PCR-RFLP) of the small subunit rRNA gene. Fifty-nine I. palifera colonies harboured Symbiodinium clade C, while eight colonies were associated with Symbiodinium clade D, and only one colony hosted members of both clades C and D. All Symbiodinium could be further assigned to either type C3 or D1 by polymorphism screening of the ITS1–ITS2 region using denaturing gradient gel electrophoresis (DGGE, Fig. 1B) and sequencing of excised bands (data not shown).

Subsequently, a combination of isotopic labelling with 15N-ammonium and 13C-bicarbonate and NanoSIMS imaging of tissue sections was used to quantify the assimilation of dissolved inorganic carbon and nitrogen in the coral tissues. The experiment was performed on six different coral colonies, which associates with Symbiodinium types from clades C and D, as described in D–I. The distribution of 15N/14N and 13C/12C isotopic ratio was imaged directly in the coral tissue and quantified using NanoSIMS. The Hue ratio (0.0370, 10 times the natural ratio for 15N/14N and 0.1100, 10 times the natural ratio for 13C/12C). Quantification of 15N and 13C enrichment Saturation Intensity images are mapping the 15N/14N (D, F, H) and 13C/12C (E, G, I) isotopic ratio. The rainbow scale corresponds to the isotopic ratios and ranges from blue, set to natural ratio (0.0037 for 15N/14N and 0.0110 for 13C/12C), to red, where the ratio is several fold above natural (Zooxanthellae, Zx) or host tissue (Ht). Scale bars, 10 μm.

Fig. 1. NanoSIMS quantification of 15N and 13C assimilation by two different Symbiodinium types naturally associated with the reef-building coral Isopora palifera.

A. A colony of I. palifera collected on Heron Island in November 2011.
B. Symbiodinium types were identified in 68 coral samples by amplification of the ITS1-ITS2 region followed by screening of polymorphisms using denaturing gradient gel electrophoresis (DGGE). In this panel DGGE data for four representative coral colonies are depicted. Selected corals containing the different Symbiodinium types (C3 or D1) were incubated in ASW containing 15NH4Cl (5 μM) and NaH13CO3 (2 mM) for 48 h.

D–I. The distribution of 15N/14N and 13C/12C isotopic ratio was imaged directly in the coral tissue and quantified using NanoSIMS. The Hue Saturation Intensity images are mapping the 15N/14N (D, F, H) and 13C/12C (E, G, I) isotopic ratio. The rainbow scale corresponds to the isotopic ratios and ranges from blue, set to natural ratio (0.0037 for 15N/14N and 0.0110 for 13C/12C), to red, where the ratio is several fold above natural ratio (0.0370, 10 times the natural ratio for 15N/14N and 0.1100, 10 times the natural ratio for 13C/12C). Quantification of 15N and 13C enrichment of individual dinoflagellate cells and coral host tissue was obtained by selecting Regions of Interest (ROIs) that were defined in Open_MIMS by drawing directly on the NanoSIMS images (i) the contours of the dinoflagellate cells and (ii) circles of about 8–10 μm diameter, covering host tissue (as described in D to I).
C. For each NanoSIMS image, the mean 15N/14N and 13C/12C ratio are given for each individual ROI corresponding to dinoflagellate symbiont (Zooxanthellae, Zx) or host tissue (Ht). Scale bars, 10 μm.

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### Figure C

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**$^{15}$N-ammonium & $^{13}$C-bicarbonate**

- **Symbiodinium clade C**
  - [Image D](#)
  - [Image E](#)

- **Symbiodinium clade D**
  - [Image F](#)
  - [Image G](#)

- **Symbiodinium clade C**
  - [Image H](#)
  - [Image I](#)
symbiotic dinoflagellate cells from colonies hosting either *Symbiodinium* type C3 or D1. NanoSIMS analyses revealed substantial incorporation of the $^{13}$C and $^{15}$N isotopic tracers into both coral and dinoflagellate cells in corals incubated in isotope-enriched artificial sea water (ASW) (Fig. 1D–G) in contrast to unlabelled control samples (Fig. 1H and I). Quantitative analysis based on tissue sections from two different coral colonies for each samples (Fig. 1H and I). Quantitative analysis based on tissue sections from two different coral colonies for each samples (Fig. 1H and I). Quantitative analysis based on tissue sections from two different coral colonies for each samples (Fig. 1H and I). Quantitative analysis based on tissue sections from two different coral colonies for each samples (Fig. 1H and I).

In the incubation period, the incorporation of $^{15}$N-ammonium and $^{13}$C-bicarbonate by individual dinoflagellate cells was significantly higher in *Symbiodinium* type C3 than type D1 ($\delta^{15}$N: 6704 ± 438‰ for type C3 and 4159 ± 379‰ for type D1, $t$-test: final $P$-value = 0.006; $\delta^{13}$C: 3613 ± 134‰ for type C3 and 2773 ± 115‰ for type D1, final $P$-value < 0.001; Fig. 2A and B). These results provide evidence that in hospite, within the same coral host species and under the same environmental conditions, *Symbiodinium* type C3 assimilated more dissolved inorganic carbon and nitrogen than *Symbiodinium* type D1. These significant differences observed in the metabolism of *Symbiodinium* type C3 and D1 in hospite could be explained by (i) substantial variability in their efficiency to fix carbon via photosynthesis and provide carbon-skeleton for ammonia assimilation from seawater or by (ii) potential differences in the respiration rate of fixed carbon, but also in the translocation rate of both carbon (Cantin et al., 2009) and nitrogen compounds between symbiont and host.

Differential assimilation rates of carbon and nitrogen may reflect differential growth rates amongst taxonomically distinct intracellular *Symbiodinium* as suggested previously (Fitt, 1985; Wooldridge, 2012). On this note, Wooldridge (2012) predicted that the bleaching-tolerant clade D symbionts would possess a slower division rate and a larger cell size than clade C. Our data indicate that *Symbiodinium* types hosted by *I. palifera* displayed differences in relative size, with *Symbiodinium* type C3 being on average smaller than type D1 (Supporting Information Table S1; 7.1 ± 0.8 μm diameter for type C3 versus 8.2 ± 1.8 μm diameter for type D1; $t$-test: $t = -2.831$; $df = 65$; $P = 0.006$). There was no significant difference in the density of the symbionts per surface area of coral branch (Supporting Information Fig. S1; $t$-test: $t = 1.501$; $df = 18$; $P = 0.151$) or in their size distribution (Supporting Information Fig. S2A and B; Kolmogorov–Smirnov test: $P = 0.769$). Both these latter features suggest similar division rates amongst these *Symbiodinium* types under the imposed experimental conditions. Furthermore, additional analyses showed that there was no significant correlation between the apparent size of *Symbiodinium* cells analysed by NanoSIMS and their incorporation of nitrogen and carbon during the 48 h labelling experiment ($^{15}$N, $R^2 = 0.02$, $P = 0.742$, Supporting Information Fig. S3A; $^{13}$C, $R^2 < 0.001$, $P = 0.995$; Supporting Information Fig. S3B). Taken together, these data suggest that greater assimilation rates of $^{15}$N-ammonium and $^{13}$C-bicarbonate by *Symbiodinium* type C3 over D1 symbionts did not enhance symbiont proliferation rates. In this respect, future studies including longer experiments are needed in order to investigate the relationships between metabolism and proliferation rates.

On the other hand, D1 symbionts may have reduced concentrations of labelled carbon and nitrogen isotopes because assimilated carbon and nitrogen are more rapidly transferred to the host compared with C3 symbionts. To clarify this issue, the incorporation of inorganic carbon and nitrogen was quantified within the coral host cells by measuring $^{15}$N and $^{13}$C-enrichment in 8–10 μm diameter regions of interests (ROI) located in direct proximity to the symbionts (Fig. 1D–I). Compared with corals containing *Symbiodinium* type D1, the *I. palifera* tissue associated with *Symbiodinium* type C3 incorporated substantially more carbon ($\delta^{13}$C: 460 ± 37‰ for corals associated with type C3 and 177 ± 23‰ for corals associated with type D1, $t$-test: final $P$-value < 0.0001) and nitrogen ($\delta^{15}$N: 1614 ± 160‰ for corals associated with type C3 and 730 ± 112‰ for corals associated with type D1, $t$-test: final $P$-value = 0.022; Fig. 2C and D). The results support the hypothesis that C3 symbionts are more efficient at both assimilating carbon and nitrogen from the environment and transferring...
acquired carbon and nitrogen to their hosts, than D1 symbionts. Previous studies have indicated that an undefined Symbiodinium type belonging to clade D might be considered less mutualistic than Symbiodinium type C1 since it provides less energy to juvenile corals (Cantin et al., 2009) and can result in reduced growth (Little et al., 2004), lipid storage and reproductive output for adult corals (Jones and Berkelmans, 2011). Consistent with this finding, our study shows a stronger capability of Symbiodinium type C3 compared with type D1 for fixing inorganic carbon and ammonium and for subsequent contribution of these newly assimilated compounds to their coral host nutrition (including potential additional carbon fixation via carboxylation reactions).

In the following section, we would like to discuss several points that are of relevance for the interpretation of the data obtained in this study. First, sample preparation for transmission electron microscopy (TEM) and NanoSIMS analyses can cause biases. For example, the successive steps of sample rinsing, ethanol dehydration and acetone storage could have resulted in partial extraction of sugars and other soluble molecules poor in amino groups. Note that this extraction can be minimized by rapid osmium post-fixation before rinsing and by avoiding acetone storage. In addition, fixation and embedding of the samples add additional 13C atoms and thus dilute the 13C signal. Therefore the 13C concentrations reported in our NanoSIMS analyses are underestimates, but the relative isotopic enrichment ratios remain valid. Second, corals are unusual animals because they can directly and rapidly assimilate ammonium into amino acids using GS/GOGAT cycle (Wang and Douglas, 1999; Grover et al., 2002; Pernice et al., 2012). Consequently, the presence of 15N in either the host or the symbiont may originate from two different sources of ammonium assimilation: the animal host or the symbiont. However, because the coral colonies were collected from the same reef site, and subsequently exposed to the same experimental conditions, one would, unless host assimilation of ammonium is stimulated differently by the presence of different symbionts, not expect this unusual aspect of host physiology to effect the interpretation of the 15N data provided above. Third, coral symbiosis can assimilate inorganic nitrogen dissolved in seawater as ammonium and nitrate. In this study, 15N-ammonium was used as a substrate for the labelling experiment because it is recognized as the preferred source of inorganic nitrogen for corals (Grover et al., 2002). However, it is important to note that nitrate is also an important factor for growth of symbiotic dinoflagellates (Fagoonee et al., 1999) and is generally more abundant on coral reefs than ammonium. Nitrate can be used as a substrate for isotopic labelling experiments combined with NanoSIMS analysis (Kopp et al., 2013), and offers some interesting insights as only the symbionts possess nitrate and nitrite reductase enzymes for assimilating nitrate into ammonium and subsequently into organic products (Crossland and Barnes, 1977). A first disadvantage of using nitrate as a substrate for labelling experiments and for quantifying metabolic rates is that uptake rate by Symbiodinium cell is known to be significantly slower for nitrate than for ammonium (D’Elia et al., 1983). Second, nitrate transporters can generally regulate ion uptake by switching from a low-affinity to a high-affinity mode depending on the availability of nitrate (Parker and Newstead, 2014) and, consequently, cellular uptake of nitrate would likely be variable, depending on the nitrate concentration in which the symbiotic cnidarian were maintained prior to the isotopic incubation experiments. Ammonium transport across biological membranes is known to be mediated by Amt proteins and several corresponding genes have been identified in Symbiodinium previously (Leggat et al., 2007). However, it is still unclear if these genes are subjected to nitrogen control or not in symbiotic dinoflagellate, and if cellular uptake is more or less variable for ammonium than for nitrate. In the future, more detailed studies are clearly needed to (i) assess more precisely the advantages and disadvantages of using ammonium or nitrate for isotopic labelling experiments in coral symbiosis and to (ii) establish to what extent the rate of assimilation of these different substrates is affected by different concentrations of nitrogen and by different feeding regime of the coral hosts.

The large standard deviation observed in our study for carbon and nitrogen enrichment ratios (i.e. high value of standard deviation indicated in Supporting Information Tables S1 and S2 and illustrated by the difference in colour scale within individual cells in Fig. 1D–G), reflects a strong spatial heterogeneity of carbon and nitrogen enrichment. Such a spatial heterogeneity is likely to be related either to variability in physiological activity between individual cells or to the thin (100 nm) section orientation through specific subcellular structures. For instance, in symbiotic dinoflagellate, Kopp and colleagues (2013) previously demonstrated that nitrogen storage into cytosolic crystals of uric acid could largely explain the spatial heterogeneity of 15N-enrichment observed in Symbiodinium cells after incorporation of 15N-ammonium and 15N-nitrate. The substantial cell-to-cell variation observed in our study could also reflect the presence of physiologically distinct populations of cells in the tissue of I. palifera. In the case of symbiotic dinoflagellates, it is possible that the observed individual Symbiodinium cells were in different physiological state as a result of different life histories, including mitotic activity. However, additional analyses failed to show any significant correlation between the apparent size of Symbiodinium cells analysed by NanoSIMS and their incorporation of nitrogen.
and carbon ($^{15}$N, $R^2 = 0.02$, $P = 0.742$, Supporting Information Fig. S3A; $^{13}$C, $R^2 < 0.001$, $P = 0.995$; Supporting Information Fig. S3B). In order to more accurately reveal the mechanisms driving the differences in metabolic rates between Symbiodinium populations, future isotopic labelling studies in corals should also include determination of Symbiodinium growth rates and population doubling times.

Nevertheless, our results are consistent with a recent study based on isotopic incubations and bulk analysis (Baker et al., 2013) as well as with previous data on coral growth and reproductive outputs (Little et al., 2004; Jones and Berkelmans, 2011) linked to symbiont identity. Indeed, although the substrates employed for the $^{15}$N labelling were different (nitrate versus ammonium in our study), it is remarkable to note that our results match closely the differences between symbiont clades published by Baker and colleagues (2013). More specifically, in our study, Symbiodinium type C3 acquired 30% more $^{15}$N than type D1, while in Baker’s (2013), Symbiodinium clade C incorporated 22% more $^{15}$N than clade D. In congruence with these previous studies, the work presented here advances the current understanding of trophic relationships between corals and their symbionts by highlighting reduced fixation and transfer rates of carbon (fixed via photosynthesis) and nitrogen (fixed via assimilation of ammonium dissolved in seawater) for Symbiodinium type D1 compared with type C3.

There is increasing evidence that environmental conditions interact with symbiont diversity and host factors to co-determine the delicate balance of compounds exchanged within the symbiosis, affecting its functioning and stability (Mieog et al., 2009; Wiedenmann et al., 2012; Beraud et al., 2013). Given the trophic role of the coral symbionts, the intriguing question arises as to whether coral bleaching may in part reflect a shift in the balance of supply versus demand for nitrogen and carbon within the host–symbiont interaction (Wooldridge, 2012). In this respect, previous studies highlighted a strong correlation between heterotrophic feeding, nutrient enrichment and bleaching (Grottoli et al., 2006; Ferrier-Pages et al., 2010; Wiedenmann et al., 2012). Whilst the genetic diversity of Symbiodinium has attracted particular interest because of its contribution to the thermal tolerance of corals (Rowan, 2004; Berkelmans and van Oppen, 2006; Jones et al., 2008) very little is known about the impact of different Symbiodinium types on the nutritional status of the cnidarian–dinoflagellate symbiosis. Studies based on bulk analysis (Baker et al., 2013) recently indicated that, when exposed to seawater enriched in nitrate, Symbiodinium type C1 and an undefined Symbiodinium type from clade D displayed different patterns of nutrient acquisition and these patterns could be reversed at elevated temperature. Our study demonstrates a stronger capability of Symbiodinium type C3 versus type D1, both naturally associated with I. palifera for fixing inorganic carbon and ammonium and passing these newly assimilated nutrients to their coral host cells at local ambient temperature (24°C). These findings strengthen the evidence that, although more temperature-tolerant (LaJeunesse et al., 2009; Oliver and Palumbi, 2009), clade D symbionts may be less metabolically efficient than clade C members under non-stressful conditions.

### Experimental procedures

#### Collection, Symbiodinium genotyping and maintenance of corals

Sixty-eight adult colonies of I. palifera were assessed at different sites adjacent to Heron Island Research Station (231330S 1511540E) before November 2011, when corals were sampled for the labelling experiment. For each of the 68 coral colonies, total genomic DNA was extracted from a single branch using the modified protocol of Wilson and colleagues’ (2002). Symbiodinium clades associated with the different coral colonies were first identified by PCR-RFLP of the SSU rRNA gene, using a combination of the universal eukaryotic ‘ss5’ and Symbiodinium-specific ‘ss3Z’ primers (Rowan and Powers, 1991b) for PCR amplification, and a combination of TaqI and DpnII restriction enzymes for digestion of the PCR products (Bythell et al., 1997). The single colony hosting a mix of both clade C and clade D Symbiodinium was excluded from the experiment.

Individual coral branches (5–6 cm long) were carefully fragmented from six I. palifera colonies (three replicates hosting C3 and three replicates hosting D1 type symbionts) using bone cutters and immediately transferred to flow-through aquaria at Heron Island Research Station. Coral fragments were then attached to fine nylon fishing line and acclimatized to the mean local ambient temperature (24°C) for a week in natural seawater in six independent flow-through aquarium tanks (60 l; three tank replicates per treatment). Within the period of the experiment, the sunrise time was 4:45 a.m. and the sunset time was 6:28 p.m. The ambient light levels were controlled throughout the experiment using shade cloths to mimic natural reef flat conditions [noon irradiance ranging from 700 to 1000 μmol photons m$^{-2}$ S$^{-1}$ as measured by Odyssey light logger (Dataflow, Christchurch, New Zealand).

For each coral fragment used for the isotopic labelling experiment and NanoSIMS analysis, a subsample was used to confirm the identity of Symbiodinium types as C3 and D1 by amplification of the ITS1–ITS2 region using primers ‘ITSinf for’ and ‘ITS 2 clamp’ (Sheffield et al., 1989; LaJeunesse and Trench, 2000), followed by screening of polymorphisms using DGGE (Fig. 1B) on a C.B.S. Scientific system (USA) (LaJeunesse, 2001). Subsequently, bands were excised and sequenced. In addition, identification of the coral host was confirmed for each sample by analyses of the skeleton morphology.

#### Isotopic labelling experiment

In order to quantify the assimilation of dissolved inorganic carbon and ammonium in symbiotic dinoflagellate cells, coral
fragments were exposed to isotopically enriched ASW (and control non-enriched ASW). Coral fragments were randomly distributed among the treatment and control aquaria (10 l; closed-water system; continuously stirred using one power head pump for each tank and aerated with air stones, previous experiments demonstrated that sufficient isotopic labeling was obtained using this outdoor aquaria system). The temperature of the tanks was maintained at 24°C by placing experimental and control tanks in flow-through ambient seawater. Isotopically enriched ASW was made in accordance with Dunn and colleagues' (2012) with the addition of 15NH4Cl (15N isotopic abundance of 98%, Sigma) and NaH13CO3 (13C isotopic abundance of 99%, Sigma) to a final concentration of 0.65 M sucrose, 2.5 mM CaCl2), pH 7.5 at 4°C for 3 days until sufficient isotopic enrichment was obtained. Control non-enriched ASW contained 14NH4Cl and NaH12CO3 in the same concentrations as enriched media. Treatment and control media were changed every 12 h throughout the experiment. A subset of coral branches (n = 3) for each colony was randomly removed from the treatment and control tanks at T = 0 and 48 h, respectively. Each coral branch was chemically fixed for 24 h at 4°C by immersion in a solution containing 2.5% glutaraldehyde and 1% formaldehyde in PBS-sucrose buffer (0.1 M phosphate, 0.65 M sucrose, 2.5 mM CaCl2), pH 7.5. Samples were then stored in PBS-sucrose buffer (0.1 M phosphate, 0.65 M sucrose, 2.5 mM CaCl2), pH 7.5 at 4°C for 3 days until further processing for TEM and NanoSIMS analyses.

Tissue preparation for TEM and NanoSIMS analyses

Coral fragments were embedded in 1.5% agarose prior to decalcification at 4°C and pH 7.5 in Sörensen 0.1 M buffer (Sörensen, 1909) containing 0.5 M EDTA, with solution changed every 12 h until the skeleton was completely dissolved. After decalcification, coral samples were dissected under a stereomicroscope into small pieces containing two or three individual polyps from the lateral part of the coral branch, and post-fixed 1 h at RT in 1% OsO4 in Sörensen phosphate buffer (0.1 M). Tissue samples were then dehydrated in an increasing series of ethanol concentrations (50%, 70%, 90% and 100%) and stored in acetone until resin embedded. Tissue samples were then stored in acetone solution until resin embedding. Samples were embedded in Spurr resin, cut to 100–120 nm sections using an Ultracut E microtome (Leica Microsystems, Australia), mounted onto finder grids for TEM (Proscitech, Australia) and counterstained with uranyl acetate 2% (10 min) and Reynold’s lead citrate (10 min).

TEM analyses

Regions of interest within the tissue sections were mapped and imaged at the Centre for Microscopy and Microanalysis (University of Sydney, Sydney, Australia) using a JEOL JEM1400 Transmission Electron Microscope (JEOL, Korea) operated at 80 kV accelerating voltage. Then the TEM grids were mounted on 10 mm diameter aluminium stubs and gold-coated for subsequent NanoSIMS analyses.

NanoSIMS analyses

The same regions of interest mapped with TEM within the tissue sections were imaged with the NanoSIMS ion probe at the Core Facility of Advanced Isotope Research (University of Vienna) in order to quantify the distribution of newly fixed 13C and 15N within I. palifera. Samples were bombarded with a 16 keV primary ion beam of (1–3 pA) Cs+ focused to a spot size of about 100–150 nm on the sample surface. Secondary molecular ions 13C12C-, 12C13C-, 12C14N- and 12C15N- were simultaneously collected in electron multipliers at a mass resolution (ΔM/M, according to Cameca’s definition) of about 9000, enough to resolve the 13C14N- and 12C15N- ions from potentially problematic interferences. Charge compensation was not necessary. Typical images of 35 × 35 μm with 256 × 256 pixels for 13C12C-, 12C13C-, 12C14N- and 12C15N- respectively were obtained by rastering the primary beam across the sample with a dwell-time of 5 ms. After drift correction, the 13C/12C or 15N/14N maps were obtained by taking the ratio between the 12C13C- and 12C12C images or the 15N14N- and 12C14N- images respectively. Additional samples were analysed with the NanoSIMS ion probe at the Max Planck Institute for Chemistry (Mainz) using the following secondary molecular ions: 12C-, 13C-, 12C13C- and 12C14N-. 13C and 15N enrichments were expressed in the delta notation (δ13C and δ15N in ‰) as follows:

\[
\delta^{13}C = \left( \frac{C_{\text{meas}}}{C_{\text{nat}}} - 1 \right) \times 10^3
\]

Where:

\[C_{\text{meas}}\] is the measured 13C/12C ratio and \[C_{\text{nat}}\] is the natural 13C/12C ratio measured in non-labelled control coral samples.

\[
\delta^{15}N = \left( \frac{N_{\text{meas}}}{N_{\text{nat}}} - 1 \right) \times 10^3
\]

Where \[N_{\text{meas}}\] is the measured 15N/14N ratio and \[N_{\text{nat}}\] is the natural 15N/14N ratio measured in non-labelled control coral samples. \[C_{\text{meas}}\] and \[N_{\text{meas}}\] were quantified on unlabelled coral tissue each day of the NanoSIMS analyses and served as internal standard. Under the analytical conditions applied, signal ranged from 113 to 829 counts per pixel for 12C13C-, 10–62 counts per pixel for 12C14N-, 270–2195 counts per pixel for 12C15N- and 1–56 counts per pixel for 13C14N-. Each coral sample analysed by NanoSIMS was obtained from an individual coral branch (5–6 cm long) that was incubated with seawater–enriched, either (i) with unlabelled ammonium and bicarbonate (for unlabelled samples) or (ii) with seawater-enriched in 15N-ammonium and 13C-bicarbonate (for labelled samples).

For each Symbiodinium type, two branches, each one coming from a different colony, were analysed using NanoSIMS (as described above). Coral sections were prepared in an area corresponding to the polyp oral tissue. Levels of 15N and 13C enrichment were quantified for individual dinoflagellate and host cells by using ROIs defined in Open_MIMS (http://nrims.harvard.edu/software) by drawing the contours of (i) the dinoflagellate cells and (ii) circles of about 8–10 μm diameter, covering host tissue directly on the NanoSIMS maps (as illustrated in Fig. 1D–I). For cell quantification purpose, ROIs were drawn on the NanoSIMS maps of the 12C14N-ions, which allow finding the contours of the dinoflagellate cells without any 15N- or 13C-label present. The isotopic ratios and enrichments measured for each ROI are reported in Supporting Information Tables S1 and S2. For each ROI, the standard deviation given directly by the software OpenMIMS is indicated.

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**Cell densities of Symbiodinium**

Three coral branches were used per colony per *Symbiodinium* type in order to assess differences in the density of endosymbiotic dinoflagellates within *I. palifera*. Coral tissue was removed from the samples previously stored at -80°C by using an airbrush and phosphate buffer solution (0.06 M solution KH2PO4, pH 6.65) (Pernice et al., 2011). *Symbiodinium* cells were separated from coral tissue by centrifugation (4000 g for 5 min at 4°C). The pellet containing *Symbiodinium* cells was then washed three times to minimize tissue contamination and re-suspended in phosphate buffer solution. The population density of dinoflagellates was determined using a SEDGEWICK rafter cell 550 haemocytometer (ProSciTech S8050) by counting and averaging the number of dinoflagellate cells present in 10 subsamples. Dinoflagellate density was then normalized to the skeletal surface area (cm²) of each coral branch by using the modified foil wrapping technique (Marsh, 1970).

**Size distribution of Symbiodinium**

The apparent diameter of dinoflagellate cells analysed with NanoSIMS was determined using the ‘Analyse particles’ function of IMAGEJ software (http://rsbweb.nih.gov/ij/). A difference method of back substitution was then used as described in (Eisenhour, 1996) to correct for biases resulting from non-equatorial sectioning and obtain size distribution of the different *Symbiodinium* types.

**Statistical analyses**

Statistical analyses comparing nitrogen and carbon assimilation between corals associated with *Symbiodinium* type C3 and D1 respectively were conducted using the computer software package R (R Core Development Team, 2008). Because greatly varying cell numbers were measured for each sample, we used a bootstrap-based method of resampling to obtain a balanced dataset. More specifically, we used resampling to select randomly (without replacement) a subset of eight cells within each coral branch. This resampling method allowed obtaining a balanced experimental design (i.e. n = 8 for each group). Resampling was done 100 000 times. We then ran t-tests to compare assimilation of nitrogen and carbon between the two groups of corals (corals associated with *Symbiodinium* type C3 or D1) for each of the 100 000 simulations. The frequency distribution of P-value is indicated in Supporting Information Fig. S4 and the final P-value was calculated as the 95th percentile of the P-values obtained in each t-test.

All the other statistical analyses were conducted using Systat 13 software package (Statsoft). Kolmogorov–Smirnov and Levene’s test were used first to test the normality and homoscedasticity of the data concerning symbiont cell density and size. Once normality and homoscedasticity of the data were confirmed, we compared the two groups of corals (corals associated with *Symbiodinium* type C3 or D1) for (i) the density and size of their symbiont using t-test and for (ii) the size distribution of their symbiont using Kolmogorov–Smirnov test. Finally, least-squares regressions were conducted to investigate the relationship between the apparent size of *Symbiodinium* cells analysed by NanoSIMS and their incorporation of nitrogen and carbon. Throughout the paper, results were considered significant at the 5% level.

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**References**


**Supporting information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Fig. S1.** Mean areal density of dinoflagellate cells in *I. palifera* corals associated with *Symbiodinium* C3 (in black, *n* = 11) and *Symbiodinium* D1 (in white, *n* = 9). Bars in the histograms indicate the standard error. No significant difference was detected between C3 and D1 (*t*-test: *t* = 1.501; df = 18; *P* = 0.151).

**Fig. S2.** Size distribution of dinoflagellate cells analysed by NanoSIMS:

A. *Symbiodinium* C3

B. *Symbiodinium* D1.

No significant difference was detected between the size distribution of *Symbiodinium* type C3 and D1 (Kolmogorov–Smirnov test: *P* = 0.769).

**Fig. S3.** Least square regressions between apparent diameter of dinoflagellate cells and 15N (A) and 13C (B) enrichment. The linear models were were checked by an F-test and did not display any significant correlation (15N, *R*2 = 0.02, *P* = 0.742 Supporting Information Fig. S3A; 13C, *R*2 < 0.001, *P* = 0.995; Supporting Information Fig. S3B). The blue lines indicate 95% confidence interval predicting the distribution of estimates of the present population of symbiotic dinoflagellate, the brown lines indicate prediction interval, which forecast the distribution of future observations.

**Fig. S4.** Frequency distribution of *P*-value for *t*-tests comparing corals associated with *Symbiodinium* type C3 or D1. The distribution of *P*-value for *t*-tests comparing the two groups of corals is indicated after resampling for:

A. Enrichment in 15N in dinoflagellate cells.

B. Enrichment in 13C in dinoflagellate cells.

C. Enrichment in 15N in coral tissue.

D. Enrichment in 13C in coral tissue.

Resampling was done 100 000 times.

**Table S1.** Increase in 14N/15N and 13C/12C ratio of individual dinoflagellate symbionts within the reef-building coral *Isopora palifera* during the experiment. Regions of interest (ROI) refer to cells analysed and marked in the respective figure. For each cell the standard deviation given directly by the software OpenMIMS is indicated. The high values of standard deviations indicate a strong spatial heterogeneity of C and N enrichment, related to specific subcellular structures. *Cells not shown in figures.*

**Table S2.** Increase in 15N/14N and 13C/12C ratio in *Isopora palifera* tissue during the experiment. Regions of interest (ROI) refer to circles of about 8–10 µm diameter, covering the host tissue in the direct proximity of the symbionts, analysed and marked in the respective figure. For each cell the standard deviation given directly by the software OpenMIMS is indicated. The high values of standard deviations indicate a strong spatial heterogeneity of C and N enrichment, related to specific subcellular structures. *Cells not shown in figures.*