

# 1 **Aiptasia as a model to study metabolic diversity and specificity in cnidarian-dinoflagellate** 2 **symbioses**

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## 21 **Keywords**

22 metaorganism, holobiont, carbon translocation, nitrogen uptake, *Symbiodinium*, selfish

23 symbiont

24

## 25    **Abstract**

26    The symbiosis between cnidarian hosts and microalgae of the genus *Symbiodinium* provides  
 27    the foundation of coral reefs in oligotrophic waters. Understanding the nutrient-exchange  
 28    between these partners is key to identifying the fundamental mechanisms behind this  
 29    symbiosis. However, deciphering the individual role of host and algal partners in the uptake  
 30    and cycling of nutrients has proven difficult, given the endosymbiotic nature of this  
 31    relationship. In this study, we highlight the advantages of the emerging model system  
 32    *Aiptasia* to investigate the metabolic diversity and specificity of cnidarian – dinoflagellate  
 33    symbiosis. For this, we combined traditional measurements with nano-scale secondary ion  
 34    mass spectrometry (NanoSIMS) and stable isotope labeling to investigate carbon and  
 35    nitrogen cycling both at the organismal scale and the cellular scale. Our results suggest that  
 36    the individual nutrient assimilation by hosts and symbionts depends on the identity of their  
 37    respective symbiotic partner. Further,  $\delta^{13}\text{C}$  enrichment patterns revealed that alterations in  
 38    carbon fixation rates only affected carbon assimilation in the cnidarian host but not the algal  
 39    symbiont, suggesting a ‘selfish’ character of this symbiotic association. Based on our  
 40    findings, we identify new venues for future research regarding the role and regulation of  
 41    nutrient exchange in the cnidarian - dinoflagellate symbiosis. In this context, the model  
 42    system approach outlined in this study constitutes a powerful tool set to address these  
 43    questions.

44

## 45     **Introduction**

46     Coral reefs thrive in nutrient poor waters (1) and their ecological success fully relies on the  
 47     nutrient-exchange between cnidarians and dinoflagellate algae of the genus *Symbiodinium*  
 48     living in the host's tissues (2, 3). In this association, the endosymbiotic algae translocate the  
 49     majority of their photosynthetically-fixed carbon to the host, which in turn provides  
 50     inorganic nutrients from its metabolism to sustain algal productivity (2, 4–6). The efficient  
 51     recycling of organic as well as inorganic nutrients within this symbiosis underpins the high  
 52     productivity of coral reefs in the absence of major sources of allochthonous nutrients (7, 8).  
 53     Yet, this ecosystem is in global decline as anthropogenic environmental change impedes the  
 54     role of cnidarians as key ecosystem engineers (9). Mass bleaching events, i.e. the disruption  
 55     of cnidarian - dinoflagellate symbiosis signified by the expulsion of symbionts and physical  
 56     whitening of corals on broad scales, are among the dominant drivers of this decline (10, 11).  
 57     Understanding the causes of this symbiotic breakdown requires considering these symbiotic  
 58     organisms as holobionts. Holobionts constitute complex metaorganisms that arise from the  
 59     interaction of the hosts and their associated microorganisms such as protists, bacteria, and  
 60     archaea (12). A crucial attribute of cnidarian holobionts is the ability to take up, assimilate,  
 61     and exchange nutrients (13). In particular, nitrogen cycling appears to be key to the  
 62     functioning of these holobionts (14, 15), since growth of *Symbiodinium* is nitrogen-limited in  
 63     a stable symbiosis (14, 16–18). Nitrogen limitation might stabilize symbiont populations and  
 64     facilitate the translocation of photosynthates to the host (19), a process providing most of  
 65     the energy required for the host's metabolism (2, 20). Yet, it is unclear whether the host can  
 66     exert control over this translocation of nutrients (21–23).  
 67     Despite the importance of the individual contribution of host and symbionts to holobiont  
 68     nutrient cycling (14, 23–25), studying these processes in scleractinian corals has proven

69 difficult due to the complex and interwoven nature of the coral holobiont. As most corals  
70 are associated with a diverse *Symbiodinium* community and cannot be maintained in a  
71 symbiont-free stage, identifying underlying processes within these symbiotic interactions is  
72 challenging. In contrast, the emerging model organism *Aiptasia* (*sensu Exaiptasia pallida*  
73 (26)) offers distinct advantages to study the cnidarian-dinoflagellate symbiosis (27–29): (I.)  
74 this sea anemone can be reared in clonal lines, enabling the study of processes in the  
75 absence of biological variation (30); (II.) animals can be maintained in a symbiont-free stage,  
76 allowing to study host processes in the absence of symbionts (29); (III.) symbiont-free  
77 *Aiptasia* can be re-infected with specific symbiont strains, enabling the comparison of  
78 different symbionts (including those commonly associated with corals) in the same host  
79 background *in hospite* (31); (IV.) an extensive array of genetic resources is available in  
80 *Aiptasia*, allowing to link genetic and physiological traits (27). These distinct advantages will  
81 prove especially powerful to study metabolic interactions between host and symbionts,  
82 particularly if combined with state of the art imaging techniques such as nano-scale  
83 secondary ion mass spectrometry (NanoSIMS), which allow precise quantification of  
84 element distribution at high spatial resolution (32, 33). Coupled with stable isotope labeling,  
85 this technology enables imaging of metabolic processes at subcellular resolution and  
86 consequently quantification of nutrients assimilation at the single-cell level for each  
87 symbiotic partner (33). NanoSIMS has opened doors to an unprecedented level of  
88 information across all fields of biology and has been successfully applied to corals (34–38).  
89 In this study, we combined the advantages of the *Aiptasia* model system with high  
90 resolution NanoSIMS to showcase the advantages of this model approach for the study of  
91 metabolic interactions and diversity in the cnidarian – dinoflagellate symbiosis.

92

## 93 **Material & methods**

### 94 *Maintenance of Aiptasia*

95 Four different host–symbiont pairings were maintained in separate batches. These  
 96 combinations involved two different host clonal lines (CC7 (41) and H2 (39)) as well as two  
 97 different symbiont populations (A4 and B1 dominated (68)). Whilst CC7 Aiptasia can form  
 98 stable associations with a diversity of *Symbiodinium* types, H2 Aiptasia show high fidelity to  
 99 their native symbionts suggesting a higher selectivity and/or specificity with their symbionts  
 100 (40). This specificity of H2 Aiptasia hinders reinfection with other symbionts thereby  
 101 preventing a full factorial design in this study. Nevertheless, these host clonal lines provide  
 102 an ideal basis for the comparison of symbiont diversity and specificity.

103 To allow comparison of symbiont types within the same host line and to compare  
 104 performance of the same symbiont type within different host lines, CC7 Aiptasia were  
 105 bleached and reinfected with type B1 (strain SSBO1) symbionts, previously isolated from H2  
 106 Aiptasia. For this, non-symbiotic CC7 Aiptasia were generated and reinfected as described  
 107 by Baumgarten *et al.* (27). In brief, animals were repeatedly bleached by incubation in 4°C  
 108 sterile seawater for 4 h, followed by 1-2 days at 25°C in sterile seawater containing the  
 109 photosynthesis inhibitor diuron. Non-symbiotic animals were maintained for at least 1  
 110 month prior to reinfection to confirm absence of residual symbionts. For reinfection, non-  
 111 symbiotic animals were subjected to three cycles of incubation for one day in sterile  
 112 seawater containing  $10^5$  *Symbiodinium* cells mL<sup>-1</sup> followed by *Artemia salina* nauplii feeding  
 113 the next day. Thus, the four combinations were: non-symbiotic CC7 Aiptasia, CC7 Aiptasia  
 114 with its native A4 symbionts; CC7 Aiptasia reinfected with B1 symbionts and H2 Aiptasia  
 115 with its native B1 symbionts (Fig. 1A-D). Animals were reared in sterile seawater (35 PSU, 25  
 116 °C, ~80 μmol photons m<sup>-2</sup> s<sup>-1</sup> on a 12h:12h light:dark schedule) and fed with freshly hatched

*Artemia salina* nauplii three times per week. Animal cultures were propagated under these conditions for more than one year to ensure anemones recovered from bleaching and reinfection procedures and to confirm the stability of native and introduced symbiotic associations. Stability of *Symbiodinium* communities was monitored using qPCR as outlined by Correa *et al.* (69). Any feeding was abandoned three days prior to measurements to exclude potential confounding effects. Thereby this experimental design allowed us to disentangle the contribution of host and symbionts to holobiont nutrient cycling in three interesting comparisons: (1.) between different symbionts within the same host line, (2.) between different hosts lines with the same symbiont, and (3.) between symbiotic and non-symbiotic states within the same host line.

#### *Oxygen flux measurements*

Net photosynthesis and respiration rates were measured via oxygen (O<sub>2</sub>) evolution and consumption measurements during light and dark incubations, respectively. For this purpose, five specimens of each host-symbiont combination were transferred into 25 ml glass chambers filled with sterile seawater. Specimens were left to settle for 30 min in the dark, before magnetic stirrers were turned on to prevent stratification of the water column. Subsequently, O<sub>2</sub> concentrations were recorded once per second over the course of 30 min incubations during the light (~80 μmol photons m<sup>-2</sup> s<sup>-1</sup>, 25°C) and dark (<1 μmol photons m<sup>-2</sup> s<sup>-1</sup>, 25°C) using FireSting O2 optical oxygen meters (PyroScience, Germany). Following incubation all specimens were immediately flash frozen and stored at -20°C until further analysis. Net photosynthesis (inferred from light incubations) as well as respiration (inferred from dark incubations) rates were corrected for seawater controls and normalized to total protein content and *Symbiodinium* densities of specimens. O<sub>2</sub> fluxes of net photosynthesis

and respiration rates were transformed into their carbon equivalents using the photosynthetic and respiration quotients of 1.1. and 0.9 as proposed by Muscatine *et al.* (4). Gross photosynthesis rates were calculated according to: gross photosynthesis = net photosynthesis + |respiration| rate.

#### *Quantification of $\text{NH}_4^+$ uptake and release*

Net uptake rates were assessed on the holobiont levels during light ( $\sim 80 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ , 25°C) and dark ( $< 1 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ , 25°C) conditions using the depletion technique (70). Five specimens of each host–symbiont combination were incubated for 60 min in 25 ml chambers filled with  $\text{NH}_4^+$ -enriched artificial seawater (ASW) with a final concentration of 5  $\mu\text{M}$  (71). 10 ml water samples were collected before and after the incubation, filtered (45  $\mu\text{m}$ ) and immediately analyzed for ammonium concentrations using an autoanalyzer (SA3000/5000 Chemistry Unit, SKALAR, Netherlands). Differences in  $\text{NH}_4^+$  concentrations were corrected for seawater controls and normalized to incubation time, total host protein content and *Symbiodinium* densities of specimens to obtain net uptake rates during both light and dark incubations.

#### *Protein content, Symbiodinium density, and chlorophyll concentrations*

Frozen specimens were defrosted in 500  $\mu\text{l}$  sterile saline water and homogenized using a Micro DisTec Homogenizer 125 (Kinematica, Switzerland). Aliquots of the homogenate were immediately analyzed for total protein content as well as symbiont concentrations. For total host protein content, *Symbiodinium* cells were removed by brief centrifugation and the supernatant was analyzed with the Micro BCA Protein Assay Kit (Thermo Scientific, USA) using 150  $\mu\text{l}$  of 15x diluted tissue slurry as per manufacturer instructions. Likewise,

*Symbiodinium* density was quantified with fluorescence assisted cell sorting (BD LSRFortessa, BD Biosciences, USA) using 100 µl of strained tissue slurry.

# *Isotope labeling and sample preparation*

To verify nitrogen and carbon assimilation rates on the holobiont level, an isotopic labeling experiment was conducted for subsequent Nanoscale secondary ion mass spectrometry (NanoSIMS) analysis. Individual specimens of each host–symbiont combination were incubated for 24 h (12h:12 light dark cycle) in 25 ml incubation chambers containing ASW. For isotopic enrichment, freshly prepared ASW, essentially free from bicarbonate and ammonium, was supplemented with NaH<sup>13</sup>CO<sub>3</sub> (isotopic abundance of 99%) as well as <sup>15</sup>NH<sub>4</sub>Cl (isotopic abundance of 99%) at a final concentration of 2mM and 5 µM, respectively (adapted from Harrison *et al.* (71)). Following incubation, all specimens were immediately transferred to a fixative solution (2.5% glutaraldehyde, 1 M cacodylate) and stored at 4°C until further processing (within 14 days).

Individual tentacles were collected from each anemone under a stereomicroscope for further sample preparation adapted after Pernice *et al.* (34) and Kopp *et al.* (46). First, samples were post-fixed for 1h at RT in 1% OsO<sub>4</sub> on Sörensen phosphate buffer (0.1 M). Samples were dehydrated in a series of increasing ethanol concentrations (50%, 70%, 90%, 100%) followed by 100% acetone. Tissues were then gradually infiltrated with SPURR resin of increasing concentrations (25%, 50%, 75%, 100%). Subsequently, tissues were embedded in SPURR resin and cut into 100 nm sections using an Ultracut E microtome (Leica Microsystems, Germany) and mounted on finder grids for Transmission Electron Microscopy (ProsciTech, Australia).



# 187    *NanoSIMS imaging*

188    Gold-coated sections were imaged with the NanoSIMS 50 ion probe at the Center for  
189    Microscopy, Characterisation and Analysis at the University of Western Australia. Samples  
190    surfaces were bombarded with a 16 keV primary Cs<sup>+</sup> beam focused to a spot size of about  
191    100 nm, with a current of approximately 2 pA. Secondary molecular ions <sup>12</sup>C<sup>12</sup>C<sup>-</sup>, <sup>12</sup>C<sup>13</sup>C<sup>-</sup>,  
192    <sup>12</sup>C<sup>14</sup>N<sup>-</sup> and <sup>12</sup>C<sup>15</sup>N<sup>-</sup> were simultaneously collected in electron multipliers at a mass  
193    resolution (M/ΔM) of about 8,000, enough to resolve the <sup>12</sup>C<sup>13</sup>C<sup>-</sup> from the <sup>12</sup>C<sub>2</sub><sup>1</sup>H<sup>-</sup> peak and  
194    the <sup>13</sup>C<sup>14</sup>N<sup>-</sup> and <sup>12</sup>C<sup>15</sup>N<sup>-</sup> peaks from one another. Charge compensation was not necessary.  
195    Five images of different areas within the gastrodermis of the tentacle (25 - 45 μm raster  
196    with 256 × 256 pixels) were recorded for all targeted secondary molecular ions by rastering  
197    the primary beam across the sample with a dwell-time of 10-20 ms per pixel. After drift  
198    correction, the <sup>13</sup>C/<sup>12</sup>C or <sup>15</sup>N/<sup>14</sup>N maps were expressed as a hue-saturation-intensity image  
199    (HSI), where the color scale represents the isotope ratio. Image processing was performed  
200    using the ImageJ plugin OpenMIMS (National Resource for Imaging Mass Spectrometry,  
201    <https://github.com/BWHCNI/OpenMIMS/wiki>).  
202    Enrichment of the isotope labels was quantified for 20 ROIs (circles of 2-10 μm) per category  
203    (symbiont cells, gastrodermal host tissue and gastrodermal vesicles) for each host-symbiont  
204    combination, and expressed using δ<sup>13</sup>C and δ<sup>15</sup>N notation. Gastrodermal host tissue was  
205    quantified in the form for ROIs placed adjacent to symbiont cells as clear cell boundaries  
206    were not always identifiable.  
207    Unlabeled Aiptasia served as unlabeled controls. δ<sup>13</sup>C and δ<sup>15</sup>N enrichment was quantified  
208    as follows:

$$209 \quad \delta^{13}\text{C} = \left( \left( \frac{C_{\text{sample}}}{C_{\text{unlabelled}}} \right) - 1 \right) * 10^3 \text{ and, } \delta^{15}\text{N} = \left( \left( \frac{N_{\text{sample}}}{N_{\text{unlabelled}}} \right) - 1 \right) * 10^3$$

where N is the  $^{15}\text{N}/^{14}\text{N}$  ratio of sample or unlabeled control and C is the  $^{13}\text{C}/^{12}\text{C}$  ratio (measured as  $^{12}\text{C}^{13}\text{C}^- / ^{12}\text{C}^{12}\text{C}^-$  ions) of sample or unlabeled control, respectively. In this context, it is important to note that carbon and nitrogen incorporation at the cellular level was likely underestimated in our study as sample preparation for NanoSIMS may result in partial extraction of biomolecules.

### *Statistical analysis*

All statistical analyses were conducted with R version 3.2.2 (72). Data were tested for normal distribution using the Shapiro-Wilk test. All measurements on the holobiont level (gross photosynthesis, respiration, net  $\text{NH}_4^+$  uptake) followed normal distribution and were analyzed with a one-way analysis of variance (ANOVA) using host-symbiont combination as explanatory variable; only gross photosynthesis rates normalized by symbiont density did not follow a normal distribution and hence were analyzed with a generalized linear model (GLM) using host-symbiont combination as the explanatory variable. Similarly,  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  enrichment data did not follow a normal distribution and were analyzed in two-factorial GLMs using additive as well as interactive effects of host-symbiont combination as well as holobiont compartment (host, lipid body, symbiont). All GLMs were fitted with Gamma distribution and 'log' function to optimize the fit of the model. Fit of model residuals were confirmed using the qqPlot() function as implemented in the 'car' package for R (73). An overview of model results is provided in the Supplementary Information Table S1. Adjustment for multiple comparisons between host-symbiont combinations and holobiont compartments was done following the Bonferroni procedure. Significant differences identified via the post hoc comparison are indicated in the figures as different letters above bars.

234

## 235 **Results**

236 We investigated the relative contribution of cnidarian hosts genotypes and their  
 237 dinoflagellate symbionts to assimilate dissolved inorganic nitrogen (as ammonium ( $\text{NH}_4^+$ ))  
 238 and carbon (as bicarbonate) both at the organismal and at the cellular level in Aiptasia by  
 239 assaying four different associations of hosts and symbionts (Fig. 1A-D). Taken together,  
 240 these four host–symbiont combinations allowed us to identify nutrient dynamics in  
 241 symbiotic and non-symbiotic Aiptasia and to address the following questions: (a.) whether  
 242 different *Symbiodinium* types possess different metabolic capabilities within the same host  
 243 strain and (b.) to what extent different host strains affect the metabolic performance of the  
 244 same algal symbiont type.

245

### 246 *Carbon assimilation and translocation*

247 Host–symbiont combination of Aiptasia showed distinct differences in carbon fixation both  
 248 at the holobiont (Fig. 1E,G) as well as at the cellular level (Fig. 2A-H, see Supplementary  
 249 Information Table S1 for an overview of statistical model results). While fixation rates were  
 250 highly variable between the three groups of symbiotic Aiptasia, no carbon fixation was  
 251 detectable in non-symbiotic Aiptasia, confirming that carbon assimilation was  
 252 photosynthetically driven. At the holobiont level, gross carbon fixation (measured as gross  
 253 photosynthesis) was highest in Aiptasia of the clonal line CC7 with their native symbiont  
 254 community (*Symbiodinium* type A4) after normalization to symbiont density (Fig. 1E) or host  
 255 protein content (Fig. 1G). In contrast, CC7 Aiptasia symbiotic with Clade B (SSBO1)  
 256 *Symbiodinium* showed the lowest gross photosynthesis rates of all symbiotic Aiptasia  
 257 groupings. In particular, rates were lower than H2 Aiptasia with the same type B1

dominated symbiont community. Photosynthetic carbon fixation was more than three-fold higher than dark respiratory carbon consumption in all symbiotic Aiptasia groupings. Overall dark respiration rates followed a less defined yet similar pattern as gross photosynthesis rates of host–symbiont combinations (Supplementary Information Fig. S1A,C), with animal holobionts showing a strong positive correlation between gross photosynthesis and respiration rates (Spearman's correlation,  $r_s=0.930$ ,  $p<0.001$ ).

Isotope labeling and NanoSIMS imaging revealed that these observed differences on the holobiont level translated into an intricate picture at the cellular level (Fig. 2A-H). First,  $\delta^{13}\text{C}$  enrichment was evident in both host and symbiont cells in all symbiotic Aiptasia groupings (Fig. 2B-D). Second, although enrichment was highest in *Symbiodinium* cells, localized regions of  $< 5\ \mu\text{m}$  diameter in the host tissue (referred to as ‘lipid bodies’ from this point on) also showed significantly higher rates of enrichment compared to the surrounding host tissue. Third, Clade B *Symbiodinium* showed no differences in  $^{13}\text{C}$ -incorporation depending on the host, and incorporation rates were 30-40 % lower than in Clade A symbionts. Host lipid bodies, on the contrary, showed a reversed picture with Clade B associated H2 Aiptasia having the highest and Clade B associated CC7 Aiptasia having the lowest  $^{13}\text{C}$  assimilation rates, despite harboring the same symbiont types.

#### *NH<sub>4</sub><sup>+</sup> assimilation and release*

Similar to carbon fixation, strong differences in ammonium ( $\text{NH}_4^+$ ) assimilation were evident between the experimental groups of Aiptasia at both holobiont ( $\chi_{(3,16)}^2 = 87.44$ ,  $p < 0.01$ ) and cellular levels ( $\chi_{(3,352)}^2 = 64.06$ ,  $p < 0.01$ ). At the holobiont level, all four host—symbiont combinations showed higher  $\text{NH}_4^+$  uptake/release rates during the light (Fig. 1F,H), compared to dark conditions (Supplementary Information Fig. S1B,D). When normalized to

host protein content, non-symbiotic Aiptasia showed the highest net release of  $\text{NH}_4^+$  at the holobiont level both during light (Fig. 1H) and dark incubations (Supplementary Information Fig. S1D). Albeit significantly lower, symbiotic H2 Aiptasia also had a net release of  $\text{NH}_4^+$  into the surrounding seawater during the light incubations. In contrast, both groups of symbiotic CC7 Aiptasia showed a net uptake of  $\text{NH}_4^+$  by the holobiont during both light and dark conditions. Further, the uptake rate was affected by the associated symbiont community, with Clade A dominated CC7 holobionts taking up more  $\text{NH}_4^+$  than their Clade B infected counterparts (Fig. 1F,H).

Although  $\text{NH}_4^+$  assimilation ranged from net uptake to net release in the different experimental groups, NanoSIMS imaging confirmed that all four host–symbiont combinations incorporated  $^{15}\text{N}$  into their cells (Fig. 2I-P). Whilst  $\delta^{15}\text{N}$  signatures were highest in *Symbiodinium* cells,  $^{15}\text{N}$  assimilation was also observed within the cnidarian host tissue including that of non-symbiotic Aiptasia. Similar to  $\delta^{13}\text{C}$  patterns,  $\delta^{15}\text{N}$  enrichment in *Symbiodinium* cells aligned with algal symbiont type rather than host identity, and Clade B symbionts showed lower rates of incorporation than Clade A. Conversely,  $^{15}\text{N}$  incorporation into host cells was not significantly different between symbiotic Aiptasia groupings, irrespective of their symbiont type. Non-symbiotic CC7 Aiptasia had the lowest overall  $^{15}\text{N}$  incorporation into their tissue, yet showed small (< 5  $\mu\text{m}$  in diameter) and localized regions of high enrichment. In contrast, the afore-mentioned lipid bodies of high  $\delta^{13}\text{C}$ -enrichment showed consistently lower  $\delta^{15}\text{N}$ -signatures than surrounding host tissues in all three symbiotic Aiptasia strains.

## Discussion

Aiptasia has proven to be a powerful emerging tool for the genetic and molecular study of the cnidarian – alga symbiosis (27, 41, 43). Beyond these realms, only few studies have begun to exploit the advantages that Aiptasia has to offer (24, 25, 44, 45). Here, we set out to assess the use of Aiptasia as a model to study nutrient cycling in the cnidarian – alga symbiosis. Whilst NanoSIMS has been successfully used previously to study nutrient uptake in corals (46–48), the flexibility of the Aiptasia model enables for the first time to decouple the relative contribution of the host and symbionts to nutrient cycling. Although the methodology outlined in our approach was optimized to trace carbon and nitrogen assimilation within coral or Aiptasia holobiont (34, 46), the method can be easily modified depending on the experimental requirements. Specific labeled compounds can also be used as tracers to follow the translocation and uptake of specific molecules in complex systems by coupling the spatial resolution of NanoSIMS with the molecular characterization afforded by time-of-flight secondary ion mass spectrometry (ToF-SIMS) (49). Also, as shown here, detailed cellular insights gained from NanoSIMS will prove most powerful when integrated with traditional holobiont based measurements to identify the complexity of processes. Using this integrative approach, differences in nutrient assimilation across different host–symbiont associations became evident, both at the holobiont as well as the cellular level. Yet, only the integration of both levels of biological organization allowed to comprehensively disentangle some of the intricacies of nutrient cycling in the Aiptasia holobiont.

### *Carbon cycling in Aiptasia*

All three groups of symbiotic Aiptasia showed high rates of gross photosynthesis that exceeded their respiratory carbon requirements thereby supporting net productivity of the

holobiont required for stable symbiotic associations (4). Yet, differences in gross photosynthesis between host–symbiont combinations were evident at the holobiont level. Gross photosynthesis rates differed between the same host infected with different algal symbionts and between different hosts infected with the same algal symbionts. Thereby our findings support the findings by Stazark *et al.* (24) who reported differences in carbon flux depending on symbiont type and between heterologous and homologous symbionts in *Aiptasia*, confirming previous observations that carbon fixation depends on the interaction of both host and symbionts (24, 25, 36, 50).

At the cellular level, we observed particular areas of  $\delta^{13}\text{C}$  enrichment (hotspots) in the host tissue similar to previous observations (36, 51). This high  $\delta^{13}\text{C}$  enrichment is further coupled with lower  $\delta^{15}\text{N}$  enrichment, suggesting that these hotspots likely constitute a form of carbon storage compartments in the host tissue. Based on shape, size, and location in the tissue, these compartments are most likely lipid bodies (52). These cellular organelles are abundant in symbiotic cnidarians as they allow for rapid short-term carbon storage and remobilization depending on cellular carbon availability (53). Hence, amount, size and enrichment of these lipid bodies may be an excellent proxy to assess the amount of carbon translocated by *Symbiodinium* to the host, but further studies are needed to unequivocally determine their nature. Lipid body enrichment in the host was highest in H2 *Aiptasia* and lowest in CC7 *Aiptasia*, both associated with *Symbiodinium* type B1. Yet,  $\delta^{13}\text{C}$  enrichment in algal cells was unaffected by host identity. At the same time, our results revealed that Clade A and B symbionts had distinctly different  $\delta^{13}\text{C}$  enrichment, even in the same clonal *Aiptasia* host line. These differences are likely the consequence of differential metabolic requirements by the specific symbionts. Thus,  $\delta^{13}\text{C}$  enrichment may be a powerful tool to differentiate between symbiont types *in hospite*.

Taken together, observed differences in gross carbon fixation at the holobiont level were reflected in the combined  $\delta^{13}\text{C}$  enrichment (host tissue + lipid bodies + symbionts) at the cellular level. However, NanoSIMS data revealed that these patterns were only caused by differences in enrichment of the host lipid bodies (a proxy of carbon translocation to the host). In contrast,  $\delta^{13}\text{C}$  enrichment in algal cells differed depending on symbiont type (i.e., showed stable  $\delta^{13}\text{C}$  enrichment within the same symbiont types), but was unaffected by host identity. This apparent contradiction may have important implications for our understanding of symbiosis functioning. The fact that  $\delta^{13}\text{C}$  enrichment in algal cells differed only depending on symbiont type but was unaffected by host identity implies that symbionts retained the same amount of fixed carbon regardless of overall fixed carbon availability. Hence, only excess carbon, not consumed by algal metabolism, appears to be available for translocation to the host. Therefore, factors reducing the availability of excess carbon in the symbiont, may potentially deprive the host of its main energy source, despite harboring viable symbionts in its tissue. This ‘selfish’ aspect of the symbiosis may pose a potential threat to the stability of the holobiont under conditions of reduced fixed-carbon availability, such as those imposed by environmental stress (54, 55).

### *Nitrogen cycling in Aiptasia*

The observation of drastically different carbon fixation and translocation rates between different host–symbiont combinations raises questions regarding the underlying regulatory mechanisms of carbon cycling within these symbioses (13). Importantly, nitrogen availability *in hospite* has been proposed to be among the environmental controls of these processes (14, 15, 19, 56). Indeed, drastic differences in nitrogen assimilation became evident when comparing different host–symbiont combinations. Strikingly, the two different host lines



Aiptasia H2 and CC7 showed net  $\text{NH}_4^+$  release and  $\text{NH}_4^+$  uptake during the light, respectively, even when hosting the same algal symbionts. These findings suggest that the *in hospite* nutrient availability for the symbiont may be drastically different depending on the associated Aiptasia host. Hence, differences in gross photosynthetic activity and translocation may be partly attributed to variations in availability of nitrogen derived from the host metabolism. Interestingly, while CC7 Aiptasia showed light-enhanced  $\text{NH}_4^+$  uptake as previously reported for corals (57), H2 Aiptasia showed net release of  $\text{NH}_4^+$  during the light, contrasted by slight uptake during the dark. While we cannot explain this discrepancy at this point, it illustrates the drastic effects of host identity on nitrogen assimilation of the holobiont. At any rate, our results highlight the functional diversity and specificity of cnidarian-dinoflagellate symbioses, prompting research across a range of host–symbiont combinations.

At this point it is not possible to distinguish whether the increased  $\delta^{15}\text{N}$  enrichment of host tissues in symbiotic animals are due to direct  $\text{NH}_4^+$  fixation by the host or the translocation of fixed nitrogen by the symbiont. However, nitrogen assimilation was observed even in the absence of algal symbionts, as evidenced by non-symbiotic Aiptasia. Although these animals showed a high net release of  $\text{NH}_4^+$  at the holobiont level, NanoSIMS imaging confirmed the incorporation of  $^{15}\text{N}$  within localized hotspots of their tissue at low rates. While the exact nature of these hotspots remains unknown at this point, our results confirm that Aiptasia also has the ability to assimilate inorganic nitrogen from seawater as previously reported for corals (34). However, it remains to be determined whether this capability is intrinsic to the host cellular machinery or a function of associated bacterial symbionts or both (48).

In contrast to  $\delta^{15}\text{N}$  enrichment of their hosts, *Symbiodinium* types showed characteristic  $\delta^{15}\text{N}$  enrichment patterns regardless of the identity of their host. Hence,  $\delta^{15}\text{N}$  enrichment

may prove a useful tool to identify symbiont identity *in hospite*, especially when combined with  $\delta^{13}\text{C}$  measurements.

Different to carbon fixation measurement, patterns of  $\text{NH}_4^+$  uptake on the holobiont level were not directly reflected in the overall  $\delta^{15}\text{N}$  enrichment on the cellular level. Specifically, symbiont-free CC7 Aiptasia as well as symbiotic H2 Aiptasia showed net release of  $\text{NH}_4^+$  from the holobiont during light conditions, yet NanoSIMS analysis confirmed the incorporation of  $^{15}\text{N}$  from surrounding seawater. While these differences may be partly attributed to differences in incubation time and light availability for the two measurements, they further suggest that uptake and release of  $\text{NH}_4^+$  appear to be in a dynamic equilibrium in Aiptasia. Hence, the stable  $\delta^{15}\text{N}$  enrichment of the same symbiont type in CC7 and H2 suggests that the contribution of nitrogen derived from host metabolism was negligible compared to the incorporation of nitrogen from seawater under these conditions. Under natural oligotrophic conditions, however, host metabolism may make a significant contribution to the nitrogen supply of the symbiont.

#### *Deciphering the role of nutrient cycling in cnidarian holobionts*

Our results suggest (I.) that nutrient cycling is drastically altered between symbiotic and non-symbiotic Aiptasia; (II.) that different *Symbiodinium* types possess different metabolic capabilities within the same Aiptasia strain and (III.) that different Aiptasia strains affect the metabolic performance of the same algal symbiont. Although our results require further validation with regard to their wider applicability beyond the Aiptasia model system, our findings showcase the distinct advantages of a model system approach for the study of nutrient cycling in the the cnidarian – dinoflagellate symbiosis. However, questions remain regarding the precise nature of nutrients exchanged in this symbiosis and the underlying

processes involved. Since nutrient exchange is arguably the functional basis of mutualistic association (7), providing answers to these questions is one of the keys to understanding holobiont functioning (14). Furthermore, nutrient cycling is likely a dominant driver of holobiont fitness under varying environmental conditions (58–61). Understanding environmental controls of nutrient cycling may therefore help to provide novel insights on the mechanisms of symbiosis establishment, maintenance, and disruption.

In this context, we formulate three important questions that are relevant for future studies of nutrient cycling in cnidarian holobionts:

**(I.) How does symbiont diversity affect nutrient exchange within the symbiosis and how does it influence holobiont success under varying environmental conditions?** It has been previously observed that the performance of symbionts depends on the environmental conditions (e.g. during coral bleaching) (62). While many studies have investigated the role of oxidative stress in these phenomena (63, 64), nutrient cycling is likely another important factor involved.

**(II.) How is nutrient cycling regulated during symbiosis establishment and maintenance?** In contrast to mature coral holobionts, carbon translocation by symbionts appears to be negligible in early stages of symbiosis establishment (51, 65). Understanding the processes around initiating and stabilizing this nutrient exchange during symbiosis development will advance our understanding of the factors underlying the success of this symbiosis.

**(III.) What is the role of bacteria and other microbes in holobiont nutrient cycling?** It is widely acknowledged that carbon, nitrogen, and sulfur cycling microbes are ubiquitous members of the cnidarian microbiome (32, 66, 67). However, questions remain regarding their relevance and contribution to holobiont function. Studying nutrient exchange between

these microbes and other members of the holobiont is necessary to evaluate the importance of the microbiome for holobiont fitness. Future research efforts incorporating a model system approach with field-based coral studies, will transform our understanding of the mechanisms underlying this symbiosis and may prompt new solutions to prevent further loss and degradation of reef ecosystem.

#### **Conflict of interest**

None declared.

#### **Author contributions**

NR, MA and CRV conceived and designed the experiment. NR, JBR, MP and GP conducted the experiment. PG and MRK carried out NanoSIMS data acquisition. All authors wrote, revised and approved the manuscript.

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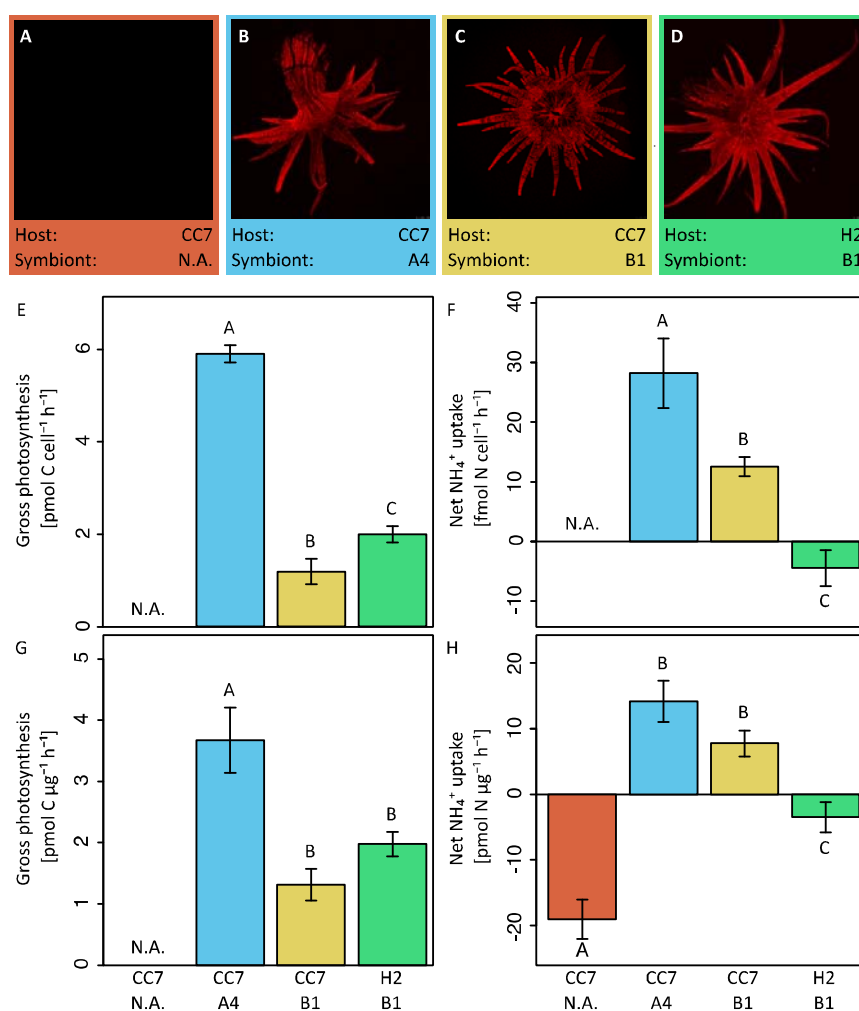


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## Figures



**Fig. 1** Ammonium (NH<sub>4</sub><sup>+</sup>) uptake and carbon fixation (gross photosynthesis) of Aiptasia.

Fluorescence microscopy overview of the four host–symbiont combinations (A–D) to

visualizes chlorophyll autofluorescence of endosymbiotic *Symbiodinium*. Gross

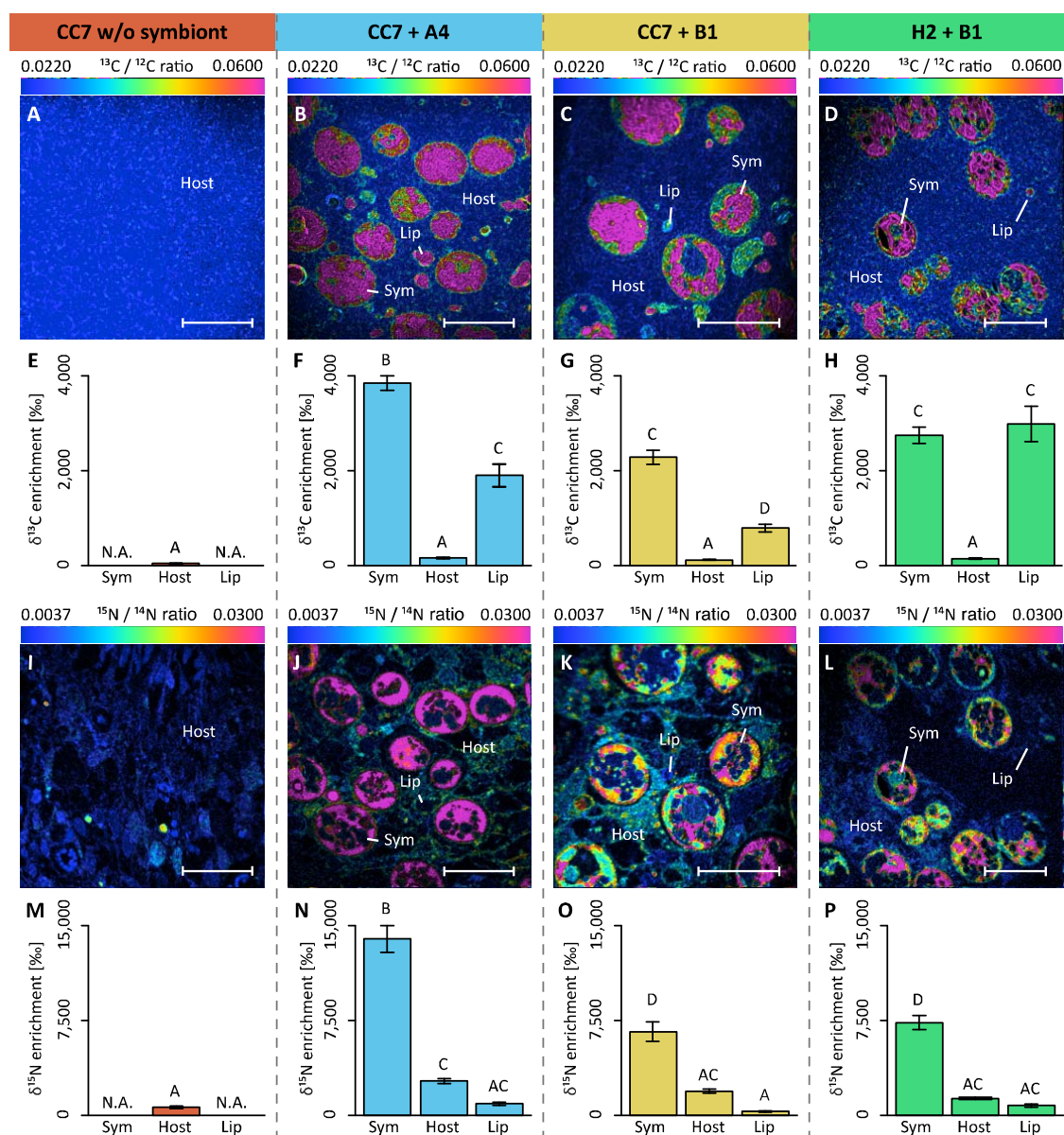
photosynthesis (E,G) and net NH<sub>4</sub><sup>+</sup> uptake (F,H) of Aiptasia were normalized either to

symbiont density (E,F) or total host protein content (G,H). Gross photosynthesis rates were

calculated as the sum of net photosynthesis and respiration rates ( $P_G = P_N + R$ ). Net NH<sub>4</sub><sup>+</sup>

uptake was quantified with the ammonium depletion method. All data shown as mean ± SE.

Different letters above bars indicate significant differences between groups ( $p < 0.05$ ).



**Fig. 2** NanoSIMS imaging and quantification of cell specific carbon (as  $^{13}\text{C}$ -bicarbonate) and nitrogen (as  $^{15}\text{N}$ -ammonium) assimilation within the Aiptasia—*Symbiodinium* symbiosis. Representative images of the distribution of  $^{13}\text{C}/^{12}\text{C}$  ratio (**A-D**) and of  $^{15}\text{N}/^{14}\text{N}$  ratio (**I-L**) within the Aiptasia holobiont are displayed as Hue Saturation Intensity (HSI). The rainbow scale indicates the  $^{13}\text{C}/^{12}\text{C}$  and  $^{15}\text{N}/^{14}\text{N}$  ratio, respectively. Blue colors indicate natural abundance isotope ratios shifting towards pink with increasing  $^{13}\text{C}$  and  $^{15}\text{N}$  incorporation levels, respectively. Corresponding  $\delta^{13}\text{C}$  enrichment (**E-F**) and  $\delta^{15}\text{N}$  enrichment (**M-P**) in the

710 tissues of the four host–symbiont combinations. For each NanoSIMS image, the  $\delta^{13}\text{C}$  (E-F)  
 711 and  $\delta^{15}\text{N}$  (M-P) enrichment were quantified for individual Regions Of Interest (ROIs) that  
 712 were defined in OpenMIMS by drawing (I) the contours of the symbionts, and circles  
 713 covering (II) the adjacent host tissue and (III) the host lipid bodies. Scale bars represent 10  
 714  $\mu\text{m}$ . Abbreviations: Sym = *Symbiodinium* cell, Host = tissue (host) & Lip = lipid body (host).  
 715 All data shown as mean  $\pm$  SE. Different letters above bars indicate significant differences  
 716 between groups ( $p < 0.05$ ).  
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