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Chemical communication in the symbiotic interaction between the *anemone Exaiptasia diaphana* (ex *Aiptasia pallida*) Rapp and the dinoflagellate *Symbiodinium* spp.

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ABSTRACT
The dinoflagellate *Symbiodinium* sp. establishes symbiotic relationships with the anemone *Exaiptasia diaphana* Rapp. The process that leads to the reciprocal recognition of the two symbiotic partners is still not very well understood. We hypothesize that chemical signals are exchanged between the *Exaiptasia diaphana-Symbiodinium* holosymbiont and aposymbiotic anemones or between free living *Symbiodinium* and holo- and aposymbiotic *Exaiptasia*, leading to changes in organic and elemental compositions in the aposymbiotic anemones. In order to test these hypotheses, bleached anemones were exposed to the presence of either free living *Symbiodinium,* previously extracted from the same *Exaiptasia* clone, or to holobionts. The *ex-hospite* algae and the holobionts were included in dialyses membranes with a cut-off of 14 000 Da. In the control treatments, the experimental samples were exposed to the presence of empty dialysis tubes. The organic composition and the elemental composition of the anemones were determined by Fourier Transform Infrared Spectroscopy and Total Reflection X-Ray Fluorescence Spectroscopy/Gas Chromatography, respectively. The fact that both the organic and elemental composition of the experimental aposymbiotic anemones differed significantly from the controls, in the absence of any obvious nutritional effect, is suggestive of an exchange of chemical signals between the aposymbiotic and holosymbiotic anemones.

KEY WORDS
Cell composition, elemental stoichiometry, FTIR, infochemicals, symbiosis.

RÉSUMÉ

Communication chimique dans l'interaction symbiotique entre l'anémone Exaiptasia diaphana *(ex Aiptasia pallida) et le dinoflagellé* Symbiodinium *spp.*

Les dinoflagellés *Symbiodinium* sp. établissent des relations symbiotiques avec l'anémone *Exaiptasia diaphana* Rapp. Le processus menant à la reconnaissance réciproque des deux partenaires symbiotiques n'est pas encore très bien compris. Nous émettons l'hypothèse que des signaux chimiques sont échangés entre les holosymbiontes *Exaiptasia diaphana-Symbiodinium* et les anémones aposymbiontiques ou entre les *Symbiodinium* libres et les *Exaiptasia* holo- et aposymbiotiques, entraînant des modifications de la composition organique et élémentaire des anémones aposymbiontiques. Afin de vérifier ces hypothèses, des anémones blanchies ont été exposées à la présence de *Symbiodinium* libres, préalablement extrait du même clone d'*Exaiptasia*, ou à des holobiontes. Les algues *ex-hospite* et les holobiontes ont été inclus dans les membranes de dialyse ayant un seuil de coupure de 14 000 Da. Dans les traitements de contrôle, les échantillons expérimentaux ont été exposés à la présence de tubes de dialyse vides. La composition organique et la composition élémentaire des anémones ont été déterminées respectivement par spectroscopie infrarouge à transformée de Fourier et par spectroscopie de fluorescence X à rayons X à réflexion totale. Le fait que la composition organique et élémentaire des anémones aposymbiontiques expérimentales diffèrent significativement des témoins, en l'absence d'un effet nutritionnel évident, suggère un échange de signaux chimiques entre les anémones aposymbiontiques et holosymbiontiques.

MOTS CLÉS
Composition cellulaire, stoechiométrie des éléments, spectroscopie IRTF, messager chimique, symbiose.

INTRODUCTION

The dinoflagellates that establish symbiosis, commonly called zooxanthellae, mainly belong to the genus *Symbiodinium* Freudenthal; they can establish relations with a variety of hosts, including protists, sponges, cnidarian and mollusks (Trench 1993; Glynn 1996; Rowan 1998; Lobban *et al.* 2002) The relationship is extremely dynamic and zooxanthellae frequently enter and exit the symbiosis (or are acquired and expelled). Smith & Muscatine (1999) reported that 106 zooxanthellae leave a typical coral host every day and expulsion/escape rates increase in situations of nutritional stress.

Symbioses can be affected and regulated by infochemicals. For instance, communication between plants and fungi occurs primarily through the release and detection of infochemicals that diffuse through the soil matrix (Barto *et al.* 2012). Glycan-lectin signaling is present in several symbiotic relationships (e.g., the nod factors in *Rhizobium*-leguminous plant symbioses; Cooper 2007); it is believed that this kind of chemical signaling also plays a role in the host-symbiont recognition in cnidarian-dinoflagellate associations (Lin *et al.* 2000; McGuinness *et al.* 2003; Koike *et al.* 2004; Wood-Charlson *et al.* 2006; Jimbo *et al.* 2010; Davy *et al.* 2012). Inducible and membrane associated C-type lectins are commonly found in cnidarians (McGuinness *et al.* 2003) and glycans have been found on the external surface of *Symbiodinium* cells (Lin *et al.* 2000; Davy *et al.* 2012). It has been shown that glycan removal from *Symbiodinium* cells significantly decreases the infection rates in anemones from genus *Exaiptasia* (Lin *et al.* 2000; Wood-Charlson *et al.* 2006). Furthermore, lectins have been shown to induce the transformation of flagellated *Symbiodinium* cells into a coccoid stage, which is the morphotype present in the symbioses (Koike *et al.* 2004). Although the glycan-lectin recognition probably requires a physical contact between host and symbiont (Lin *et al.* 2000), Hagedorn *et al.* (2015) showed that trehalose acts as chemical signals in the establishment of cnidarians-algae symbiosis, in the absence of direct contact. Upon the establishment of symbioses, the mode of nutrition of the anemone changes, with consequent modification in the animal cells composition. We therefore designed experiments to monitor the composition of aposymbiotic specimens of the anemone *Exaiptasia diaphana* Rapp, previously known as *Aiptasia diaphana*, which we used as a signal of the onset of the symbiotic process. These compositional changes were employed to understand if the relationship between free living *Symbiodinium* or the holosymbiont and the aposymbiotic anemone is initiated by the exchange of infochemicals prior to any direct physical interaction. The symbiotic pair *Symbiodinium-Exaiptasia* was selected because it is a well-established experimental model for dinoflagellate-cnidarian interaction (Muller-Parker 1985; Davy & Cook 2001; Mobley & Gleason 2003; Bachar *et al.* 2007).

MATERIAL AND METHODS

Experimental design

Bleached, aposymbiotic *Exaiptasia diaphana* anemones were exposed to the presence of either the holobiont or *Symbiodinium* cells extracted from the same monoclonal *Exaiptasia* population from which the bleached specimens were obtained. The parties involved in the alleged communication were separated by dialysis membrane with a cut-off of 14 kDa (Medicell Membranes Ltd, London, UK); either the free *Symbiodinium* (*Symbiodinium* treatment) or the holobiont (*Exaiptasia* treat-

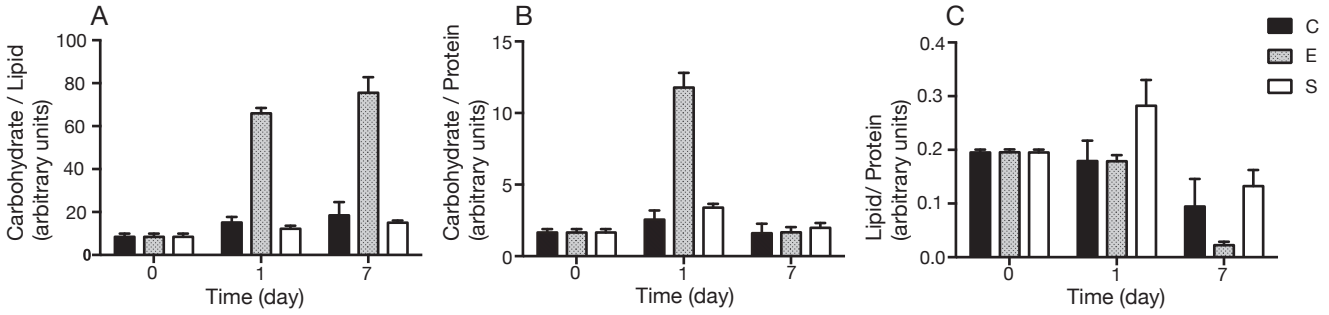

Fig. 1. — Organic composition in bleached *Exaiptasia diaphana* Rapp samples. The error bars represent the standard deviation (n ≥ 3). Abbreviations: **C**, Aposymbiotic *Exaiptasia* samples exposed to an empty dialysis tube (control group); **E**, Aposymbiotic *Exaiptasia* samples exposed to a dialysis tube containing the holobionts; **S**, Aposymbiotic *Exaiptasia* samples exposed to a dialysis tube containing *Symbiodinium* cells.

ment) were put in a dialysis tube; the aposymbiotic specimens were maintained outside of the dialysis tube.

Light (PAR) was provided by fluorescent lamps (Feilo Sylvania Europe Limited, London, UK) with a color temperature of 10 000 K; the photon flux density at the bottom of the tank was 30 µmol m^2 s^{-1}; the photoperiod was 12 h light:12 h dark. The temperature in the tank was 27°C, the salinity was 30, the pH 8.2 and NO_2 and NH_3 concentrations were below 0.03 ppm.

The experiments were conducted in 300 ml tanks, each containing 4 aposymbiotic anemones. Changes in composition (see below) of the aposymbiotic *Exaiptasia* were monitored at the beginning of the incubation (day 0), after 1 day and after 7 days. For each treatment and sampling time, we used three tanks of anemones (total number of tanks = 18). For each tank, one anemone was used to assess the organic composition by FTIR spectrometry, one to investigate the elemental compositions with a CHN analyzer and one to quantify the presence of additional elements by Total Reflection X-ray Fluorescence spectrometry (TXRF). The fourth anemone was used as a spare, in case of problems during the preparation for the analyses or death during the experimental treatment (death never occurred).

Rearing of the anemones

The *Exaiptasia* population used for all the experiments was obtained from a single individual, which was cut in 4 parts that were then allowed to grow and asexually generate new individuals. Monoclonal *Exaiptasia* individuals were used in order to minimize the influence of genetic heterogeneity among samples.

The anemones were maintained in glass tanks with artificial seawater (ASW; Prodac Ocean Reef, Prodac International, Cittadella, Italy). Temperature and light conditions in the rearing tanks were the same as those described for the experiments. The anemones were fed 2 times per week with *Artemia salina* nauplii obtained from INVE, Belgium. In order to avoid regurgitation, nutrition was carried out according to Leal *et al.* (2012), with 1 naplius·mL^{-1}; the artificial seawater was filtered to avoid the presence of particulate. The medium was replaced 24 hours after feeding. Every 2 weeks, the anemones were transferred to a clean tank.

Symbiont extraction

Symbiodinium cells were isolated from 20 specimens of *Exaiptasia diaphana*, randomly selected from our experimental monoclonal population. *Symbiodinium* extraction was done according to Yacobovitch *et al.* (2004). The extracted algal cells were checked for integrity under a microscope (Leitz hm-lux 3, Leica Microsystem, Wetzlar, Germany) and their physiological conditions were assessed via PSII quantum efficiency measurements effected with a Pulse Amplitude Modulated (PAM) fluorometer (Dual-PAM-100, Walz GmbH, Effeltrich, Germany); Fv/Fm values of *c.* 0.5 were consistently obtained.

Bleaching protocol

Our bleaching protocol, derived from Lehnert *et al.* (2012), was as follows: sterile ASW was cooled to 4°C in the dark. The anemones were then placed in the tank. After 4 h, the 4°C water was replaced with water at 27°C, which was replaced daily. As soon as the anemones started to open and move their tentacles again, extend their foot and react to tactile stimuli, the thermal shock was repeated. This procedure produced animals apparently deprived of algae in about two weeks. Observation with a fluorescence microscope (ZOE Fluorescent Cell Imager, Bio-Rad, Hercules, California, USA) revealed that few *Symbiodinium* cells were still present; therefore, the anemones were incubated in the dark for 30 min, in the presence of 50 µM of 3-(3,4-Dichlorophenyl)-1-1dimethylurea (DCMU) and then irradiated with a photon flux density of 240 µmol m^{-2} s^{-1} for 30 min. Until completely bleached, the animals were kept in the dark and not fed, to avoid food regurgitation and death. The anemones were subsequently transferred to the same culture conditions described above for holosymbiotic *Exaiptasia*, with the only difference that they were kept in the dark and fed weekly.

Organic composition

The organic composition of aposymbiotic *Exaiptasia diaphana* was assessed using a Tensor 27 Fourier Transform InfraRed (FTIR) spectrometer (Bruker Optik GmbH, Ettlingen, Germany). Anemones were frozen, homogenized with a ceramic mortar and a pestle and then resuspended in 200 µl of Milli-Q water; 50 µl of homogenate were deposited on a silicon

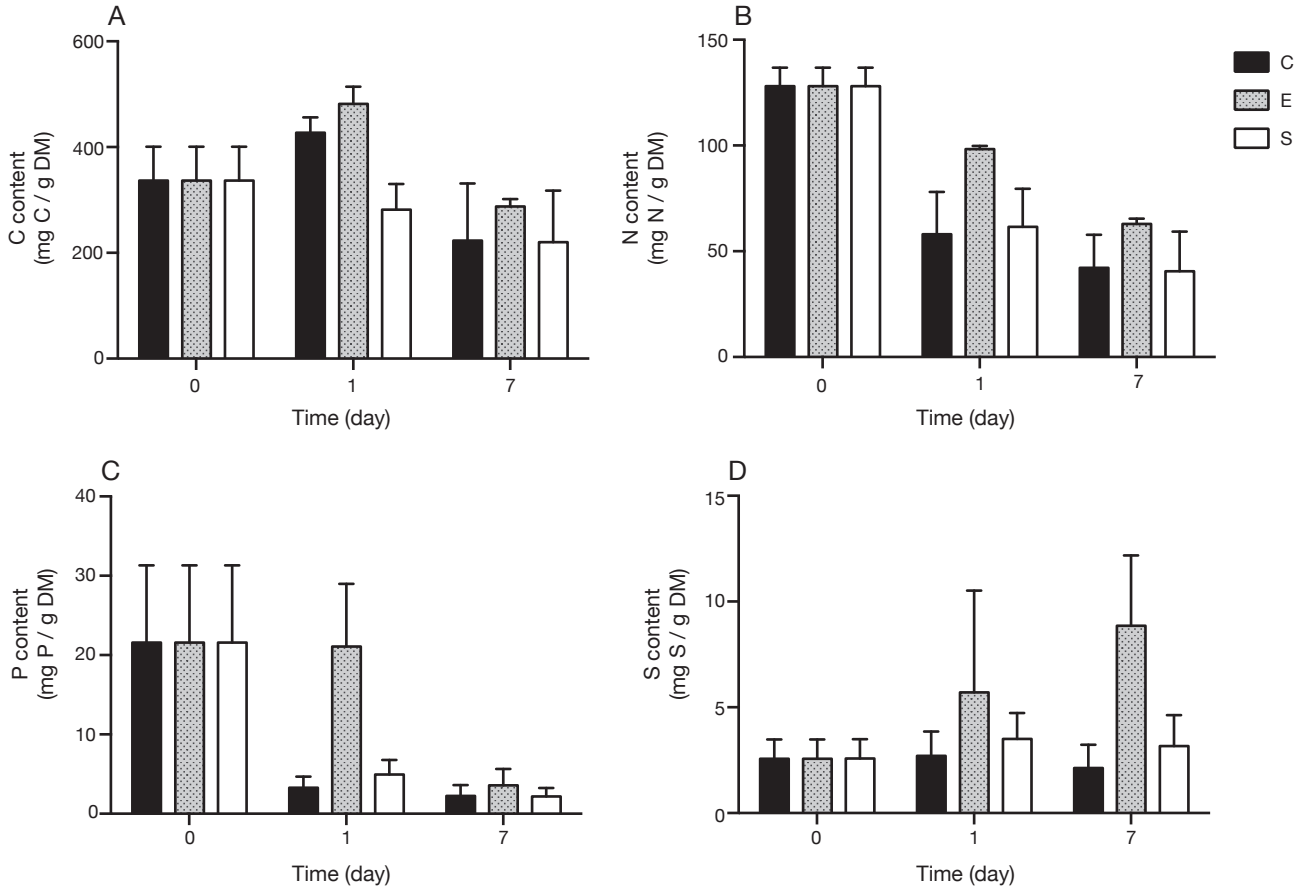

FIG. 2. — Carbon (**A**), Nitrogen (**B**), Phosphorus (**C**), Sulphur (**D**) mean contents in bleached *Exaiptasia diaphana* Rapp samples. The error bars represent the standard deviation (n ≥ 3). Abbreviations: **C**, Aposymbiotic *Exaiptasia* samples exposed to an empty dialysis tube (control group); **E**, Aposymbiotic *Exaiptasia* samples exposed to a dialysis tube containing the holobionts; **S**, Aposymbiotic *Exaiptasia* samples exposed to a dialysis tube containing *Symbiodinium* cells.

window (Silicon FZ 13 mm diameter × 1 mm thickness, polished, Crystran Limited, Poole, UK). Blanks were prepared by depositing 50 µl Milli-Q water on silicon windows. Samples and blanks were then dehydrated in an oven at 80°C, for 6 h. Spectra acquisitions, normalizations and analysis were done according to Domenighini & Giordano (2009), Marchetti *et al.* (2010) and Palmucci *et al.* (2011).

Elemental composition

The C and N contents were measured with a CHN analyzer (Carlo Erba EA-1108) according to Montechiaro & Giordano (2010).

The content of P, S and of main trace elements was determined by Total Reflection X-ray Fluorescence spectrometry (TXRF). For these measurements, the procedure by Fanesi *et al.* (2014) was followed with some modifications. All material for sample preparation and elemental analysis was acid-washed in 10% trace-metal grade hydrochloric acid at 60°C, for at least 12 hours. It was then thoroughly rinsed in Milli-Q H2O. In order to minimize the contamination from ambient metals, we washed our sample with an Oxalate-EDTA (OE) solution prepared as follows: for a final volume of 100 ml, 1.95 grams EDTA-Na_2:$2H_2O$ and 0.5 grams NaCl were added to about 60 ml milli-Q water. This solution was brought to pH 6.5 with 10 M NaOH. Next, 1.26 grams of oxalic acid ($C_2H_2O_4$:$2H_2O$) were dissolved in the solution and the pH was adjusted to pH 6.5 with 10 M NaOH. The resultant solution was brought to 100 ml volume and stored at 4°C for a maximum of 60 days. Prior to the measurements, the anemones were soaked for 5 min in the OE solution; they were then washed in 0.5 M NaCl for further 5 min to eliminate any residue of the OA solution, and in Milli-Q water for few seconds, to wash away excess of NaCl.

Statistics

Statistical tests were conducted using the Graph Pad Prism software, version 6.05 (GraphPad Software, San Diego, California, United States) and the R software (version 3.5.1, R Development Core Team 2018). Differences between treatments and times were compared after testing the normality of the distributions with the Shapiro-Wilk normality test (Royston 1995) and the homogeneity of variances with the Levene's Test of Equality of Variances from the Lawstat package for R (version 3.2; Levene 1960). The dataset was transformed as log+x before being analyzed with two-way analyses of variance (ANOVA; Chambers *et al.* 1992), followed by TukeyHSD post-hoc tests (Yandell 1997). The level

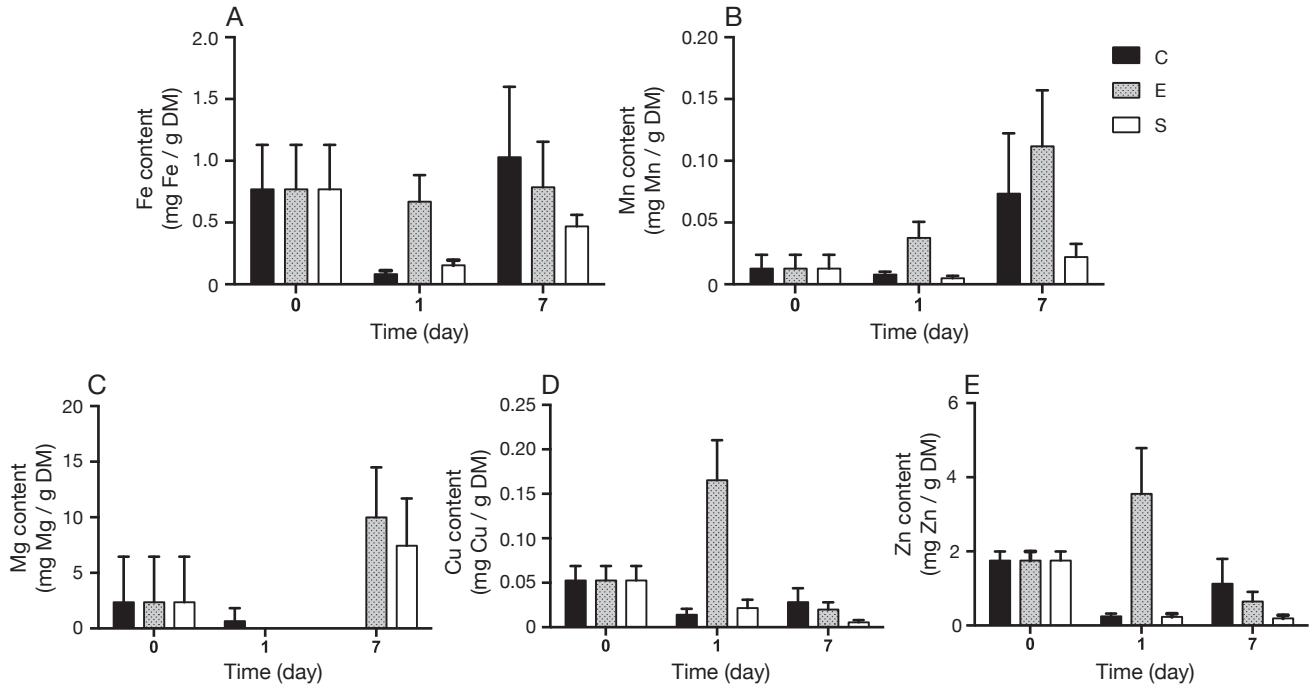

Fig. 3. — Iron (**A**), Manganese (**B**), Magnesium (**C**), Copper (**D**), Zinc (**E**) mean contents in bleached *Exaiptasia diaphana* Rapp samples. The error bars represent the standard deviation (n ≥ 3). Abbreviations: **C**, Aposymbiotic *Exaiptasia* samples exposed to an empty dialysis tube (control group); **E**, Aposymbiotic *Exaiptasia* samples exposed to a dialysis tube containing the holobionts; **S**, Aposymbiotic *Exaiptasia* samples exposed to a dialysis tube containing *Symbiodinium* cells.

of significance was set at 95%. Tuckey's post-hoc tests were only performed and described for those analyses and factors that showed significant results. All results were expressed as the mean of at least three biological replicates and standard deviations.

RESULTS

Relative size of the main organic pools

The relative sizes of the main organic pools are shown in Fig. 1. Carboidrate:Lipid ratio, the Carboidrate:Protein ratio and the Lipid:Protein ratio differed significantly between the two treatments ($p<0.001$; Appendix 1). Carboidrate:Lipid for the whole duration of the treatment, and Carboidrate:Protein at day 1, were higher in the *Exaiptasia* treatment, whereas these ratios were not appreciably different between the *Symbiodinium* treatment and control (Carboidrate:Lipid, *Exaiptasia* vs Control: $p<0.001$; Carboidrate:Protein *Exaiptasia* vs Control: $p<0.001$). Significant changes over time were observed for the Carboidrate:Protein ratio ($p<0.001$) and for the Lipid:Protein ratio ($p<0.001$; Appendix 1). Significant interactions between the two factors (time and treatments) were detected for the Carboidrate:Protein ratio ($p<0.001$; Appendix 1). Tuckey's post-hoc test revealed that the differences between the Carboidrate:Lipid ratio and the Carboidrate:Protein ratio were significant only in the *Exaiptasia* treatment (Carboidrate:Lipid, *Exaiptasia* vs Control: $p<0.001$; Carboidrate:Protein Exaiptasia vs Control: $p<0.001$). The differences in the Lipid:Protein ratios were confirmed by the statistical analysis only for the *Symbiodinium* treatment (Lipid:Protein, *Symbiodinium* vs Control: $p<0.05$. Appendix 1). No change was detected in the holosymbiont, during the experiments.

Elemental composition

The elemental composition of the aposymbiotic *Exaiptasia* exposed to holosymbiotic *Exaiptasia*, free *Symbiodinium* cells and control conditions are depicted in Fig. 2 (C, N, P, S) and Fig. 3 (Fe, Mn, Mg, Cu, and Zn).

ANOVA showed significant differences between the treatments for all the macronutrients in Fig. 1 (C, $p<0.05$; N, $p<0.05$; P, $p<0.001$; S, $p<0.05$; see Appendix 2 for the outcome of the statistical tests). Time was a significant factor for C ($p<0.01$), N ($p<0.05$), and P ($p<0.001$), but not for S ($p>0.05$; Appendix 2). Significant interactions between time and treatment existed for P ($p<0.001$; Appendix 2). The amount of P decreased with time for all treatments and for the controls (Fig. 2C); the only exception was the *Exaiptasia* treatment at day 1 ($p<0.001$). By day 7, the amount of P was equal in all the experimental groups. The Tuckey's post-hoc test did not show significant differences among treatments ($p>0.05$) for C and revealed that only the *Exaiptasia* treatment different significantly from the control for the N, P and S contents (Fig. 2; *Exaiptasia* vs Control for N, S: $p<0.05$. *Exaiptasia* vs Control for P: $p<0.001$).

For the data in Fig. 2, significant differences between the *Exaiptasia* and *Symbiodinium* treatment existed for the cell content of Mg ($p<0.01$), Cu ($p<0.001$) and Zn ($p<0.001$);

the same was not true for Fe and Mn (ANOVA; Appendix 3). Mg became significantly higher in both the treatments at day 7 (Fig. 3C). The levels of Cu and Zn showed a higher value in the *Exaiptasia* treatment, at day 1 (Fig. 3D, E). Time was a significant factor for all the elements, Fe ($p<0.01$), Mn ($p<0.01$), Mg ($p<0.001$), Cu ($p<0.001$), and Zn ($p<0.05$; Appendix 3). Significant interactions between time and treatments existed for Mg ($p<0.01$), Cu ($p<0.001$), and Zn ($p>0.001$) (Appendix 3). The Tuckey's post-hoc test revealed that Cu and Zn were influenced by the *Exaiptasia* treatment (*Exaiptasia* vs Control for Cu: $p<0.001$. *Exaiptasia* vs Control for Zn: $p<0.01$), but were not affected by the *Symbiodiniu*m treatment. Both the *Exaiptasia* and the *Symbiodinium* treatments were different from the Controls for the amount of Mg (*Exaiptasia* vs Control: $p<0.01$. *Symbiodinium* vs Control: $p<0.05$).

DISCUSSION

Our initial hypothesis was that chemical communication plays a role in the establishment of symbiosis between *Exaiptasia* and *Symbiodinum*. A chemical interaction between the partners of the symbiosis (i.e. aposymbiotic *Exaiptasia* and free living *Symbiodinium*) was in our expectations. However, our experiments did not show evidence of such interactions, whereas holosymbiotic *Exaiptasia* appeared to have an effect on the aposymbiotic *Exaiptasia*. This short communication was meant to be an initial step in the investigation of the chemical interaction between the partners of the cnidarian-dinoflagellate symbiosis and a proof of concept for our experimental approach. Especially the lack of response of aposymbiotic *Exaiptasia* to the exposure to free *Symbiodinium* cells leaves a number of questions open and a lot of space for improvement in future experiments. For instance, the extracted *Symbiodinium* cells differed from those typically found in nature, because they were mostly in a coccoid stage (while in nature they are usually flagellated). It may thus be possible that they were not in a stage that allowed chemical communication.

More revealing are the data from the interaction between holosymbiotic and aposymbiotic *Exaiptasia*: our experiments indicated that the holosymbiont is able to elicit a compositional response in the aposymbiotic individuals. It is possible that this happens through the simple transfer of compounds from the holosymbiont to the aposymbiotic anemones. This transfer, if the molecules that are acquired by the aposymbiont are assimilated, should also lead to a nutritional effect resulting in growth stimulation. The main change that we observed in the "*Exaiptasia* treatment" was an increase in the carbohydrate to protein ratio. If the increase of this ratio was due to a higher carbohydrate content, it may be possible that carbohydrates released by the holosymbiont (Markell & Trench 1993; Brown & Bythell 2005) were acquired by our free of symbionts *Exaiptasia*. In fact, the increase of the carbohydrate to protein ratio observed in the "*Exaiptasia* treatment" at day 1 seems to be due to a decline in the protein content and not to an increase in carbohydrates, since the C/N ratio was appreciably lower at day 2 than at day 0 (especially) and day 7 (Fig. 2). The fact that no exogenous food source was received by the anemones during the "*Exaiptasia* treatment" is also confirmed by the fact that no obvious specific nutritional effect was observed in the "*Exaiptasia* treatment": for a nutritional effect to be demonstrated, it is necessary that the acquired exogenous compounds are assimilated and cause an increase in the biomass of the target organisms. This did not happen in our experiments: anemones from both treatments and from control experiments showed similar carbon contents (mg C / g DW). The changes that occurred in the composition of the aposymbiotic anemones are thus most likely generated by an endogenous reorganization of the organic pools elicited by signals of non-nutritional nature. Although the increase in the carbohydrate to protein ratio increase in our experiments seems to be mostly due to a decline in the protein content, modest amounts of carbohydrates may be acquired. Consequently, we cannot exclude that, similarly to what was proposed by Hagedorn *et al.* (2015) for trehalose in coral symbioses, small non-reducing sugars constitute the signal that triggers the response we observed, at low concentration.

Speculatively, it may be proposed that the reorganization of internal pools requires the intervention of metallo-proteins using Fe, Cu and Zn, elements that were also found to increase in parallel to the carbohydrate:protein ratio. This would have to occur at the expenses of other proteins, since, overall, protein decrease. *Ad hoc* experiments are required to directly test this hypothesis.

In conclusion, the cell composition of aposymbiotic *Exaiptasia diaphana* changed significantly when the experimental organisms were exposed to symbiotic anemones; this does not seem to be due to transfer of material from the holosymbionts nor to nutritional effects; although further confirmation is required, our observation suggest that chemical communication occurs between symbiotic and non-symbiotic *Exaiptasia.* The molecule(s) that trigger the interactions between the holobiont and the aposymbiotic anemones have to be small enough to diffuse through a dialysis membrane with a cut off of 14 000 Da. For logical reasons, it is unlikely that the onset of symbiosis requires the pre-existence of holobionts. It is however not uncommon that infochemicals are used: a) to distribute a response throughout a population; and b) in feed forward mechanisms that allow the acceleration of a response enactment till steady state is attained (Venuleo *et al.* 2017). We thus propose that the establishment of symbiotic relations between *Exaiptasia* and *Symbiodinium* facilitates the propagation of the symbiosis throughout the population, predisposing *Exaiptasia* individuals that have not yet been colonized to accept *Symbiodinium* cells.

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APPENDICES

Appendix 1. — Summary of ANOVA results for the measurement of the Carbohydrates:Lipids ratio, Proteins:Lipids ratio, Lipids:Proteins ratio. The analyses were conducted on transformed data (log+x). Abbreviations: **Df**, degrees of freedom; **Sum Sqs**, sum of squares; **Mean Sqs**, mean of squares. Asterisks indicate statistical significances.

	Df	Sum Sqs	Mean Sqs	F value	P
Carbohydrates:Lipids					
Treatment	2	5.556	2.778	136.133	5.66 E-09 ***
Time	1	0.068	0.069	3.356	0.092
Treatment:Time	2	0.000	0.000	0.004	0.997
Residuals	12	0.245	0.020		
Carbohydrates:Proteins					
Treatment	2	0.266	0.132	78.180	1.31 E-07 ***
Time	1	0.333	0.333	196.270	8.47 E-09 ***
Treatment:Time	2	0.280	0.140	82.460	9.74 E-08 ***
Residuals	12	0.020	0.002		
Lipids:Proteins					
Treatment	2	0.000	0.000	13.942	0.000737 ***
Time	1	0.001	0.001	62.708	4.17 E-06 ***
Treatment:Time	2	0.000	0.000	2.076	0.168
Residuals	12	0.000	0.000		

Appendix 2. — Summary of ANOVA for the measurements of the amounts of Carbon, Nitrogen, Phosphorus, and Sulfur. The analyses were conducted on transformed data (log+x). Abbreviations: **Df**, degrees of freedom; **Sum Sqs**, sum of squares; **Mean Sqs**, mean of squares. Asterisks indicate statistical significances.

	Df	Sum Sqs	Mean Sqs	F value	P
Carbon					
Treatment	2	0.745	0.372	4.735	0.03049 *
Time	1	0.832	0.832	10.574	0.00693 **
Treatment:Time	2	0.036	0.018	0.226	0.80118
Residuals	12	0.944	0.079		
Nitrogen					
Treatment	2	0.735	0.368	5.103	0.0249 *
Time	1	0.543	0.543	7.536	0.0178 *
Treatment:Time	2	0.016	0.008	0.108	0.899
Residuals	12	0.865	0.072		
Phosphorus					
Treatment	2	1.188	0.594	30.430	1.99 E-05 ***
Time	1	0.853	0.853	43.730	2.49 E-05 ***
Treatment:Time	2	0.788	0.394	20.200	0.000144 ***
Residuals	12	0.234	0.020		
Sulfur					
Treatment	2	0.316	0.158	6.200	0.0142 *
Time	1	0.008	0.008	0.328	0.577
Treatment:Time	2	0.057	0.028	1.116	0.359
Residuals	12	0.306	0.026		

Appendix 3. — Summary of ANOVA results for the measurements of the amounts of Iron, Manganese, Magnesium, Copper, and Zinc. The analyses were conducted on transformed data (log+x). Abbreviations: **Df**, degrees of freedom; **Sum Sqs**, sum of squares; **Mean Sqs**, mean of squares. Asterisks indicate statistical significances.

	Df	Sum Sqs	Mean Sqs	F value	P
Iron					
Treatment	2	0.005	0.0023	3.161	0.0789
Time	1	0.008	0.0085	11.503	0.00535 **
Treatment:Time	2	0.005	0.0025	3.401	0.0676
Residuals	12	0.009	0.0007		
Manganese					
Treatment	2	1.0110 E-04	5.0550 E-05	6.531	0.01205 *
Time	1	1.1610 E-04	1.1610 E-04	14.999	0.00222 **
Treatment:Time	2	2.6960 E-05	1.3480 E-05	1.742	0.2168
Residuals	12	9.2880 E-05	7.7400 E-06		
Magnesium					
Treatment	2	0.3118	0.1559	7.117	0.009162 **
Time	1	0.6616	0.6616	30.199	0.000137 ***
Treatment:Time	2	0.4590	0.2295	10.476	0.002332 **
Residuals	12	0.2629	0.0219		
Copper					
Treatment	2	2.2220 E-04	1.1110 E-04	27.040	3.59 E-05 ***
Time	1	1.0080 E-04	1.0080 E-04	24.530	0.000335 ***
Treatment:Time	2	2.0690 E-04	1.0340 E-04	25.170	5.09 E-05 ***
Residuals	12	4.9310 E-05	4.1100 E-06		
Zinc					
Treatment	2	0.0827	0.0414	19.060	0.000188 ***
Time	1	0.0130	0.0130	5.994	0.030695 *
Treatment:Time	2	0.0821	0.0410	16.841	0.000195 ***
Residuals	12	0.0260	0.0022		