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Enhancement of coral calcification via the interplay of nickel and urease

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ABSTRACT

Corals are the main reef builders through the formation of calcium carbonate skeletons. In recent decades, coral calcification has however been impacted by many global (climate change) and local stressors (such as destructive fishing practices and changes in water quality). In this particular context, it is crucial to identify and characterize the various factors that promote coral calcification. We thus performed the first investigation of the effect of nickel and urea enrichment on the calcification rates of three coral species. These two factors may indeed interact with calcification through the activity of urease, which catalyzes the hydrolysis of urea to produce inorganic carbon and ammonia that are involved in the calcification process. Experiments were performed with the asymbiotic coral *Dendrophyllia arbuscula* and, to further assess if urea and/or nickel has an indirect link with calcification through photosynthesis, results were compared with those obtained with two symbiotic corals, *Acropora muricata* and *Pocillopora damicornis*, for which we also measured photosynthetic rates. Ambient and enriched nickel (0.12 and 3.50 $\mu g\,L^{-1}$) combined with ambient and enriched urea concentrations (0.26 and 5.52 $\mu mol\,L^{-1}$) were tested during 4 weeks in aquaria. We demonstrate in the study that a nickel enrichment alone or combined with a urea enrichment strongly stimulated urea uptake rates of the three tested species. In addition, this enhancement of urea uptake and hydrolysis significantly increased the long-term calcification rates (i.e. growth) of the three coral species investigated, inducing a 1.49-fold to 1.64-fold increase, respectively for *D. arbuscula* and *P. damicornis*. Since calcification was greatly enhanced by nickel in the asymbiotic coral species – i.e. in absence of photosynthesis – we concluded that the effect of increased urease activity on calcification was mainly direct. According to our results, it can be assumed that corals in some fringing reefs, benefiting from seawater enriched in nickel may have advantages and might be able to use urea more effectively as a carbon and nitrogen source. It can also be suggested that urea, for which hotspots are regularly measured in reef waters may alleviate the negative consequences of thermal stress on corals.

1. Introduction

Scleractinian corals are the main reef builders through their calcification process, and reefs are critically important for providing goods and services to the tropical and subtropical nations (Moberg and Folke, 1999). Over the past 30 years, coral reefs have suffered extensive degradation worldwide (Hughes, 2003). These disturbances are caused by a complex combination of global (climate change) and local (destructive fishing practices, and changes in water quality) stressors (Cooper et al., 2009). The worsening quality of reef waters is mainly due to deforestation, agricultural pollution, dredging operations but also to mining operations, which are increasingly more frequent. Mining activities, leading to sedimentation, metal and chemical inputs, concern many reef areas (e.g. Costa Rica, Panama, Red Sea, Thailand, Tuvalu, Puerto Rico; New Caledonia) (Ali et al., 2011; Biscéré et al., 2017; Fujita et al., 2014; Guzmán and Jiménez, 1992; Tanaka et al., 2013; Whitall et al., 2014). In particular, tropical waters are often enriched in nickel (Ni), due to its geological origin: nickel laterites (Bobicki et al., 2014; Elias, 2002; Mudd, 2010; van der Ent et al., 2013). For example, Indonesia and New Caledonia, which are two coral biodiversity hotspots, have several important deposits of nickel laterites and are two of the top producers of nickel worldwide (U.S. Geological Survey, 2016). In New Caledonia, several open cut mines contribute, through erosion, to nickel discharges in coral reefs (Fichez et al., 2005; Hédouin et al., 2006; Metian et al., 2008). As a consequence, nickel concentrations increase from 0.1 to 1.0 $\mu g\ L^{-1}$ in open waters (Barceloux, 1999;

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Srichandan et al., 2016; Sutherland and Costa, 2002) to 20 µg L^{-1} in some impacted areas of the lagoon (Moreton et al., 2009). Few studies have investigated the impacts of nickel on corals and are mainly concerned with their early life stages. They highlighted the negative role of nickel concentrations around 1500 µg L^{-1}, on the fertilisation success and planulae stage of corals (Gissi et al., 2017; Goh, 1991; Reichelt-Brushett and Hudspith, 2016). This level is however well above actual environmental concentrations. Although those results are important in determining the toxicity threshold of nickel for the early life stages, they give no information on the physiological response of adult corals at an ecological concentration. Only one study focused on the response of coral colonies to relevant nickel enrichment (3.5 µg L^{-1}) (Biscéré et al., 2017). This last experiment suggested a beneficial effect of nickel at ambient temperature, increasing the calcification rates of *Acropora muricata* and *Pocillopora damicornis* by up to 47%, but so far, the processes involved in the nickel enhancement of calcification in corals remain unclear. Also, whether this effect of nickel is reproducible with other coral species and/or environmental conditions still needs to be tested.

Calcification relies on the transport of calcium (Ca^{2+}) and dissolved inorganic carbon (DIC), such as bicarbonate (HCO_3^-), from ambient seawater to the calcification site, and on the withdrawal of protons (H^+) resulting from the mineralization process, all summarized in the following equation $Ca^{2+} + HCO_3^- \Leftrightarrow CaCO_3 + H^+$. Any process, which increases the supply of Ca^{2+} and DIC to the calcification site, or the removal of H^+ from this site, will likely enhance calcification. One stimulus can be urease, which catalyzes the hydrolysis of urea to produce inorganic carbon and ammonia (Krajewska, 2009). Ammonia can help neutralizing protons emitted during the calcification process, and thus help increasing the pH at the calcification site (Crossland and Barnes, 1974). Inorganic carbon produced by urease activity can stimulate calcification directly or indirectly, via increased photosynthesis, which also requires DIC (Allemand et al., 2004; Gattuso et al., 1999). Urease, which is a nickel-dependent enzyme, has however been poorly studied in corals (Barnes and Crossland, 1976). The same applies to the link between nickel or urea availability and coral calcification and photosynthesis.

Corals can get urea through different ways, including *(i)* the recycling of animal wastes by the *Symbiodinium* and the subsequent transfer to the host as amino acids (Ferrier, 1991), ammonium or urea (Furla et al., 2000), *(ii)* the direct uptake of urea and amino acids (considered dissolved organic nitrogen, DON) by coral hosts (Grover et al., 2006, 2008). DON is generally available in high concentrations in reef waters (from 5 to 20 µmol L^{-1}). External supply comes from anthropogenic terrestrial sources (Glibert et al., 2006; Lomas et al., 2002), from the atmosphere (Cornell et al., 2003) or from sedimentary production (Lund and Blackburn, 1989; Therkildsen and Lomstein, 1994). Biological *in situ* production of urea comes from zooplankton and fish excretion (Conover and Gustavson, 1999; McDonald et al., 2006; Walsh et al., 2001) and regeneration by microheterotrophs (Cho et al., 1996; Satoh, 1980). To date, very few works have been performed on the link between urea and corals. First, pioneering studies revealed that the carbon (^{14}C) contained in urea was incorporated into the skeleton of corals and that urease was present both in *Symbiodinium* and host tissue (Barnes and Crossland, 1976; Crossland and Barnes, 1974). Thereafter, other experiments investigated the urea uptake rates by isolated *Symbiodinium* or the entire association (Grover et al., 2006; Wafar et al., 1985, 1993). A significant transfer of nitrogen from urea to coral tissue and *Symbiodinium* compartments has been measured and urea seems to be preferentially or more easily absorbed by corals than nitrate (Grover et al., 2006). However, the direct role of urea in coral calcification, has never been studied.

The overall aim of our work was to investigate the effects of nickel enrichment alone or in combination with urea enrichment, on coral calcification, growth and urea uptake rates. For this purpose, the experiments were performed with the asymbiotic coral *Dendrophyllia arbuscula,* and to further assess if urea and/or nickel enrichment has an indirect link with calcification through photosynthesis, we compared the previous results with those obtained with two symbiotic corals, *Acropora muricata* and *Pocillopora damicornis*, for which we also measured photosynthetic rates.

2. Methods

2.1. Coral collection and experimental setup

The individual and combined effects of seawater enrichment in urea and nickel were assessed on *Acropora muricata* and *Pocillopora damicornis,* two major scleractinian coral species of New Caledonia. To avoid the natural enrichment of New Caledonian seawater in urea or nickel, experiments were performed on corals originally collected in the Gulf of Aqaba (Red Sea) more than ten years ago and which were maintained during all this time in the aquaria of the Centre Scientifique de Monaco under the same controlled conditions as described below (T: 25 °C, light: 180 ± 10 µmol photons m^{-2} s^{-1} (photoperiod 12h:12 h light:dark) and low nutrient conditions (Ezzat et al., 2015, 2016). Two hundred terminal portions (i.e., nubbins) of branches (2-cm long) were cut from five different mother colonies of each species (40 nubbins/colonies). Nubbins were then evenly distributed in eight 20 L aquaria (5 nubbins/colony/species) and allowed to heal for three weeks under controlled conditions as described below. Tanks were supplied with seawater at a renewal rate of 288 L day^{-1} and water was mixed using a submersible pump (Aquarium system, micro-jet MC 320, Mentor, OH, USA). Temperature (25 ± 0.2 °C) was kept constant using heaters connected to Elli- Well PC 902/T controllers. Corals received a constant irradiance of 180 ± 10 µmol photons m^{-2} s^{-1} (photoperiod was 12h:12 h light:dark) using 400 W metal halide lamps (HPIT, Philips) and light was monitored using a LI-COR data logger (LI- 1000) connected to a spherical quantum sensor (LI-193). Corals were fed twice a week during the recovery period of one month with nauplii of *Artemia salina*. After the recovery, feeding was stopped and four experimental conditions (two nickel and two urea concentrations) were tested in duplicate in a cross factor design (Fig. 1). Four tanks were supplied with seawater at ambient nickel concentration (Ambient: 0.12 ± 0.05 µg L^{-1}) (Boyle et al., 1985), while the remaining four others were enriched in nickel (Enriched: 3.50 ± 0.1 µg L^{-1}). This nickel enriched condition represented one of the highest concentrations found on a reef in the lagoon of New Caledonia (Moreton et al., 2009). For each nickel condition, urea concentration was either set up to ambient (0.26 ± 0.07 µmol L^{-1}) or, enriched concentrations at 5.52 ± 0.13 µmol L^{-1}. To exacerbate the role of urea in enriched conditions, concentrations of urea usually found in reefs were doubled (Crandall and Teece, 2012). Thus, four different conditions were tested: control (C), nickel supplied (Ni), urea supplied (Ur) and both nickel and urea supplied (NiUr). To enrich the seawater in nickel or urea, a peristaltic pump (ISMATEC) continuously supplied the experimental tanks with a solution of stable nickel ($NiCl_2$, Humeau, France) and/or a solution of urea (NH_2CONH_2, Sigma-Aldrich, Missouri, United States) at a rate of 15 mL h^{-1}, together with a 12 L h^{-1} seawater flow-through. Corals were incubated under such experimental conditions for a total of 4 weeks. Photosynthetic efficiency, growth and calcification rates, urea incorporation and tissue parameters were measured at the beginning of the experiment and, these measurements, together with the photosynthetic and respiration rates were measured again after one week and at the end of the experiment.

In parallel, a second experiment with exactly the same experimental design, except that the incubations were performed in the dark was run on a third coral species: *Dendrophyllia arbuscula*. *D. arbuscula* is an asymbiotic coral, colonies of this species have been collected in Indonesia and have been acclimated to laboratory conditions for at least three months before the beginning of the experiments. Colonies were maintained at the Centre Scientifique of Monaco for three weeks in the dark at 25 °C before to start the experiment. 32 micro-colonies composed by 5–10 polyps according to their size have been glued (Holdfast

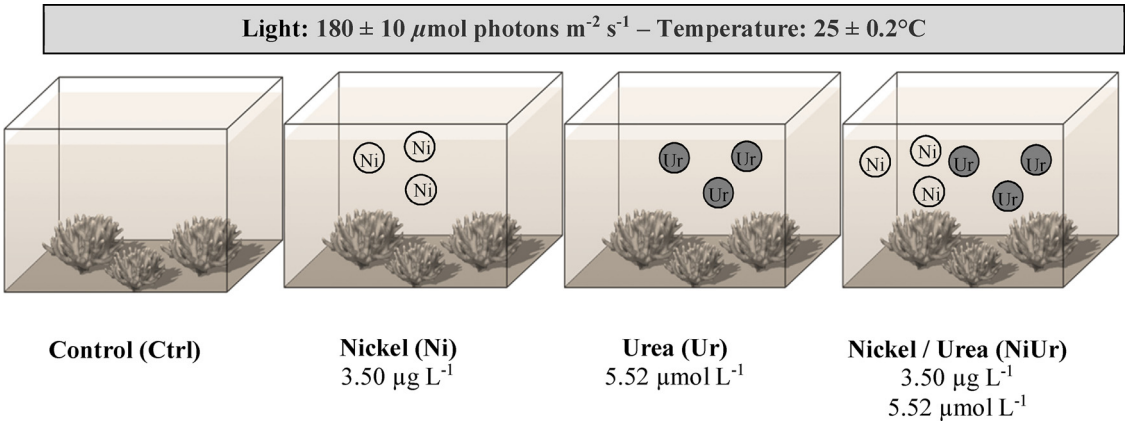

Fig. 1. Experimental set up with four conditions in a crossed factor design. Every condition was in duplicate. Control (Ctrl): ambient concentrations of nickel and urea; Nickel (Ni): higher nickel concentrations; Urea (Ur): higher urea concentrations; Nickel/Urea: higher nickel and urea concentrations.

epoxy) on plastic plates, randomly assigned (n = 4 per tank) to one of the eight tanks and set up at the bottom of the aquaria. *D. arbuscula* colonies have been submitted to the same conditions of temperature and enrichment as the first experiment but they were fed twice a week with nauplii of *Artemia salina* even during the experiment. As *D. arbuscula* is an asymbiotic coral, only growth, calcification and urea uptake rates were measured.

2.2. Photosynthetic efficiency measurements

Photosynthetic efficiency (F_v/F_m) and the relative electron transport rate (rETR) of the Photosystem II (PSII) of *Symbiodinium in hospite* were measured in aquaria using a DIVING-PAM fluorometer (Walz, Germany) ($n = 5$ for each tank). Measurements were performed the morning before the lights were switch on, when corals were adapted to dark. The initial fluorescence (F_0) was measured by applying a weak pulsed red light (3 μs, LED 650 nm) on dark-adapted colonies. A saturating pulse (800 ms) of bright actinic light (8000 μmol photons m^{-2} s^{-1}) was then applied to give the maximum fluorescence value (F_m). Variable fluorescence (F_v) was calculated as F_m-F_0. F_v/F_m represents the maximum quantum yield of the PSII. rETR were obtained through the rapid light curves (RLC) according to Ralph and Gademann (Ralph and Gademann, 2005) protocol. Light intensities used for the RLC were calibrated with an external PAR sensor (LI-COR data logger (LI- 1000) connected to a spherical quantum sensor (LI-193). The RLC were generated by illuminating corals for 10-s periods, incremented in eight steps from 0 to ca. 1800 μmol photons m^{-2} s^{-1}. To quantitatively compare RLCs, they were characterized by their $rETR_{max}$. During measurements, the 8 mm optical fiber was maintained perpendicular to the coral's surface using a black-jacket at a fixed distance of 5 mm (Biscéré et al., 2017).

2.3. Growth rates

Buoyant weight gives an estimation of the calcification rates of corals over long time scales and takes into account both daytime and nighttime calcification rates. Nubbins were weighed ($n = 5$ for each tank for symbiotic corals and $n = 4$ for *D. arbuscula*) using the buoyant weight technique (Davies, 1989). Samples, hung on a nylon wire, were weighed using a Mettler XP205 electronic balance (readability 0.01 mg) in seawater of known density as temperature is controlled by a water bath at 25 °C and salinity continuously measured. The net buoyant weight of the corals was converted into dry weight using the density of pure aragonite (2.94 g cm^{-3}). Their growth rates were calculated as the daily change in dry weight between the initial and the final weight and expressed in mg g^{-1} d^{-1}.

2.4. Calcification rates

Calcification rates were estimated using the alkalinity anomaly technique (Chisholm and Gattuso, 1991) as the change in the total alkalinity (AT) values during each incubation. This technique gives an estimation of short-term daytime calcification. Three nubbins from each tank were incubated for 3 h in individual small glass beakers, filled with 250 mL of 0.2 μm filtered seawater which were semi-immersed in a thermostat water bath at 25 °C, under a saturating light of 200 ± 5 μmol photons m^{-2} s^{-1} using a 400 W metal halide lamp (HPIT, Philips). The water was sampled at the beginning and at the end of the incubation period and alkalinity was measured using a 888 Titrando titrator (Metrohm). Data were expressed as μmol $CaCO_3$ cm^{-2} d^{-1}.

2.5. Urea uptake

At the beginning and end of each incubation for calcification measurement (see details above), 40 mL of seawater were collected to measure urea concentrations. They were determined using a colorimetric method based on diacetyl monoxime and thiosemicarbazide, in presence of sulphuric acid and ferric chloride (Grover et al., 2006; Rahmatullah and Boyde, 1980). Absorbance was read at 525 nm after 30 min at 80 °C and 10 min in water at ambient temperature. Urea uptake rates were calculated as the decrease in urea concentration during the 3 h incubation. Data were normalized per unit surface area and expressed as nmol urea cm^{-2} h^{-1}.

2.6. Photosynthetic and respiration rates

Measurements were always performed early morning to avoid any confounding effect of metabolic diurnal variations (Edmunds and Davies, 1988). Respiration (R) and gross photosynthesis rates (Pg) were assessed, respectively, in dark, and at 200 μmoles photons m^{-2} s^{-1}. Three nubbins from each tank were placed in thermostatically-controlled chambers at 25 °C, filled with seawater collected from their experimental tanks, filtered using 0.45 μm Whatman glass-fiber filters (GF/F), and continuously stirred with a stirring bar. Each chamber was equipped with a Unisense optode connected to a computer with Oxy-4 software (Chanel fiber-optic oxygen meter, PreSens, Regensburg, Germany) (Johnson et al., 2014). Optodes were calibrated against nitrogen-saturated and air-saturated seawater for the 0% and 100% oxygen, respectively. Pg and R rates were estimated by regressing oxygen data against time. Data were normalized to the skeletal surface area (cm^2) and expressed as μmol O_2 cm^{-2} h^{-1}.

2.7. Symbiodinium and chlorophyll concentrations

At the end of the photosynthesis/respiration measurements, nubbins were frozen at −20 °C. Tissue was removed using an air pick and the slurry was homogenised with a Potter tissue grinder. The number of *Symbiodinium* was quantified in a 100 µL sub-sample using a Z1 Coulter Particle Counter (Beckman Coulter) (Courtial et al., 2017). The remaining slurry was centrifuged at 3000 *g* for 10 min at 4 °C to separate the host tissue (supernatant) from the symbionts (pellet). Then, *Symbiodinium* cells were re-suspended in 10 mL of 100% acetone to extract chlorophyll *a* and c_2 in darkness (24 h at 4 °C). The extracts were centrifuged at 10,000 *g* for 15 min and the absorbance was measured at 630, 663, 750 nm using a Xenius spectrofluorometer. Chlorophyll concentrations were computed according to the spectrometric equations of (Jeffrey and Humphrey, 1975). All measurements were normalized to unit surface area. Chlorophyll *a* and c_2 are given as total chlorophyll.

2.8. Data normalization

The skeleton surfaces of *A. muricata* and *P. damicornis* have been estimated using the wax-dipping method described in (Stimson and Kinzie, 1991). A different methodology has been used for *D. arbuscula* in order to keep colonies alive. Polyp surface (PS) has been estimated, on all polyps, taking into account the mean diameter of the corallite (4–5 mm), as well as the exosarc extension around the corallite (3–5 mm), according to the following equation: $PS = \pi*(R + R')*H + \pi R^2$, where R represents the top polyp radius, R' the bottom polyp radius, and H the exosarc extension, measured with a caliper (Rodolfo-Metalpa et al., 2006).

2.9. Statistical analysis

One-way ANOVA factorial analyses were first used to test the effects of tanks (two replicates). All data were tested for the assumptions of normality and homoscedasticity using Shapiro Wilk's test and Bartlett's test respectively. Since tanks had no effect (ANOVA, $p > 0.05$), data were pooled and two-way ANOVAs were used to test the effects of nickel and/or urea concentrations on *Symbiodinium* and chlorophyll concentrations, respiration and photosynthetic rates, growth rates, calcification rates, maximum electron transport rate ($rETR_{max}$) and urea uptake. All tests were performed using R software. When the ANOVA determined a significant difference, a Tukey's honest significant difference test (HSD) was used to attribute differences between specific factors. Letters on the figures represent individual differences revealed by the Tukey test. All data are expressed as mean ± SD.

3. Results

3.1. Autotrophic parameters of A. muricata and P. damicornis

Compared to control conditions, symbiont densities remained unchanged for both coral species with Ni enrichment ($p > 0.05$; Tables 1 & 2) but decreased by 0.7-fold in *P. damicornis,* under Ur or NiUr enrichment ($p < 0.01$; Table 2) (Fig. 2A, C). No significant differences with control corals were observed under NiUr enrichment for *A. muricata* ($p > 0.05$; Table 1). For both colonies, total chlorophyll concentrations were 2-fold higher in Ni enriched colonies compared to control ones ($p < 0.05$ and $p < 0.01$; for *P. damicornis,* and *A. muricata*, Tables 1 & 2) (Fig. 2B, D), but did not change under Ur enrichment ($p > 0.05$; Tables 1 & 2). NiUr enrichment increased chlorophyll concentrations by 2- to 3-fold for *A. muricata* and *P. damicornis* respectively ($p < 0.05$ and $p < 0.01$; Tables 1 & 2).

Gross photosynthetic rates of *A. muricata* were enhanced, increasing 1.57-fold under Ni enrichment, 1.20-fold under Ur enrichment ($p < 0.05$, respectively; Table 1; Fig. 3A), and 2-fold under NiUr enrichment compared to the control condition (Tukey test; $p < 0.01$). For *P. damicornis* colonies, Ni and NiUr enrichment stimulated the gross photosynthetic rates, inducing a 1.20- to 1.30-fold increase compared to those of control colonies ($p < 0.05$; Table 2; Fig. 3B), while Ur enrichment did not affect those rates ($p > 0.05$; Table 2). Ni enrichment did not modify the respiration rates of either species ($p > 0.05$; Tables 1 & 2; Fig. 3A, B), while Ur enrichment generated a 2-fold increase of the respiration rates of *A. muricata* colonies and increased 1.35-fold those of *P. damicornis* ($p < 0.01$ and $p < 0.05$, respectively; Tables 1 & 2). Respiration rates of both species were not changed by a NiUr enrichment ($p > 0.05$; Tables 1 & 2).

For both species, F_v/F_m was not affected by any enrichment: Ni, Ur or NiUr ($p > 0.05$, $p > 0.05$ and $p > 0.05$, respectively; Tables 1, 2 & 4). F_v/F_m values were 0.521 ± 0.05 for *A. muricata* and 0.517 ± 0.04 for *P. damicornis*. The maximum electron transport rate ($rETR_{max}$) was not affected by Ni and Ur enrichment in both species (Fig. 4A & B)

Table 1
Summary of two-way ANOVAs testing the combined effect of two nickel (ambient: 0.12 µg L^{-1} and enriched: 3.50 µg L^{-1}) and two urea concentrations (ambient: 0.26 µmol L^{-1} and enriched: 5.52 µmol L^{-1}) on *Acropora muricata* physiological parameters during the 4-weeks experiment.

Acropora muricata

Source of variation	SS	df	F-ratio	*p*-Values
***Symbiodinium*.cm^{-2}**				
Nickel	3.273×10^{10}	1	5.195	0.052
Urea	3.884×10^{9}	1	0.617	0.455
Nickel x Urea	9.412×10^{8}	1	0.149	0.709
Error	5.04×10^{9}	20		
Total chlorophyll.cm^{-2}				
Nickel	0.047	1	25.082	0.001**
Urea	0.000	1	0.242	0.636
Nickel x Urea	0.021	1	3.766	0.041*
Error	0.015	20		
Gross photosynthesis				
Nickel	1.129	1	9.820	0.014*
Urea	0.993	1	8.419	0.042*
Nickel x Urea	1.922	1	16.719	0.003**
Error	0.920	20		
Respiration				
Nickel	0.004	1	0.047	0.833
Urea	1.408	1	16.796	0.003**
Nickel x Urea	0.002	1	0.019	0.893
Error	0.671	20		
F_v/F_m				
Nickel	0.006	1	3.242	0.08
Urea	0.00	1	0.919	0.343
Nickel x Urea	0.005	1	2.862	0.097
Error	0.071	36		
$rETR_{max}$				
Nickel	336.98	1	3.241	0.123
Urea	269.88	1	2.2021	0.287
Nickel x Urea	414.09	1	12.5849	0.001**
Error	1184.54	36		
Urea uptake rate				
Nickel	1.45 × 10-4	1	270.187	0.000***
Urea	1.58 × 10-5	1	29.355	0.000***
Nickel x Urea	3.84 × 10-6	1	7.14	0.015*
Error	1.08 × 10-5	20		
Calcification rate				
Nickel	25.256	1	23.368	0.000***
Urea	1.009	1	0.933	0.346
Nickel x Urea	24.464	1	24.129	0.000***
Error	21.616	20		
Growth rate				
Nickel	73.696	1	43.93	0.000***
Urea	8.73	1	5.204	0.028*
Nickel x Urea	80.439	1	46.242	0.000***
Error	60.392	36		

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 2
Summary of two-way ANOVAs testing the combined effect of two nickel (ambient: 0.12 µg L^{-1} and enriched: 3.50 µg L^{-1}) and two urea concentrations (ambient: 0.26 µmol L^{-1} and enriched: 5.52 µmol L^{-1}) on *Pocillopora damicornis* physiological parameters during the 4-weeks experiment.

Pocillopora damicornis				
Source of variation	SS	df	F-ratio	*p*-Values
***Symbiodinium*.cm^{-2}**				
Nickel	3.372×10^{10}	1	1.067	0.332
Urea	3.714×10^{11}	1	11.754	0.009**
Nickel x Urea	3.655×10^{11}	1	10.012	0.009**
Error	2.528×10^{11}	20		
Total chlorophyll.cm^{-2}				
Nickel	0.092	1	6.205	0.037*
Urea	0.011	1	0.709	0.424
Nickel x Urea	0.181	1	8.738	0.004**
Error	0.119	20		
Gross photosynthesis				
Nickel	0.812	1	8.750	0.018*
Urea	0.000	1	0.000	0.989
Nickel x Urea	0.917	1	9.258	0.012*
Error	0.743	20		
Respiration				
Nickel	0.024	1	0.548	0.480
Urea	0.351	1	8.080	0.022*
Nickel x Urea	0.012	1	0.276	0.613
Error	0.347	20		
F_v/F_m				
Nickel	0.001	1	1.006	0.322
Urea	0.001	1	0.638	0.429
Nickel x Urea	0.002	1	1.511	0.226
Error	0.067	36		
$rETR_{max}$				
Nickel	256.7	1	0.729	0.102
Urea	170.2	1	1.725	0.197
Nickel x Urea	457.7	1	4.641	0.038*
Error	3550.1	36		
Urea uptake rate				
Nickel	1.22×10^{-4}	1	145.023	0.000***
Urea	1.40×10^{-5}	1	16.728	0.000***
Nickel x Urea	8.59×10^{-6}	1	10.228	0.004**
Error	1.68×10^{-5}	20		
Calcification rate				
Nickel	93.108	1	16.039	0.000***
Urea	14.307	1	2.465	0.132
Nickel x Urea	100.691	1	17.564	0.000***
Error	116.098	20		
Growth rate				
Nickel	96.384	1	46.401	0.000***
Urea	3.342	1	1.609	0.213
Nickel x Urea	106.762	1	50.255	0.000***
Error	74.779	36		

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

($p > 0.05$; Tables 1, 2 & 4). $rETR_{max}$ values were 1.31-fold and 1.41-fold higher in colonies submitted to NiUr enrichments for *A. muricata* and *P. damicornis*, respectively (Tukey test; $p < 0.01$ and $p < 0.001$, respectively).

3.2. Heterotrophic parameters of *A. muricata, P. damicornis and D. arbuscula*

Ni enrichment induced a 2- to 3-fold increase in urea uptake rates of *D. arbuscula* and *P. damicornis* and a 7-fold increase in *A. muricata* (Tukey test; $p < 0.001$, $p < 0.001$ and $p < 0.001$, respectively; Fig. 5A–C). Ur enrichment stimulated only the urea uptake of the symbiotic species inducing a 2-fold increase of the rates of *A. muricata* and a 1.25-fold increase of those one of *P. damicornis* (Tukey test; $p < 0.001$ and $p < 0.01$, respectively). For both symbiotic and asymbiotic species, the highest rates were measured in colonies submitted to NiUr enrichment (Tukey test; $p < 0.001$), with a 15-fold and 6-fold increase for *A. muricata* and *P. damicornis* respectively, and 5-fold increase for *D. arbuscula* compared to those of control corals.

Ni enrichment enhanced the short-term day calcification rates (µmol $CaCO_3$ cm^{-2} d^{-1}) in both symbiotic species, while Ur enrichment had no effect ($p < 0.001$ and $p > 0.05$, respectively; Tables 1 & 2; Fig. 6A–C). For these species, the highest calcification rates were reached under NiUr enrichment, and were 1.42 and 1.54-fold higher compared to the control condition, for *P. damicornis* and *A. muricata*, respectively (Tukey test; $p < 0.001$ and $p < 0.001$). Calcification rates of *D. arbuscula* were 5- to 10-fold lower than for symbiotic corals (Fig. 6). Ni, Ur or NiUr enrichment had no effect on its calcification rates ($p > 0.05$; Table 3).

Ni enrichment also stimulated the long-term growth rates (mg g^{-1} d^{-1}) of all species, inducing a 1.62-, 1.64- and 1.49-fold increase, respectively, for *A. muricata*, *P. damicornis* and *D. arbuscula* ($p < 0.001$, $p < 0.001$ and $p < 0.05$; Tables 1–3; Fig. 7A–C). Only the growth rates of *A. muricata* were increased by Ur enrichment ($p < 0.05$; Table 1). Under NiUr enrichment, the growth rates were 1.27-, 1.19- and 1.11-fold higher, respectively for *A. muricata*, *P. damicornis* and *D. arbuscula*, compared to colonies only exposed to Ni enrichment (Tukey test; $p < 0.05$).

4. Discussion

This study brings new and major insights into the effects on coral physiology of *(i)* nickel, a metal widely found throughout the environment and *(ii)* urea, a nitrogen source, which was largely ignored in studies on coral physiology. The major finding is that coral calcification is enhanced under a nickel or a combined nickel and urea enrichment. The increased long-term calcification rate of the asymbiotic coral *D. arbuscula*, suggests that nickel and urea have a direct effect on the calcification process. The results obtained with symbiotic corals -an enhancement of the rates of photosynthesis, and a significant increase in both long-term and short-term calcification rates, above those measured in asymbiotic corals- further suggest an indirect effect of photosynthesis on calcification.

As only demonstrated in phytoplankton so far (Dupont et al., 2008; Morel and Price, 2003; Price and Morel, 1990), and here for corals, nickel availability is essential for the proper functioning of the urease. A shortage of nickel indeed impairs the urease catalytic activity, as previously observed in phytoplankton (Muyssen et al., 2004). Nickel enrichment strongly stimulated urea uptake rates of the three tested coral species. The maximization of the urease activity inside the coral tissue by nickel created a urea gradient from outside to inside coral tissue, which stimulated urea transport and resulted in higher incorporation rates. Since urea uptake is concentration dependent (Grover et al., 2006), we also observed an enhancement of urea uptake rates in corals maintained under urea enrichment. The highest uptake rates were logically observed when both urea (the substrate) and nickel (the enzyme-component) were simultaneously available in excess to corals.

In parallel to the stimulation of urea uptake rates, nickel significantly surged calcification and growth rates of both symbiotic coral species. It has been suggested, but never demonstrated, that urea hydrolysis by urease could enhance calcification by two mechanisms (1) by producing NH_3, which neutralizes protons formed during calcification and shifts the equilibrium towards the precipitation of $CaCO_3$ and (2) by supplying CO_2 to the calcification process (Barnes and Crossland, 1976; Campbell and Speeg, 1969; Crossland and Barnes, 1974). The incorporation of urea-derived carbon into skeleton was indeed demonstrated in a previous study with the use of ^{14}C-labelled urea (Crossland and Barnes, 1974), but the actual importance of urea degradation for calcification was not investigated. We demonstrate in this study that the enhancement of urea uptake and hydrolysis following both nickel and urea enrichment significantly stimulated

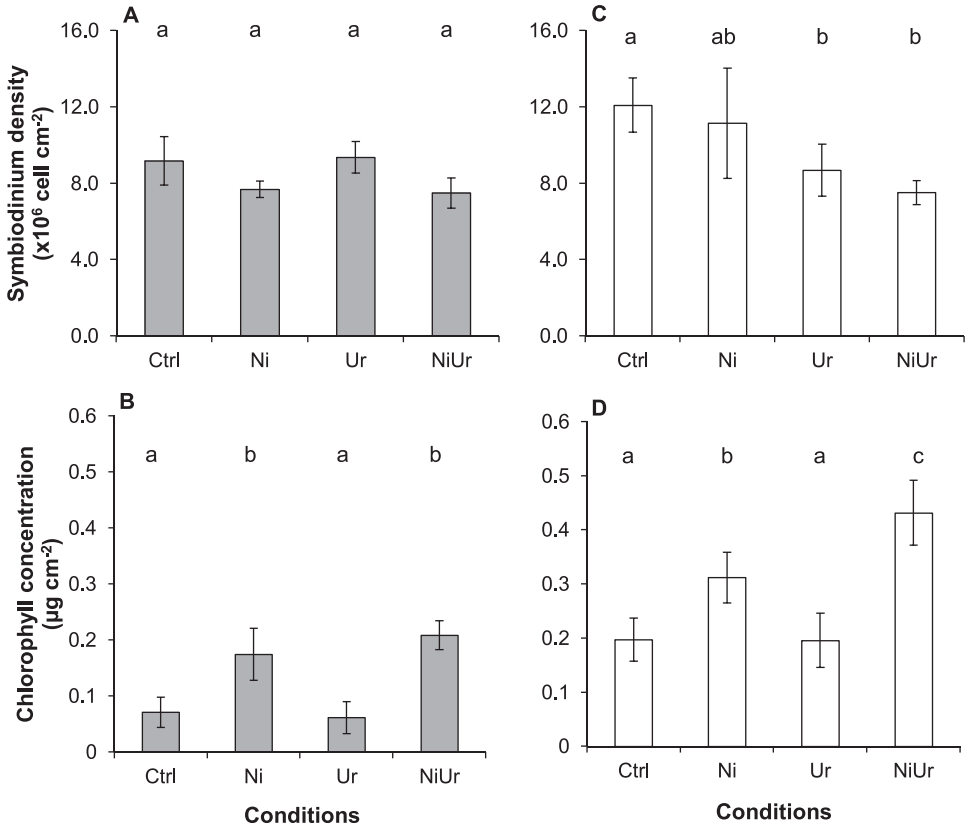

Fig. 2. *Symbiodinium* density and total chlorophyll concentration of *Acropora muricata* (A and B) and *Pocillopora damicornis* (C and D) (mean ± SD, n = 3) exposed for four weeks (chronic exposure) to ambient nickel and urea concentrations (0.12 µg L^{-1} and 0.26 µmol L^{-1}) (Ctrl); to higher concentrations in nickel (3.50 µg L^{-1}) (Ni); to higher urea concentrations (5.52 µmol L^{-1}) (Ur); and to both higher concentrations in nickel and urea (NiUr).

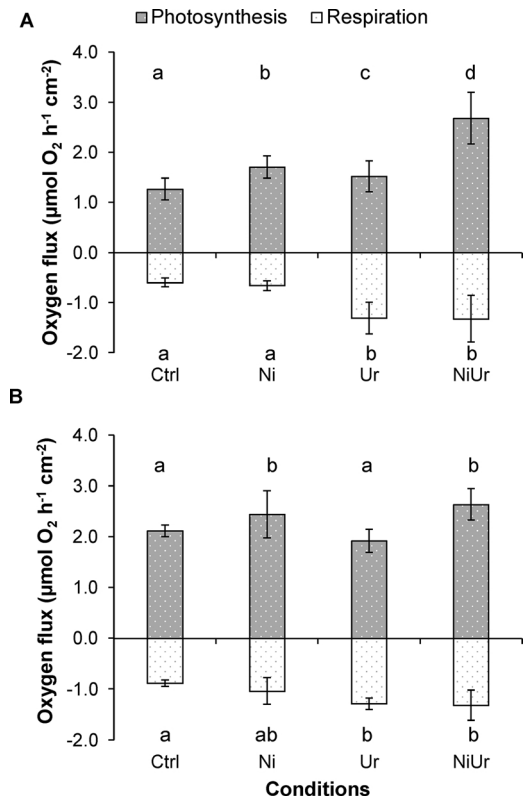

Fig. 3. *Acropora muricata* (A) and *Pocillopora damicornis* (B) gross photosynthesis and respiration rates (mean ± SD, n = 3) measured in aquaria exposed for four weeks (chronic exposure) to ambient nickel and urea concentrations (0.12 µg L^{-1} and 0.26 µmol L^{-1}) (Ctrl); to higher concentrations in nickel (3.50 µg L^{-1}) (Ni); to higher urea concentrations (5.52 µmol L^{-1}) (Ur); and to both higher concentrations in nickel and urea (NiUr).

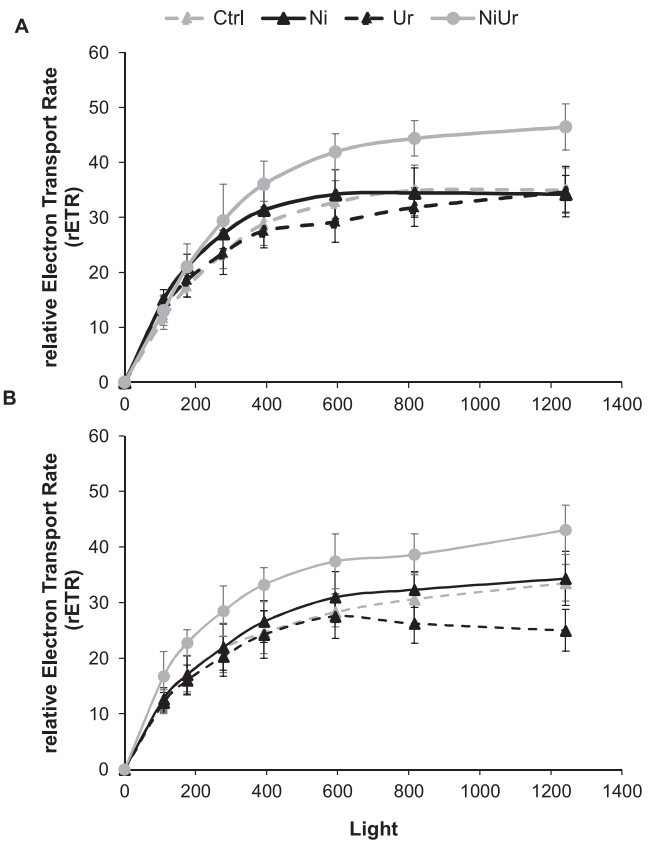

Fig. 4. Relative electron transport rate (rETR) *vs.* irradiance (PAR) for *Acropora muricata* (A) and *Pocillopora damicornis* (B) (mean ± SD, n = 5) exposed for four weeks (chronic exposure) to ambient nickel and urea concentrations (0.12 µg L^{-1} and 0.26 µmol L^{-1}) (Ctrl); to higher concentrations in nickel (3.50 µg L^{-1}) (Ni); to higher urea concentrations (5.52 µmol L^{-1}) (Ur); and to both higher concentrations in nickel and urea (NiUr).

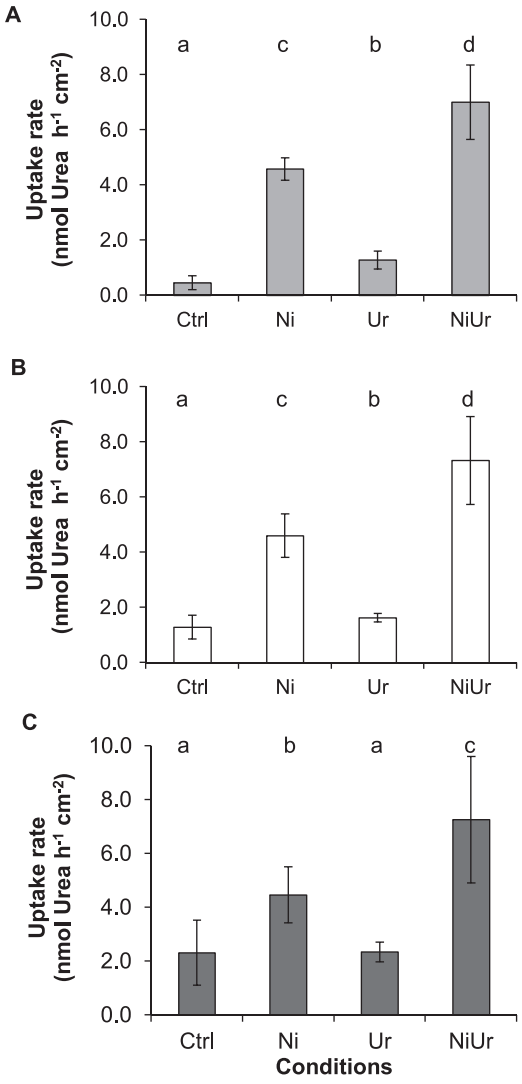

Fig. 5. *Acropora muricata* (A), *Pocillopora damicornis* (B) and *Dendrophyllia* sp. (C) uptake rates of urea (mean ± SD, n = 3) measured in aquaria after a four weeks exposure (chronic exposure) to ambient nickel and urea concentrations (0.12 µg L^{-1} and 0.26 µmol L^{-1}) (Ctrl); to higher concentrations in nickel (3.50 µg L^{-1}) (Ni); to higher urea concentrations (5.52 µmol L^{-1}) (Ur); and to both higher concentrations in nickel and urea (NiUr).

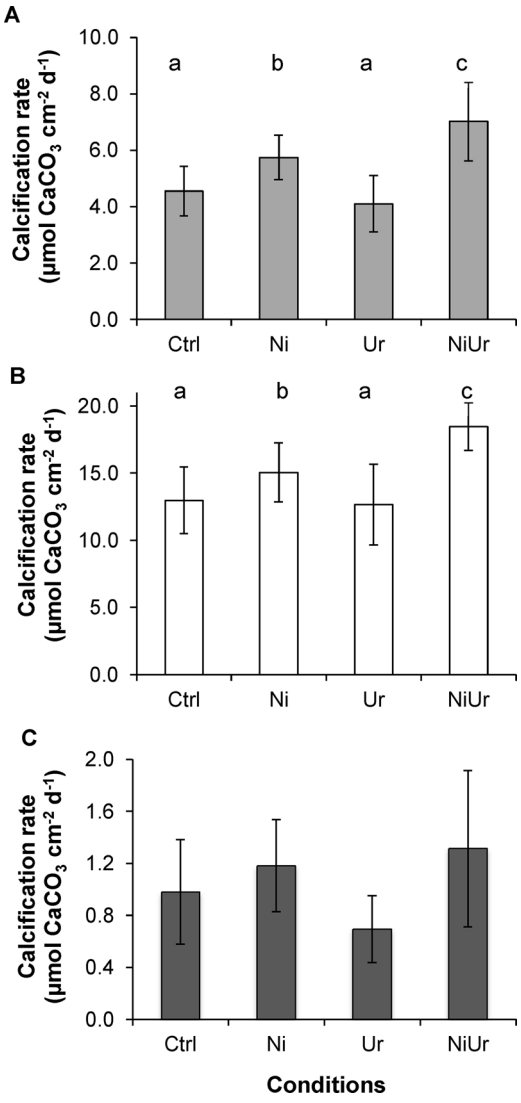

Fig. 6. Calcification rates of *Acropora muricata* (A), *Pocillopora damicornis* (B) and *Dendrophyllia* sp. (C) (mean ± SD, n = 3) measured in aquaria after four weeks exposed (chronic exposure) to ambient nickel and urea concentrations (0.12 µg L^{-1} and 0.26 µmol L^{-1}) (Ctrl); to higher concentrations in nickel (3.50 µg L^{-1}) (Ni); to higher urea concentrations (5.52 µmol L^{-1}) (Ur); and to both higher concentrations in nickel and urea (NiUr).

the long-term calcification rates (i.e. growth) of the three coral species investigated. Since calcification was greatly enhanced in the asymbiotic coral species- i.e. in absence of photosynthesis- the effect of increased urease activity on calcification was mainly direct, through one or both processes described above. These observations are in line with several studies that have examined the impact of fish excretion (inducing high concentrations of urea and ammonium) on coral growth and which have consistently measured > 50% higher growth rates when corals were surrounding by fish herds (Crandall and Teece, 2012; Holbrook et al., 2008; Liberman et al., 1995; Meyer et al., 1983; Meyer and Schultz, 1985).

Although there was a direct effect of urea degradation on coral calcification, we also observed, in symbiotic corals, a small indirect effect, through photosynthesis stimulation. Indeed, while the growth rates of the asymbiotic coral *D. arbuscula* was stimulated by 49% under urea and nickel enrichment, the rates of the symbiotic species (*P. damicornis* and *A. muricata*) were enhanced by 64% and 62% under the same conditions. These results suggest that photosynthesis may have induced an additional increase of 10–15% in calcification rates. This effect was likely through an enhancement of the photosynthetic and respiration rates under urea or urea/nickel enrichment, which provided calcification with additional photosynthates and metabolic inorganic carbon respectively (Furla et al., 2000).

This stimulation of the photosynthetic process can be explained by various factors among which, an increase in chlorophyll concentrations observed in coral colonies subjected to nickel and both nickel and urea enrichment. Chlorophyll is a molecule composed of magnesium, which constitutes its metal core. While magnesium (Mg^{2+}) is the preferential metal for chlorophyll synthesis, Küpper et al. (Küpper et al., 1996) showed that nickel, as a bivalent cation, has the ability to replace magnesium in the composition of plant chlorophyll. Thus in presence of higher nickel concentrations, chlorophyll synthesis might have been stimulated. The higher photosynthetic rates can also be explained by the stimulation of urease activity by nickel, as previously mentioned. Indeed, urea hydrolysis by urease could be involved in photosynthesis by producing NH_4^+ (resulting from the combination of NH_3 and H^+ formed during the calcification process). As it has been widely demonstrated that dissolved inorganic nitrogen (including NH_4^+) has the ability to boost coral photosynthesis (Ferrier-Pagès et al., 2000; Hoegh-Guldberg and Smith, 1989), an increased production of ammonium in

Table 3
Summary of two-way ANOVAs testing the combined effect of two nickel (ambient: 0.12 µg L^{-1} and enriched: 3.50 µg L^{-1}) and two urea concentrations (ambient: 0.26 µmol L^{-1} and enriched: 5.52 µmol L^{-1}) on *Dendrophyllia* sp. physiological parameters during the 4-weeks experiment.

Source of variation	*Dendrophyllia sp.* SS	df	F-ratio	*p*-Values
Urea uptake rate				
Nickel	8.76×10^{-5}	1	26.154	0.000***
Urea	1.09×10^{-5}	1	3.277	0.89
Nickel x Urea	6.31×10^{-5}	1	15.884	0.018*
Error	5.35×10^{-5}	16		
Calcification rate				
Nickel	1.195	1	2.167	0.166
Urea	2.554	1	4.633	0.052
Nickel x Urea	0.019	1	0.034	0.857
Error	6.616	16		
Growth rate				
Nickel	1.345	1	7.537	0.011*
Urea	0.048	1	0.271	0.606
Nickel x Urea	1.450	1	9.963	0.017*
Error	4.995	28		

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 4
Summary of $rETR_{max}$ and F_v/F_m meaning values of *Acropora muricata* and *Pocillopora damicornis* measured in aquaria after four weeks exposed (chronic exposure) to ambient nickel and urea concentrations (0.12 µg L^{-1} and 0.26 µmol L^{-1}) (Ctrl); to higher concentrations in nickel (3.50 µg L^{-1}) (Ni); to higher urea concentrations (5.52 µmol L^{-1}) (Ur); and to both higher concentrations in nickel and urea (NiUr).

	A. muricata	*P. damicornis*
$rETR_{max}$		
Ctrl	37.38 ± 6.46	35.17 ± 7.6
Ni	36.75 ± 7.15	39.16 ± 3.39
Ur	36.14 ± 3.62	32.53 ± 7.43
NiUr	48.38 ± 5.05**	50.05 ± 10.12*
F_v/F_m		
Ctrl	0.521 ± 0.03	0.518 ± 0.05
Ni	0.522 ± 0.04	0.515 ± 0.04
Ur	0.510 ± 0.05	0.512 ± 0.04
NiUr	0.531 ± 0.05	0.522 ± 0.05

* $p < 0.05$; ** $p < 0.01$.

the presence of nickel and urea could largely explain greater photosynthesis rates in corals supplied with metals compared to control corals.

The increase of photosynthesis by nickel reveals a different action mode of this metal compared to copper, for which enrichments lessen the photosynthetic rates in colonies of *A. cervicornis* (Bielmyer et al., 2010). According to these authors, this decrease of photosynthetic rates following a copper supply resulted from a loss of symbionts and a reduced carbonic anhydrase activity, which may have affected the symbiont by reducing the CO_2 available for photosynthesis.

While many studies to date have focused on the effects of dissolved inorganic nitrogen on the metabolism of corals (Dunn et al., 2012; Ezzat et al., 2015, 2016; Sawall et al., 2011), this study is one of the few to investigate the effects of dissolved organic nitrogen, urea. A previous one assessed the effects of phosphate, urea and ammonium fertilization on a patch reef of the Great Barrier Reef, but as urea and ammonium were added simultaneously, their respective role on the observed calcification decline was impossible to determine (Kinsey and Davies, 1979). The results obtained in our study show that even though both symbiotic species have increased urea incorporation rates when

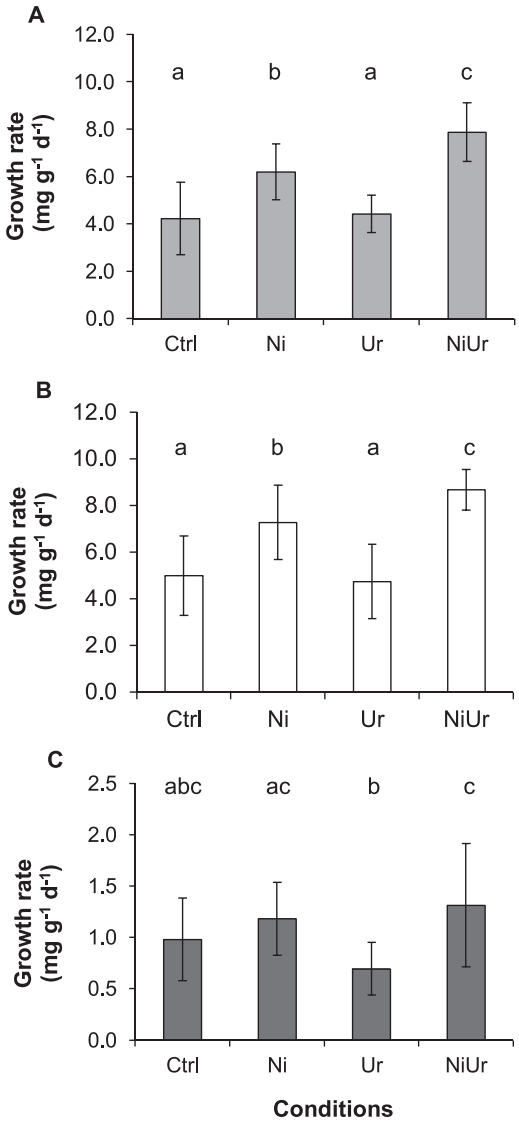

Fig. 7. Growth rates of *Acropora muricata* (A), *Pocillopora damicornis* (B) (mean ± SD, n = 5) and *Dendrophyllia* sp. (C) (mean ± SD, n = 4) measured in aquaria after four weeks exposed (chronic exposure) to ambient nickel and urea concentrations (0.12 µg L^{-1} and 0.26 µmol L^{-1}) (Ctrl); to higher concentrations in nickel (3.50 µg L^{-1}) (Ni); to higher urea concentrations (5.52 µmol L^{-1}) (Ur); and to both higher concentrations in nickel and urea (NiUr).

subjected to moderate urea enrichment alone (5.52 µmol L^{-1}), their level of sensitivity to urea is different. At this concentration of 5.52 µmol L^{-1}, colonies of *A. muricata* have significantly increased $rETR_{max}$, photosynthesis and growth values. On the contrary, colonies of *P. damicornis* and *D. arbuscula*, did not significantly changed their metabolism, likely because urea concentration was not sufficient to boost urease activity.

5. Conclusion

This study reveals for the first time the beneficial interactions between nickel and urea for coral calcification and photosynthesis. Nickel, which is toxic at very high concentrations, appears to be essential for both the symbionts and the host cells at moderate concentrations, stimulating the incorporation of urea and, as a consequence, the main physiological parameters. According to our results, it can be assumed that corals in some fringing reefs, benefiting from seawater highly enriched in nickel, as it is the case for example in New Caledonia, may

have advantages and might be able to use urea more effectively as a carbon and nitrogen source. While there are still a lot of controversial results regarding the impacts of dissolved inorganic nitrogen enrichment on coral health and resistance to climate change, this work provides evidence that a source of dissolved organic nitrogen such as urea, through its by-products, might play a key role in coral metabolism by boosting both photosynthesis and calcification processes. Thus, although additional experiments are still needed to define the urea threshold at which, coral physiological parameters are stimulated, it can be suggested that urea, for which hotspots are regularly measured in reef waters (Crandall and Teece, 2012), may alleviate the negative consequences of thermal stress on corals.

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