RESEARCH ARTICLE

Responses of Two Scleractinian Corals to Cobalt Pollution and Ocean Acidification

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Abstract

The effects of ocean acidification alone or in combination with warming on coral metabolism have been extensively investigated, whereas none of these studies consider that most coral reefs near shore are already impacted by other natural anthropogenic inputs such as metal pollution. It is likely that projected ocean acidification levels will aggravate coral reef health. We first investigated how ocean acidification interacts with one near shore locally abundant metal on the physiology of two major reef-building corals: *Stylophora pistillata* and *Acropora muricata*. Two pH levels (pH$_T$ 8.02; pCO$_2$ 366 μatm and pH$_T$ 7.75; pCO$_2$ 1140 μatm) and two cobalt concentrations (natural, 0.03 μg L^{-1} and polluted, 0.2 μg L^{-1}) were tested during five weeks in aquaria. We found that, for both species, cobalt input decreased significantly their growth rates by 28% while it stimulated their photosystem II, with higher values of rETR$_{max}$ (relative Electron Transport Rate). Elevated pCO$_2$ levels acted differently on the coral rETR$_{max}$ values and did not affect their growth rates. No consistent interaction was found between pCO$_2$ levels and cobalt concentrations. We also measured *in situ* the effect of higher cobalt concentrations (1.06 ± 0.16 μg L^{-1}) on *A. muricata* using benthic chamber experiments. At this elevated concentration, cobalt decreased simultaneously coral growth and photosynthetic rates, indicating that the toxic threshold for this pollutant has been reached for both host cells and zooxanthellae. Our results from both aquaria and *in situ* experiments, suggest that these coral species are not particularly sensitive to high pCO$_2$ conditions but they are to ecologically relevant cobalt concentrations. Our study reveals that some reefs may be yet subjected to deleterious pollution levels, and even if no interaction between pCO$_2$ levels and cobalt concentration has been found, it is likely that coral metabolism will be weakened if they are subjected to additional threats such as temperature increase, other heavy metals, and eutrophication.

Introduction

Recent studies have highlighted how coral reefs are extremely delicate systems and how they may easily be altered by human activities (e.g. [1]). Among the main sources of pollution threatening them, metals are conservative in nature, persisting in the environment for long periods [2]. Major inputs of heavy metals to the marine environment include urban storm water run-off, industrial effluents, mining operations, and atmospheric contaminants in both particulate and dissolved forms [3]. Furthermore, motor vehicle emissions, disposal of sewage sludge, dredged spoil, ash and antifouling paints for marine vessels and structures also yield appreciable concentrations of heavy metals to the ocean [2,4,5]. Metal pollution affects many reefs worldwide (*e.g.* Costa Rica, Panama, Red Sea, Thailand, Tuvalu, Puerto Rico) [5–9]. Among them, coral reefs of New Caledonia (Pacific Ocean) are particularly concerned by this issue. New Caledonia is among the five major nickel producers in the world and extended portions of its fringing reefs are impacted by extensive nickel mining activities [10], which contribute primarily to metal discharges [11–13]. Among these metals, cobalt is associated with nickel in the laterites of the mining sites and the most recent mines in New Caledonia, and throughout the world, launch the mining of cobalt, as a by-product of the treatment of nickel. Experimental studies on metal potential effects on corals mainly investigated their reproduction and early life stages. They can be summarized in the following: inhibition of coral fertilization and reduced reproductive success [14–16], decreased settlement and survival of coral larvae [17]; changes in the rates of photosynthesis resulted in a decrease in coral calcification and growth rates during the juvenile polyp stage [18,19]; loss of zooxanthellae in coral tissue (also called "coral bleaching") [18,20]; enhanced coral mortality [20,21]. All these experimental studies, using very high metal levels, highlighted the harmful role played by metals when in excess. However, it has never pointed out potential benefic effects due to metals like cobalt (Co), copper (Cu), zinc (Zn), manganese (Mn) and iron (Fe) at moderate and representative levels. These metals are indeed essential for the health and growth of corals since they are cofactors of many enzymes [22,23]. Amongst metals, cobalt might play an important role. For example, the vitamin B_{12}, also called cobalamine (due to its cobalt nucleus), is involved in the metabolism of all animals and many phytoplanktonic species [24], and stimulates *in hospite* zooxanthellae growth [25]. Carbonic anhydrases are ubiquitous enzymes known to act as catalysts for the interconversion between CO_2 and HCO_3^-, and it can play an important role when calcification is carbon limited such as under ocean acidification scenarios. However, so far no study has been performed on the direct effect of a cobalt input on coral metabolism.

In addition to waterborne pollutants, the ultimate danger that corals have to face is climate change (e.g. [26]). Atmospheric CO_2 levels have nearly doubled since pre-industrial times. As CO_2 diffuses in seawater, it behaves like a weak acid leading to a drop in pH on a global scale [27]. About a third of anthropogenic CO_2 emissions has been absorbed by the oceans, driving the process of ocean acidification during which absorbed CO_2 transforms into carbonic acid, increasing the concentrations of H^+, bicarbonate (HCO_3^-), and dissolved carbon dioxide (CO_2), while lowering carbonate (CO_3^{2-}) concentrations and seawater pH [27,28]. The current consensus estimate is that by the end of this century the rate of calcification in scleractinian corals will decrease by 17–37% as a result of reduced seawater [CO_3^{2-}] due to a doubling of pre-industrial levels of atmospheric pCO$_2$ [29,30] but coral response to acidification is in fact not unequivocal and still puzzling. It has been shown that some tropical species are able to calcify under high pCO$_2$ levels [31–34]. Comeau *et al.* [35], testing eight tropical species demonstrated that response of tropical coral reef communities to ocean acidification was heterogeneous.

To date, no work has studied the combined effect of ocean acidification and metal impacts on corals. Houlbrèque et al. [33] pointed out that zinc uptake rates in *Stylophora pistillata* were

higher under normal pH conditions (pH_T 8.1) compared to lower ones (pH_T 7.5), suggesting that ocean acidification might also change the incorporation of metals. Depending on whether metals are essential or not, changes in their uptake rates might have radical consequences on coral metabolism.

Here we tested in laboratory conditions the combined effect of ocean acidification and cobalt concentrations on two coral species separately. We also investigated *in situ* the effects of higher cobalt concentrations on coral metabolism through benthic chamber experiments.

Materials and Methods

1-Aquaria experiment

Coral collection and experimental setup. *Stylophora pistillata* and *Acropora muricata* were collected in the lagoon of New Caledonia, on the reef of Îlot Maître (22°19.702′ S; 166° 24.626′ E). A license has issued by the "Province Sud" to allow this collection. One hundred twenty terminal portions of branches (2-cm long) for each coral species were cut from 10 parent colonies. After collection, *S. pistillata* microcolonies have been hung on nylon wires and suspended on the aquaria, while *A. muricata* microcolonies have been glued (Holdfast epoxy) to 2 x 2 cm plastic plates and set up at the bottom of the aquaria. All microcolonies were positioned with the apex in front of the artificial light source. They recovered for one month in the laboratory of the Aquarium des Lagons (Nouméa) under controlled conditions as described below. Microcolonies were randomly assigned to one of the 8 experimental tanks of 20 L volume (n = 15 per species per tank) supplied with 100 μm-filtered seawater pumped from 5 meter depth in front of the Aquarium. In each tank, seawater was renewed at a rate of 16.5 L h^{-1} and mixed using a submersible pump (Aquarium system, micro-jet MC 320, Mentor, OH, USA). Temperature (26 ± 0.1°C) and salinity (35.70 ± 0.02) were kept constant using heaters connected to electronic controllers (± 0.2°C accuracy) and routinely verified using an YSI MPS 556 probe (YSI, USA). Corals received a constant irradiance of 120 ± 10 μmol photons m^{-2} s^{-1} (photoperiod was 12h:12h light:dark) using four neon Aquablue plus (Blue-white, 15000 Kelvin, Giesemann, Germany). They were fed twice a week during the recovery period and once a week during the experiment with nauplii of *Artemia salina* (ca. 120 ± 50 *nauplii* L^{-1}).

After the recovery, four tanks were set-up at ambient pH (pH_T 8.02 ± 0.03; pCO_2 366 μatm) and four others at pH level projected for the end of the century (pH_T 7.75 ± 0.06; pCO_2 1140 μatm) (IPCC 2007). pH was controlled using a pH-stat system (IKS, Karlsbad, accuracy ± 0.05 pH unit) by bubbling independently pure CO_2 in each tank that were continuously aerated with CO_2-free air. For each pCO_2 condition, two tanks received a natural cobalt concentration of the seawater pumped in front of the Aquarium (Natural: 0.035 ± 0.007 μg L^{-1}), and two others were enriched in cobalt (Polluted: 0.222 ± 0.006 μg L^{-1}). For that, a peristaltic pump (ISMA-TEC), together with seawater flow-through, continuously supplied the experimental tanks with solution of stable cobalt ($CoNO_3$, CPAchem, Bulgaria) at a rate 50 ml h^{-1}. Throughout the experiment, cobalt concentrations have been analyzed once a week by the AEL laboratory (*Analytical Environmental Laboratory*) of Nouméa, according to the protocol described in [36].

Colonies were maintained under these experimental conditions for five weeks, after which their photosynthetic efficiency and growth rates were measured.

Seawater carbonate chemistry. Seawater pH values were continuously monitored by a pH-stat system, which electrodes were calibrated using NBS solutions (Seawater National Bureau of Standards) and adjusted every day to the desired pH_T (Total Scale) using a pH meter with a glass electrode (Eutech Instruments EcoScan) calibrated with Tris/HCl referenced solutions ([37]; standards provided by A.G. Dickson, batch 13). Mean pH_T were calculated from hydrogen ion concentrations of each measurement and then re-converted back to pH [37]. Total

alkalinity was measured twice a week on water samples collected in glass bottles, filtered at 0.45 μm (GF/F Whatman) and stored in the dark at 4°C to avoid biological alteration. The pH was measured at 0.1 ml increments of 0.01 N HCl at 25°C using a Metrohm titration system (848 Titrino Plus). Three replicated 20 mL sub-samples were analyzed. Total alkalinity (A_T) was calculated from the Gran function applied to pH variations from 4.2 to 3.0 as mEq L^{-1} from the slope of the curve HCl volume versus pH. Titrations of A_T standards provided by A.G. Dickson (batch 121) were within 0.85 μmol kg^{-1} of the nominal value. Mean A_T of seawater was 2.341 ± 0.047 mmol kg^{-1} (n = 40). CO_2 and saturation states of aragonite (Ω_{ara}) were calculated from pH_T, mean A_T, temperature, and salinity using the free access CO_2 Systat package.

Photosynthetic efficiency measurements. Photosynthetic efficiency (F_v/F_m) and the relative electron transport rate (rETR) of the Photosystem II (PSII) of zooxanthellae *in hospite* were measured using a DIVING-PAM fluorometer (Walz, Germany) (n = 10 for each species and each tank). During measurements light sources were switched off. Ambient light level at the aquarium room was <10 μmol photon m^{-2} s^{-1} and did not substantially affect the coral F_v/F_m. After 15 min of dark adaptation (after [38]), the first measurements were performed. 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 (8,000 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. At the end of all F_v/F_m measurements, light was switched on and the corals were allowed to recover under light conditions for 1 h after which rapid light curves (RLC) were generated by illuminating corals for 10·s periods, eight times from 0 to ca. 3000 μ mol·photon·m^{-2}·s^{-1}, and the coral maximum relative electron transport rate (rETR$_{max}$) was assessed and compared between the different treatments.

During measurements, the 8·mm optical fibre was maintained perpendicular to the coral's surface using a black-jacket at a fixed distance of 5·mm [39]. This system created a quasi-darkness status allowing the rapid re-oxidation of primary electron acceptor [40]. The same setting was also used during F_v/F_m measurements to guarantee correct distance of the optical fiber to the coral.

It is worth noting that to calculate ETR using RLCs, some assumptions were made. First, because we did not measure the different tissue absorptances between species, we used the relative ETR (*sensu* [40]) which does not account for the fraction of incident light absorbed [41]. rETR was therefore uniquely used to compare data from different treatments and not between species. Another source of significant errors when measuring rETR is a likely different symbiont density between samples which can lead to a significant change in reflected light and thus in absorptance. As we measured ETR only one time at the end of the experiment and as the zooxanthellae density did not change between treatments (see Table 1), we are confident that our measurements of rETR reflect coral response to treatment uniquely. It should be noted that pre-configured protocols designed for measuring ETR by PAM fluorometry permit rapid assessment of the light adapted state of corals without allowing sufficient time for steady state conditions to be established, which is required to correctly evaluate the photosynthetic response to light (i.e. ETR vs Irradiance curve [42]). However, our approach was to contrast ETRs between different treatments and therefore contrast relative differences in the responses observed. In no way we measured absolute electron transport rates to contrast relative patterns in light utilization of the two studied species.

Growth rates. Microcolonies were weighted (n = 7 for each species and each tank) using the buoyant weight technique [43]. Samples were weighed using a Mettler AT200 electronic balance (readability 0.1 mg) in seawater of known density as measured by an YSI MPS 556 probe (YSI, USA). For *A. muricata*, for which samples were attached to plastic plates, they were weighed before and after attachment to tagged plastic plates, and the difference (plate and glue weight) was subtracted from the total weight. The net buoyant weight of the corals (total coral

Table 1. Summary of two-way ANOVAs testing, in aquaria, the effect of pCO_2 (366 and 1140 ppm) and cobalt concentrations (Natural, 0.03 μg L^{-1} and Polluted, 0.2 μg L^{-1}) on *Stylophora pistillata* and *Acropora muricata* main physiological parameters.

Source of variation	*Stylophora pistillata*				*Acropora muricata*			
	SS	df	F-ratio	*p*-Values	SS	df	F-ratio	*p*-Values
Zooxanthellae								
pCO_2	948x10^8	1	1.92	0.18	820x10^8	1	2.23	0.15
Cobalt	320x10^8	1	0.65	0.43	531x10^8	1	0.014	0.91
pCO_2 x cobalt	21x10^{10}	1	4.33	0.51	136x10^8	1	0.37	0.55
Error	987x10^9	20			736x10^9	20		
Chlorophyll *a*								
pCO_2	12.96	1	6.54	0.019*	2.969	1	1.837	0.19
Cobalt	6.74	1	3.40	0.08	0.627	1	0.388	0.54
pCO_2 x cobalt	24.55	1	12.38	0.002*	0.002	1	0.001	0.927
Error	39.64	20			32.31	20		
ETR$_{max}$								
pCO_2	1569	1	13.15	0.001*	253	1	4.14	0.049*
Cobalt	1950	1	16.35	0.000*	1000	1	16.38	0.000*
pCO_2 x cobalt	633	1	5.30	0.027*	8	1	0.13	0.721
Error	4295	36			2198	36		
F$_v$/F$_m$								
pCO_2	0.002	1	1.555	0.22	0.002	1	4.040	0.052
Cobalt	0.006	1	6.27	0.017*	0.001	1	1.858	0.181
pCO_2 x cobalt	0.000	1	0.012	0.912	0.000	1	0.956	0.335
Error	0.035	36			0.015	36		
Growth rate								
pCO_2	0.01	1	0.00	0.956	0.08	1	0.038	0.845
Cobalt	68.37	1	28.71	0.000*	16.45	1	8.210	0.006*
pCO_2 x cobalt	5.48	1	2.30	0.135	1.11	1	0.553	0.46
Error	123.83	52			104.19	52		

* face numbers indicate $p<0.05$.

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weight *minus* the weight of each plate) was converted into dry weight using the density of the pure aragonite (2.94 g cm^{-3}). Their net calcification 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}.

Zooxanthellae and chlorophyll content. At the end of the incubation microcolonies (n = 3 per each species and tank) were collected and frozen (-20°C) for zooxanthellae and total chlorophyll (chl) measurements. Tissue was removed from coral colonies using an air pick [44] and the slurry was homogenised with a Potter tissue grinder. For each sample, the number of zooxanthellae was counted five times by light microscopy using a Neubauer's cell. Chlorophylls were extracted twice in 100% acetone (24 h at 4°C). The extracts were centrifuged at 10,000 g for 15 min and the absorbances were read at 630, 663, 750 nm. Chlorophyll concentrations were computed according to the spectrometric equations of [45]. All measurements were normalized to the nubbin surface area, which was measured using the aluminum foil technique [46].

2—*In situ* experiment

In situ experiments were conducted during two consecutive sunny days at the end of August 2012 during the winter Austral season. The study site was located at Tabou Reef (22°28.845′ S;
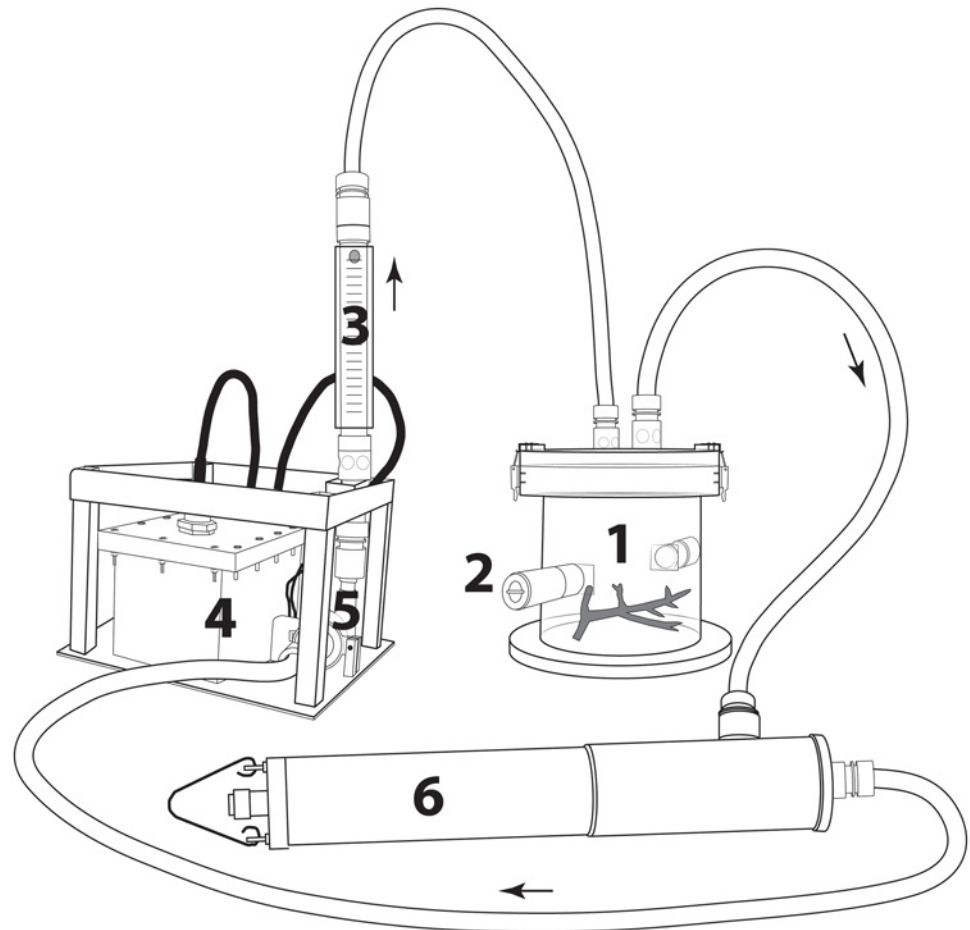
Fig 1. Schematic representation of the experimental system. 1- Benthic chamber with a coral colony, 2-Seawater sampling port, 3- Flow meter, 4- Battery in a waterproof housing, 5- Adjustable submersible pump, 6- Multiparameter probe. The arrows indicate the direction of the water flux.

166°26.806′ E) in the southwest New Caledonia lagoon at a depth of 5 m. Three colonies of ca. 15 cm long of *A. muricata* were sampled by scuba diving near to the study site. Their bare skeleton (due to the collect) was covered by a layer of Holdfast epoxy to avoid the development of algae on this part. Specimens were then transferred to benthic chambers left opened until the beginning of the experiment. Four transparent PVC benthic chambers of 0.19 m diameter and 6.4 L volume were used simultaneously, three chambers containing *A. muricata* colonies and a control one without coral colony to account for seawater microbial activity. Each chamber was hermetically connected to an YSI 6920 multiparameter probe. Seawater was recirculated between the chamber and the probe at a water flow of 2 L min^{-1} using an adjustable submersible pump alimented by waterproof batteries [47] (Fig 1). Photosynthetically active radiation (PAR, 400–700 nm) irradiance (I, μmol photons m^{-2} s^{-1}) was measured adjacent to experimental chambers using quantum sensors (LI-192 SA coupled to a Li-1400 LI-COR). Oxygen, temperature, salinity and depth were recorded every minute inside each chamber with the YSI 6920 multiparameter probe.

During two consecutive days, for each species, six successive incubations of one hour were performed from 11:00 am to 9:00 pm, to encompass the full range of daily irradiance levels, including dark. The first series of incubations has been realized at seawater natural cobalt

concentration (less than quantification limit measured by [36]). The second series of incubations have been performed on the same coral colonies but 10 ml of a 17 μmol l^{-1} cobalt solution have been injected in each chamber at the beginning of each incubation (CoNO$_3$, CPAchem, Bulgaria). Final cobalt concentration into the chambers was equal to 1.06 ± 0.16 μg l^{-1} (i.e. Polluted). Between incubations, enclosures were left open for at least 30 mn to restore ambient conditions.

Photosynthetic, respiration and calcification rates. Photosynthetic and respiration rates, as well as calcification rates associated to *A. muricata* metabolism were measured during the in situ incubations at different light levels and in the dark. At the beginning and at the end of each of the six incubations, seawater samples were collected with 450 ml syringes in each chamber for pH and total alkalinity (A$_T$) measurements. pH was measured immediately on board using a pH-meter (Radiometer pH240) calibrated with TRIS/HCl (2-amino-2-hydroxymethyl-1, 3-propanediol) and 2-aminopyridine/HCl buffer solutions in synthetic seawater of salinity 35. Samples were then filtered through 0.7 μm Whatman glass-fiber filters (GF/F) and stored in 250 ml bottles in the dark. Total alkalinity (μmol kg^{-1}) was further determined on 20 ml sub-samples (6 replicates) by Gran automatic potentiometric titration (Radiometer, Titrilab TIM 865) using 0.01 M HCl (see above "Seawater carbonate chemistry" for further details). Total alkalinity values were corrected for ammonium concentrations which were analyzed on board with the fluorometric method [48] (Turner Trilogy fluorometer). These concentrations were negligible and did not modify A$_T$ values. The mean standard deviation of replicate measurements was less than 0.003 mmol l^{-1}.

Calcification rates at each light level and in the dark were estimated using the alkalinity anomaly technique [49] as the difference between the final and the initial A$_T$ values of each incubation. Calcification rates (Cnet, μmol CaCO$_3$ h^{-1}) were estimated for each incubation using the following equation:

$$C_{net} = \frac{\Delta A_T \times v}{\Delta t \times 2} \tag{1}$$

where ΔA$_T$ is the variation of total alkalinity during incubation (μmol L^{-1}), v is the volume of the benthic chamber (L) and Δt is the incubation time (h).

Oxygen production and consumption rates were measured by the oxygen sensor of the YSI probe which was calibrated before each experiment against air saturated with moisture. Rates of photosynthesis and respiration were estimated by regressing oxygen data against time. Net photosynthetic rates were calculated according to the following equation:

$$P_{net} = (\Delta O_2 \times v) \tag{2}$$

where P$_{net}$ is the rate of net photosynthesis (μmol O$_2$ h^{-1}); v is the volume of the chamber (L); Δ[O$_2$] is the dissolved oxygen variation (μmol O$_2$ L^{-1} h^{-1}) during the 1 h incubation.

At the end of all the incubations coral colonies were frozen (-20°C) for further analyses. Photosynthesis, respiration and calcification rates were normalized by unit surface skeleton (cm^{-2}), which was measured using measured using the aluminum foil technique [46].

For each colony, a Michaelis-Menten model [50,51] was iteratively fitted using non-linear regression (Gauss-Newton algorithm) to P$_{net}$ versus *in situ* PAR irradiance (I, μmol photons m^{-2} s^{-1}), according to the following equation:

$$P_{net} = \frac{P_{max} \times I}{K_m + I} - R \tag{3}$$

where P_{max} is the gross maximal production rate (μmol O_2 cm^{-2} h^{-1}), K_m is the half saturation constant (μmol photons m^{-2} s^{-1}), and R is the respiration in the dark (μmol O_2 cm^{-2} h^{-1}).

From the equation above, P_{max} and K_m have been calculated (Eqs 4 and 5):

$$P_{\max} = \frac{(P_{net} + R) \times (K_m + I)}{I} - R \qquad (4)$$

$$K_m = \frac{(P_{max} \times I)}{C_{net} + R} - I \qquad (5)$$

The same procedure was also used to fit calcification rates (C_{net}) versus Irradiance values. Eqs 6 and 7 were therefore used to calculate maximum fitted calcification values (C_{max}) as follow:

$$C_{net} = \frac{C_{max} \times I}{K_m + I} - C_{dark} \qquad (6)$$

$$C_{\max} = \frac{(C_{net} + C_{dark}) \times (K_m + I)}{I} - C_{dark} \qquad (7)$$

where C_{max} is the gross maximal calcification rate (μmol $CaCO_3$ cm^{-2} h^{-1}), K_m is the half saturation constant (μmol photons m^{-2} s^{-1}), and C_{dark} is the calcification rate measured in the dark (i.e. dissolution, μmol $CaCO_3$ cm^{-2} h^{-1}).

Statistical analysis

For the aquaria experiment, three-way ANOVA factorial analyses were used to test the effects of pH (normal and low), cobalt (normal and polluted) and tanks (two replicates), independently for each of the two species. All data were tested for the assumptions of normality and homoscedasticity using Shapiro Wilk's test and Cochran's c-test respectively. After verification of the absence of significant differences between tanks (ANOVA, p > 0.05), data were pooled before proceeding to test for main effect using two-way ANOVAs. Two-way ANOVAs were used to test the effects of pH_T and cobalt concentrations on zooxanthellae density, chl concentration, net calcification rate, photosynthetic efficiency (F_v/F_m) and maximum electron transport rate ($rETR_{max}$). All the tests were performed using PRISM software (Statsoft). When the ANOVA determined a significant difference, a Tukey's honest significant difference test (HSD) was used to attribute differences between specific factors. All data are expressed as the mean ± SD. For each *in situ* experiment P_{max}, K_m and R, as well as C_{max} and C_{dark} were compared between cobalt concentrations (i.e. natural vs. polluted) using F-test according to [52].

Results

1-Aquaria experiment

Zooxanthellae density did not differ between experimental treatments (2-way ANOVA, p > 0.05; Table 1) for both *S. pistillata* (pooled data, 1.06 x 10^6 ± 2.26 x 10^5 cells cm^{-2}) and *A. muricata* (pooled data, 1.21 x 10^6 ± 1.84 x 10^5 cells cm^{-2}). Chl *a* concentrations slightly but significantly varied between pCO_2 (6.04 ± 1.9 and 7.10 ± 1.1 μg chl cm^{-2} at pH_T 8.1 and 7.8 respectively) and its interaction with cobalt (6.04 ± 1.9 and 7.10 ± 1.1 μg chl cm^{-2} at Natural and Polluted concentrations respectively) for *S. pistillata* (interaction pCO_2 x Metal $F_{1,20}$ = 12.38, p = 0.002; Table 1), but not for *A. muricata* (pooled data: 6.389 ± 1.242 μg chl cm^{-2}; Table 1).
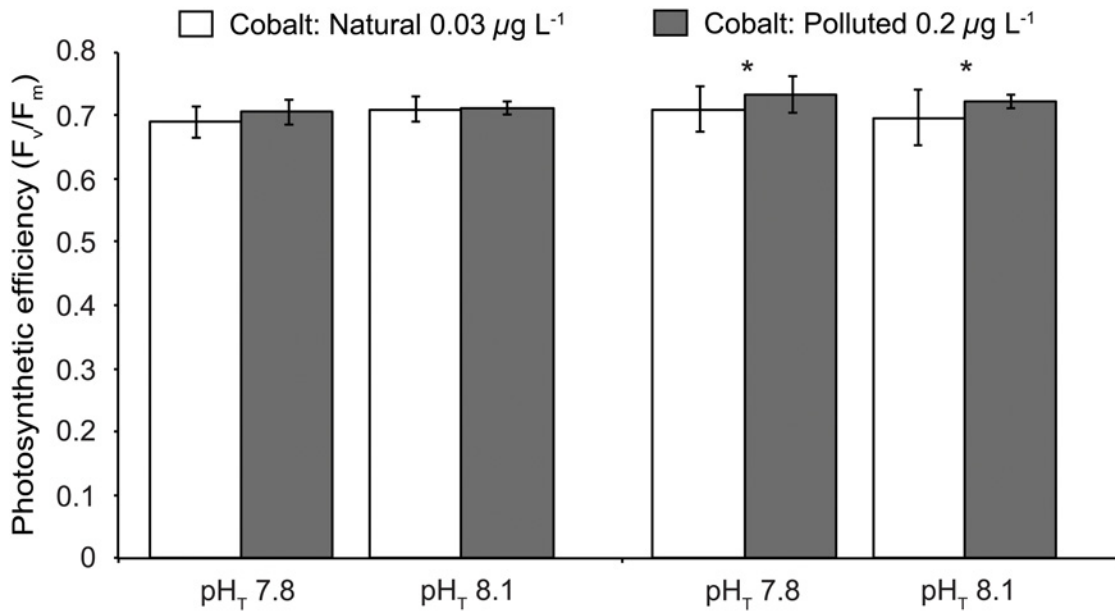
Fig 2. Effective photosynthetic efficiency (F_v/F_m) measured on dark adapted *Stylophora pistillata* and *Acropora muricata* colonies exposed during five weeks to two pH_T conditions (pH_T 7.8 or 8.1) and two cobalt concentrations (Natural and Polluted). Stars indicate statistically significant differences (Tukey's test, $p < 0.05$). Each point represents the mean ± SD ($n = 10$).

Concerning their photosynthetic properties, both coral species did not show uniform responses to experimental treatments (Figs 2 and 3). For *S. pistillata*, F_v/F_m and $rETR_{max}$ significantly differed between treatments (2-way ANOVA, $p < 0.05$, $p < 0.01$ respectively; Table 1). F_v/F_m was not affected by pCO_2 treatment (2-way ANOVA, $p > 0.05$; Table 1) but was enhanced by cobalt with higher values at the polluted metal concentration (Tukey test, $p < 0.05$), while $rETR_{max}$ values showed an interaction between pCO_2 and cobalt (2-way ANOVA, $F_{1,36} = 5.30$, $p = 0.027$; Table 1). On the opposite, *A. muricata* F_v/F_m were not affected by both cobalt concentration and pCO_2 treatments (2-way ANOVA, $p > 0.05$; Table 1), while $rETR_{max}$ values for this species significantly varied between treatments (2-way ANOVA, $p > 0.05$; Table 1). They increased at polluted cobalt concentrations but decreased at elevated pCO_2 level (Tukey test, $p < 0.01$ and $p < 0.05$ respectively) (Fig 3).

Coral growth rates for both species were not significantly affected by pCO_2 treatments but significantly by cobalt (2-way ANOVA, $p > 0.05$; Table 1 and Fig 4). Polluted cobalt concentrations significantly decreased their growth rates (Tukey test, $p < 0.01$) with values of 4.68 ± 1.44 mg g^{-1} d^{-1} and 6.88 ± 1.62 mg g^{-1} d^{-1} for *S. pistillata* and 3.57 ± 1.31 and 4.66 ± 1.48 mg g^{-1} d^{-1} for *A. muricata* at polluted and natural cobalt concentration respectively.

2—*In situ* experiment

Rates of maximal gross photosynthesis (P_{max}) per unit surface area were significantly lower on *A. muricata* incubated with cobalt than exposed to natural concentrations (0.77 vs. 1.16 µmol O$_2$ cm^{-2} h^{-1}; F-test = 15.05, $p = 0.000$; Fig 5a). Cobalt also significantly affected (F-test = 12.51, $p = 0.001$) the half saturation constant (K_m), with much lower values measured for colonies incubated with a cobalt supply (115.29 vs. 408.41 µmol photons m^{-2} s^{-1}). No significant differences in the respiration rates were found (R = -0.28 vs. -0.24 µmol O$_2$ cm^{-2} s^{-1}, respectively) (F-test = 15.05, $p > 0.05$).
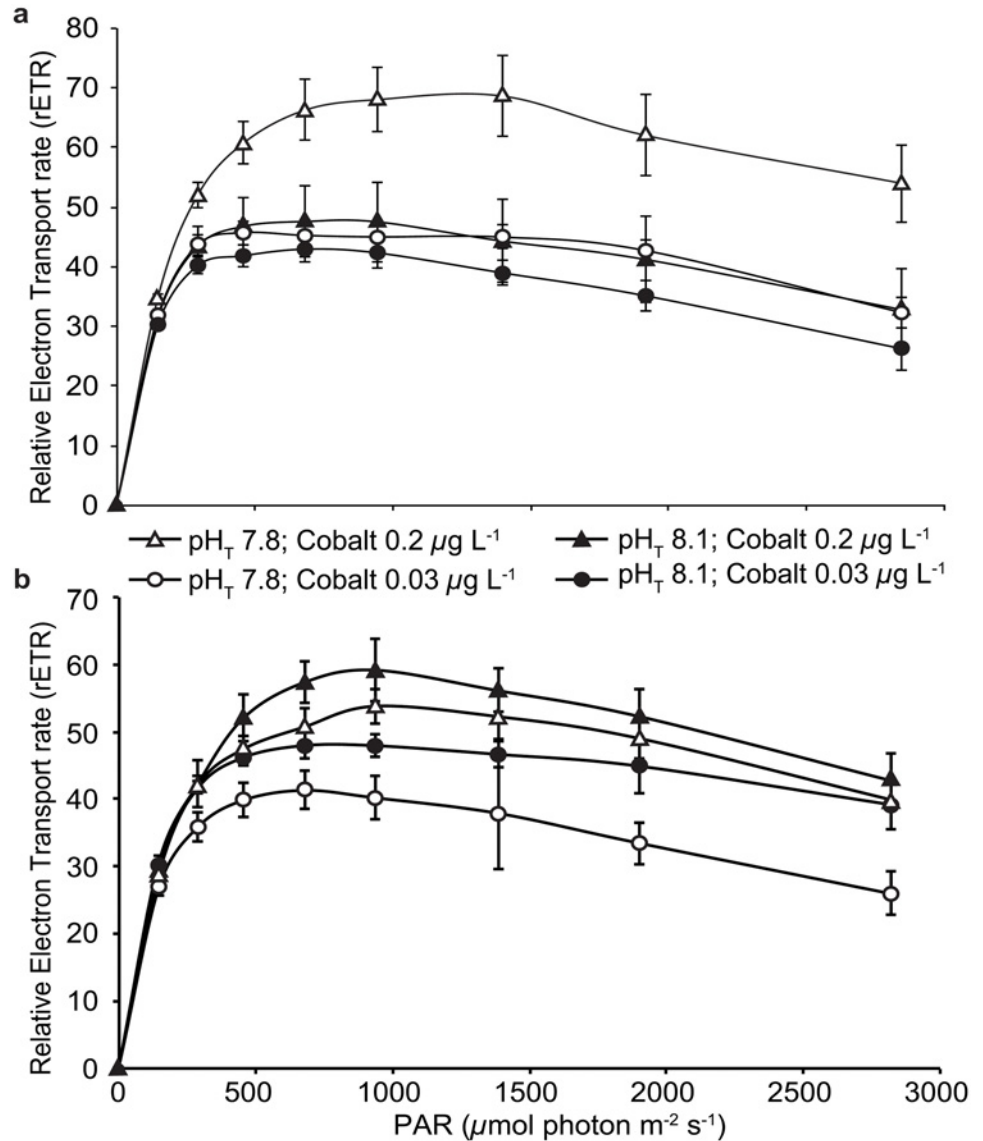
Fig 3. Relative electron transport rate (rETR) versus irradiance (PAR) for colonies of *Stylophora pistillata* (a) and *Acropora muricata* (b) (mean ± SD, *n* = 10) exposed during five weeks to two pH$_T$ conditions (pH$_T$ 7.8 or 8.1) and two cobalt concentrations (Natural and Polluted).

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Cobalt significantly affected the growth of *A. muricata* (Fig 5b), with both maximal light calcification (C_{max}: F-test = 6.33, p = 0.02) and dark calcification rates (C_{dark}: F-test = 4.80, p = 0.04) lower for corals submitted to cobalt (0.120 and 0.030 μmol $CaCO_3$ cm^{-2} h^{-1} for C_{max} and C_{dark} respectively) than in natural seawater (0.453 and 0.070 μmol $CaCO_3$ cm^{-2} h^{-1} for C_{max} and C_{dark} respectively).

Discussion

Ocean acidification is one of the major threats that tropical coral reefs are facing during this century. Key life functions in corals, such as growth and photosynthesis, have been shown to be affected by high pCO$_2$ levels (i.e. [27,30]). In addition, near shore coral reefs are often
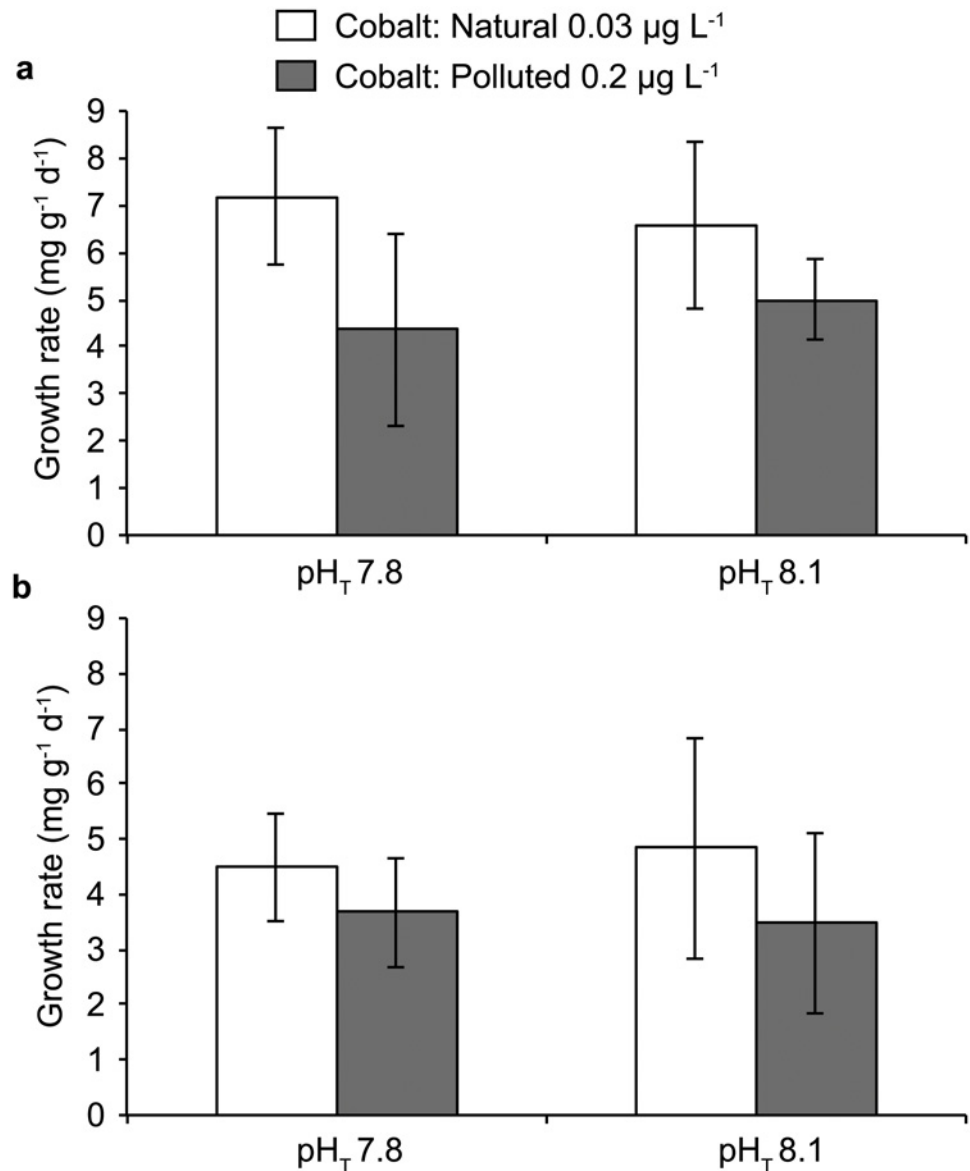
Fig 4. Growth rate (mg g⁻¹ d⁻¹) measured for *Stylophora pistillata* (a) and *Acropora muricata* (b) colonies after five weeks of incubation under two pH_T conditions (pH_T 7.8 or 8.1) and two cobalt concentrations (Natural and Polluted). Data are mean ± SD (n = 14).

already weakened by coastal human impacts, such as metal pollution, but the combination of ocean acidification and these other anthropogenic pollution forms has not been investigated yet. Thus, this study *(i)* examined, in laboratory conditions, the responses of two corals to the combination of metal pollution (here the cobalt) and ocean acidification and *(ii)* determined *in situ* whether a cobalt input alters the main coral physiological parameters. Cobalt levels added into the experimental tanks and the benthic chambers are realistic since they are in the range of maximal concentrations measured along the coasts of New Caledonia [36]. Our experiments highlighted that even a moderate cobalt concentration (0.2 μg L⁻¹), and without interaction with acidification, adversely affects the growth of both coral species and that an increase in

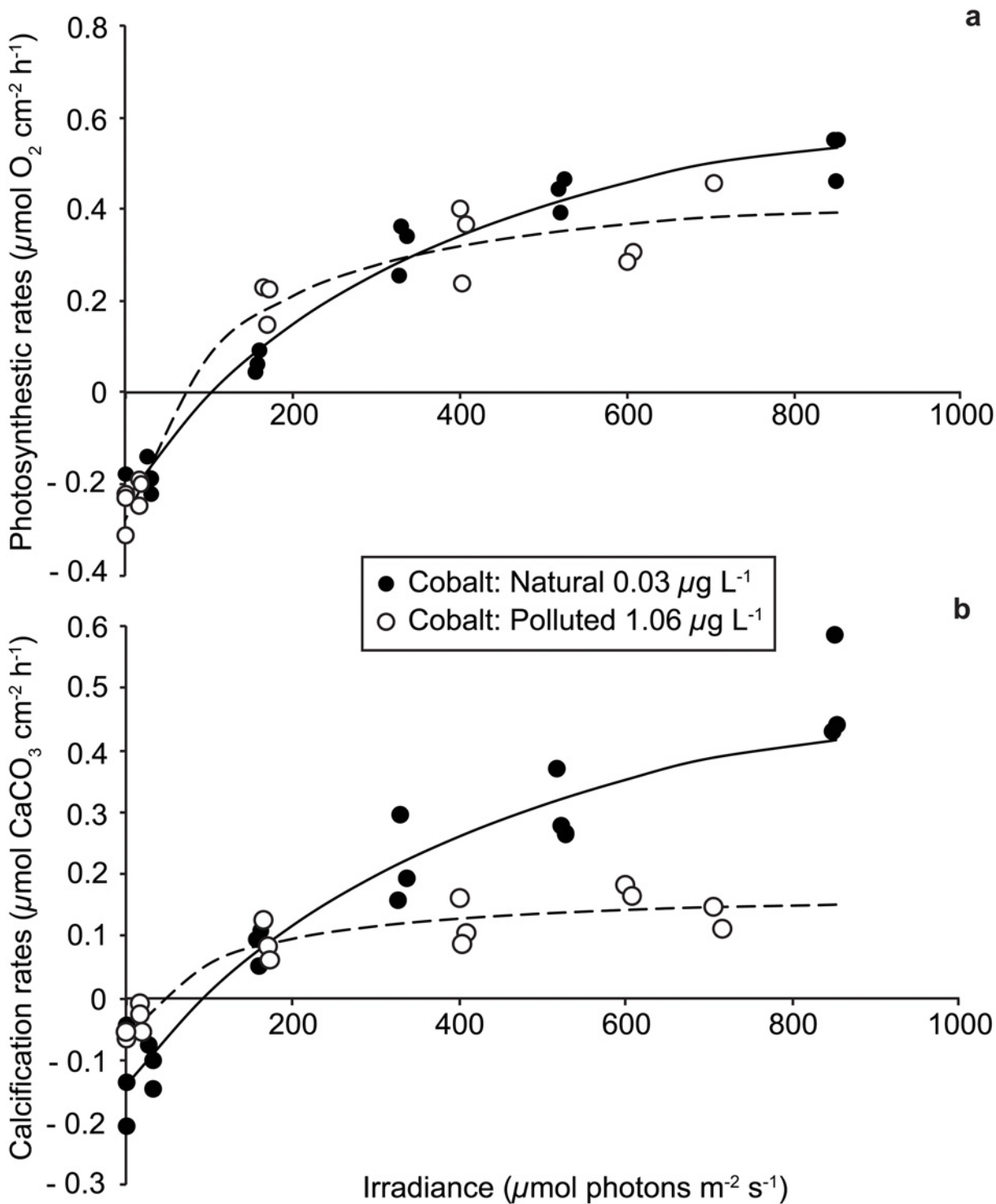Fig 5. *Acropora muricata* photosynthetic (a) and calcification rates (b) measured *in situ* during benthic chamber incubations at Natural (<0.03 μg L^{-1}) and Polluted (1.06 μg L^{-1}) cobalt concentrations.

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cobalt concentration to 1.06 μg L^{-1} leads not only to coral growth decrease, but also to an inhibition of their photosynthesis process.

Effect of cobalt enrichment on coral physiology

One of the major results of this study is that, at cobalt concentrations regularly measured along the New Caledonian coasts for example (0.2 μg L^{-1}) [36] a decrease of 28% in growth rates is already observed for both species. Furthermore, *in situ* incubations of *A. muricata* colonies at a higher concentration (ca. 1 μg L^{-1}) confirmed these results, revealing coral calcification rates of 70% lower than at normal cobalt concentrations. Although no study so far have measured the effects of cobalt on coral growth, our result is in agreement with [53] which demonstrated, for a coccolithophoridae species, *Cricosphaera carterae* that a cobalt concentration of 200 μmoles L^{-1} leaded to a decrease in calcium ion incorporation rates. Therefore, our results suggest that near shore reef corals may greatly suffer from metal pollution and, in turn, may be less prone to resist to other anthropogenic stresses.

As calcification and photosynthesis mechanisms are coupled in symbiotic corals [29], one should expect that the decrease in calcification rates observed in the present study was combined to an inhibition of the photosynthetic efficiency and/or the zooxanthellae. However, during the laboratory experiment, the F_v/F_m measured in *S. pistillata* and *A. muricata* (ranging from 0.6 to 0.7), exposed to an ecologically relevant enrichment in cobalt (0.2 μg L^{-1}), revealed that their photosynthetic processes were stimulated by such moderate cobalt input and were operating at their maximum capacity [54]. Rather, this moderate cobalt concentration stimulates the rETR$_{max}$ values in *S. pistillata* and *A. muricata*. On the other hand, their zooxanthellae concentrations did not change, suggesting that, unlike iron [19], cobalt addition did not stimulate symbiont densities but would rather stimulate the photosynthetic efficiency of these algal cells. This is consistent with previous studies performed on the same coral species, showing a stimulation of rETR$_{max}$ values in response to a moderate zinc or iron input [19,55]. Two hypotheses may explain this stimulation: *(i)* as cobalt is a cofactor of the carbonic anhydrase, an additional input would stimulate this enzyme and would bring additional inorganic carbon to the photosynthetic process [56,57]; *(ii)* as coral gastric activity harbors a bacterial community able to produce the B12 vitamin, named cobalamin (due to its cobalt core), an enhancement of this vitamin production could stimulate the photosynthetic process [58].

When cobalt was equal to ca. 1 μg L^{-1}, in the *in situ* experiments, photosynthetic rates per unit surface area decreased, suggesting that the toxic threshold for zooxanthellae in *A. muricata* was reached. This result is consistent with previous studies, in which a clear inhibition of the photosynthetic efficiency of the two branching corals *Acropora cervicornis* and *Pocillopora damicornis* was observed, when they are exposed to elevated copper levels between 4 and 20 μg L^{-1} [59]. Furthermore, as metals are mostly accumulated in coral tissues and more particularly in symbiotic algae [55], their expulsion is thought to be a mechanism of metal detoxification. Although our *in situ* incubations were too short to detect a change in the zooxanthellae density and no measurements have been performed, it is likely that the cobalt level tested reached the zooxanthellae toxic threshold affecting whole coral metabolic functions. Zooxanthellae expulsion was observed for *Acropora formosa* and *Porites lutea* exposed to extreme copper and iron concentrations, from 10 to 100 times higher than the concentrations measured in coral reef surrounding waters [18,60]. In conclusion, our results show that while extreme cobalt concentrations may be lethal for corals, reducing calcification and photosynthetic metabolism, a moderate concentration effect may be more species-specific. Two hypotheses may likely explain this antagonism: *(i)* algae and host may be in competition for inorganic carbon for photosynthesis and calcification [19,61,62] but the coral ability to compete for this substrate may be

highly species-specific with some species able to preferentially allocate inorganic carbon to the photosynthetic process; *(ii)* moderate cobalt concentration might be toxic for the host cells but not yet for the zooxanthellae. Despite these observations, identifying the mechanisms underlying the enhancement of the photosynthetic process or the calcification decrease is beyond the scope of our study and we suggest that further research should be conducted.

Effect of pCO$_2$ increase on coral physiology

Higher pCO$_2$ levels did not affect the photosynthetic efficiency of both coral species but caused an opposite effect on their rETR$_{max}$ values, stimulating *S. pistillata* and inhibiting *A. muricata*. Previous studies focused on the effects of ocean acidification on photosynthetic processes gave ambiguous results. While the lack of impact for *S. pistillata* have been already noticed during short-term studies [33,63–66] and are consistent with the assertion that corals do not rely on dissolved CO$_2$ for their photosynthesis [29]; other studies rather highlighted a detrimental effect of pCO$_2$ on the coral photosynthetic efficiency [67,68]. Response heterogeneity among corals would be linked to the symbiont types [69].

No change in the growth rates of *S. pistillata* and *A. muricata* was detected between the different pCO$_2$ conditions. These results confirm a recent study performed on *S. pistillata*, where all coral fragments submitted for one month to high pCO$_2$ conditions (2,039 μatm) survived and showed equivalent calcification rates to corals submitted to normal conditions [33]. Although these results should be taken with caution, since corals were incubated during short periods to low pH, it is consistent with some previous studies showing that some corals are able to calcify at low-pH seawater (e.g. [32,44,70–73]). Coral species like *S. pistillata* and *A. muricata* would be part of a "low-sensitivity" group to pCO$_2$ [74] and to the "potential winners" that would be able to control pH at their calcification site [73] and would outcompete species unable to do it [34]. Furthermore, ocean warming should also to be taken into consideration as [64] showed that *S. pistillata* was insensitive to doubled pCO$_2$ at 25°C but on the other hand experienced a 50% reduction in calcification rate at a higher temperature (+ 3°C). So, based on changes in temperature, some coral species like *S. pistillata* could shift between insensitivity and high sensitivity to pCO$_2$ increase.

Even if no interaction between pCO$_2$ levels and cobalt concentration has been revealed in our study for these two coral species, it is likely that coral colonies, for which growth rates already decrease by 28% when submitted to a moderate cobalt input, will be weakened if they are subjected to additional threats (i.e. temperature increase, other metal pollution, eutrophication). Indeed, when their temperature threshold has been exceeded or when corals are submitted to a poor seawater quality, it has been widely demonstrated that they stop their growth and expel their zooxanthellae (e.g. [62,75,76]) and so a cobalt pollution would exacerbate these phenomena.

It is also likely that species originally classified into the "highly sensitive group" such as *Porites lutea* or *Acropora cervicornis* for example [77] will be probably more drastically affected by metal inputs. In addition, our results only correspond to a five weeks incubation experiment and as it has been demonstrated that metals accumulate in coral tissues [55], a longer-term experiment should induce a stronger calcification inhibition and an overrun of the toxicity threshold for the zooxanthellae. Previous studies performed on other marine organisms like copepods and polychaetes demonstrated that metal pollution increases their sensibility to ocean acidification [78,79] and the present study clearly indicates that it is also the case for corals.

Acknowledgments

Author Contributions

Conceived and designed the experiments: TB RRM AL FH. Performed the experiments: TB RRM AL LC JT JC FH. Analyzed the data: TB RRM AL JC FH. Contributed reagents/materials-/analysis tools: TB RRM AL LC JT JC FH. Wrote the paper: TB RRM AL JT JC FH.

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