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Effect of iron speciation on growth and heat resistance of Symbiodiniaceae

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ABSTRACT

Iron is a limiting nutrient for Symbiodiniaceae (colloquially known as zooxanthellae), with low solubility in seawater. The use of stable, soluble, and chemically defined iron complexes is proposed as a strategy to control the supply of this metal to target organisms. In this work, we investigated the effect of iron(II) and derivatives of iron(III) (desferrioxamine, deferiprone, deferasirox, and HBED) over the growth and metal loading of five Symbiodiniaceae species. Iron supplementation did not affect growth or metal load in species with high iron stocks. In contrast, for species with low iron stocks, hydrophobic $Fe(DFX)_2$ was very efficient in inducing growth and iron loading. Also, the desferrioxamine derivative of iron Fe(DFO) appeared as an interesting, ecologically friendly source of the nutrient. The effect of iron supplementation on the growth of *Breviolum minutum* submitted to heat shock was also studied. Iron supplementation prior to the heat shock episode increased the heat tolerance of *B. minutum*. Such findings provide new insights for the strategy of iron supplementation to improve the fitness of Symbiodiniaceae, both in vitro and in the environment.

Descriptors: Chelate, Heat shock, Lipophilicity, Photosynthetic dinoflagellates, Bioinorganic chemistry.

INTRODUCTION

Iron is an essential trace element for all life and has pivotal roles in marine processes, the most important of which is arguably the modulation of ocean productivity through the control of photosynthesis (Martin and Fitzwater, 1988; Martin et al., 1990; Aumont and Bopp, 2006; Reich et al., 2020; Reich et al., 2021). For algae, iron is a crucial component of the photosynthetic apparatus (photosystems I and II) (Blaby-Haas and Merchant, 2012). The biological importance of iron stems from its versatile redox chemistry at circumneutral pH in the presence of physiological level of molecular oxygen and the possibility of forming coordination compounds (complexes) with variable degrees of stability, reactivity, and spatial geometry. This dependence of biological systems on iron is probably related to its availability on Earth and biological evolution. Iron is the fourth most abundant metal on Earth´s crust, and its ferrous (Fe^{2+}) form is rather water soluble, which would facilitate its recruitment in early biochemistry (Taylor and Konhauser, 2011). However, after molecular oxygen became abundant in the atmosphere, iron was steadily converted to its ferric (Fe^{3+}), insoluble form, which presently limits its bioavailability. Indeed, in oxygenic seawater (at a salinity of 35, pH ~ 8) it is estimated that free iron

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concentrations are 10^{-11} M (Ussher et al., 2004), posing a clear limitation for microorganism growth.

The limiting effect of iron bioavailability on ocean productivity has long been recognized (Martin and Fitzwater, 1988). Strategies for iron fertilization in marine environments have been devised and applied, mainly for CO_2 sequestration purposes. However, their efficiency is disputed (Aumont and Bopp, 2006). From a chemical point of view, one of the shortcomings of the proposed fertilization strategies is the release of simple iron salts (such as ferrous sulphate) in the water column, which promptly oxidize, precipitate, and/or acidify the surrounding environment (Gordon et al., 1998). Thus, managing the chemical speciation of the metal is important to keep it soluble with controlled reactivity, which modulates its availability to target organisms rather than benefits toxic microalgae (Trick et al., 2010).

Eukaryotic algae can assimilate iron from the environment through several mechanisms: release of siderophores, which are high affinity iron binding molecules (Krachler et al., 2019), Fe^{3+} reduction by membrane ferrireductases, assimilation of photochemically reduced Fe^{3+} or Fe^{3+}–L (L = low affinity iron binding molecules (Shaked et al., 2005)), or by assimilation of bacteria and particulate minerals (Nodwell and Price, 2001). Coral reef symbionts are photosynthetic dinoflagellates of the family Symbiodiniaceae (LaJeunesse et al., 2018), known by forming mutualistic endosymbiosis with reef building corals and marine invertebrates (Davy et al., 2012). Symbiodiniaceae aid on coral calcification, and their photosynthetic products are translocated to coral hosts (Muscatine, 1990). In exchange, they receive CO_2, nitrogen, and phosphorus in a protected environment, which guarantees coral survival and growth, even in nutrient-poor waters (Muscatine and Porter, 1977). Studies on the role of iron in the coral-symbiont model point to a positive effect of increased levels of the metal in dinoflagellate growth (Ferrier-Pages et al., 2001; Rodriguez et al., 2016; Reich et al., 2020). On the other hand, and more relevant to us, iron deficiency is associated with decreased photosynthetic and antioxidant activity in the symbionts (Shick et al., 2011;Reich et al., 2020), which may jeopardize the exchange of metabolites within the coral host. In combination with other environmental stressors related to climate change, such as ocean warming and acidification, iron deficiency might contribute to symbiosis dysfunction and lead to coral bleaching (Davy et al., 2012; Reich et al., 2021).

For a better understanding of the mechanisms of iron acquisition in Symbiodiniaceae, this work investigates the effect of well-defined and stable chemical species of iron on the growth of five coral symbiont species (*Symbiodinium microadriaticum*, *Breviolum minutum*, *Cladocopium goreaui*, *Effrenium voratum* and *Fugacium kawagutii*). Desferrioxamine (DFO) was selected as a siderophore involved in iron acquisition by bacteria (Bickel et al., 1960). Deferiprone (DFP), N-bis(2-hydroxybenzyl) ethylenediamine-N,N-diacetic acid (HBED), and deferasirox (DFX) are iron chelators that can be used in the treatment of iron-overload disorders and may be efficient redistributors of the metal in biological media (Kakhlon et al., 2010). We also investigated whether iron supplementation improves the thermal plasticity of *Breviolum minutum*. It is hypothesized that adequate control of iron supply can positively affect both the growth and resilience of coral-symbiotic dinoflagellates.

METHODS

Chemicals

The salts $FeSO_4.7H_2O$, n-octanol, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. High affinity iron chelators DFP (Apotex, Canada), DFO (Cristália, Brazil; donated by Associação Brasileira de Talassemia), DFX (Novartis, Switzerland), and HBED (gifted by Dr. Ioav Cabantchik, Hebrew University, Israel) were used as received.

Synthesis and characterization of the complexes

The iron complexes of the chelators were prepared through the direct reaction of stoichiometric amounts of ferrous sulfate in either ultrapure water (DFO) or DMSO (DFP, DFX, HBED), according to the solubility of the molecules. The chelators have different binding denticities; HBED and DFO are hexadentate, DFX are tridentate, and DFP are bidentate. As

such, to complete the hexacoordinated environment of Fe ion, a 1:1 (metal:chelator) proportion was used for hexadentate ligands, 1:2 for tridentate, and 1:3 for bidentate. The reaction was allowed to proceed for 3 h at 37°C in open air to facilitate the oxidation of Fe(II) to Fe(III). In the end, stock solutions at 10 mM (iron-based) were obtained, with the formulas Fe(DFO), Fe(HBED), $Fe(DFX)_2$, $Fe(DFP)_3$. Formation of the complexes was confirmed by UV-visible spectroscopy.

The octanol-water partition coefficient (P_{ow}) of the complexes was determined by the shake-flask method (OECD, 1995). 100 μL of the iron complexes (1 mM in DMSO) were mixed with 100 μL of water and then treated with 200 μL of water-saturated n-octanol. The tubes were vortexed for 20 min at room temperature and then centrifuged (2350 ×*g*) for 5 min. Two other phase combinations were used: 1:2 and 2:1 (volume:volume; n-octanol:water). The concentration of iron in the aqueous phase was determined photometrically in a SpectraMax M4 microplate reader, using the λ_{max} wavelengths determined by the UV-visible spectroscopy. P_{ow} was calculated according to Equation 1:

$$P_{OW} = \frac{C_0 C_W}{C_W} \qquad \text{(Eq 1)}$$

where c_0 is the initial concentration and c_w is the concentration in the aqueous phase after extraction. All determinations were carried out in duplicate.

Organisms

The Symbiodiniaceae species used were *Symbiodinium microadriaticum* (BMAK215), *Breviolum minutum* (BMAK213), *Cladocopium goreaui* (BMAK210), *Effrenium voratum* (BMAK212), and *Fugacium kawagutii* (BMAK214) cultivated in the Aidar & Kutner Microrganism Bank (BMAK) of the Oceanographic Institute, University of São Paulo. The ITS2 of the species were checked previously (Mies et al., 2018) through gDNA extraction, PCR amplification, and sequencing, and correspond to ITS2 types A1, B1, C1, E1, and F1. These clades were obtained from the Buffalo Undersea Reef Research Collection (Buffalo University) and have been kept in BMAK since 2013, and were verified by a staff member after acquisition. Before the experiments, culture identities were checked by visual characteristics such as culture color, cell size, and swimming pattern, which are known from frequent observation over time.

Inocula (10^4 cells/mL) were seeded in 150 mL erlenmeyer flasks containing 90 mL of Guillard - f/2 culture medium (Guillard, 1975) deprived of silicate and without iron supplementation (f/2-Fe), as we aimed to control the iron speciation with the complexes. The medium was prepared with autoclaved seawater (salinity 35), per standard protocols (Guillard, 1975). The organisms were kept at 23° C and lighting of 80 $\mu E.m^{-2}.s^{-1}$ (daylight type lamps) with a photoperiod of 12:12 h. After the acclimation period for some generations, growth was monitored by counting in hemocytometers (Nageotte or Fuchs-Rosenthal; accuracy: ±10% (Lund et al., 1958)). Exponential growth phase conditions were attained between 24–28 days for *C. goreaui* and *E. voratum* and 50 days for *S. microadriaticum, B. minutum,* and *F. kawagutii*.

Effect of iron on growth

An outline of this experiment is depicted in Figure 1(a). Aliquots of 1 mL of the mother culture in the exponential growth phase (*ca*. 10^4 cells/mL) were diluted to 30 mL with f/2-Fe, in triplicate. Then, 35 μL of the iron complexes (10 mM), DMSO, ferrous sulfate (10 mM), or water were added to the culture flasks. The final iron concentration was 11.7 μM in the iron-treated samples. Incubation was carried out under the same light and temperature conditions. Cells were counted on days 1, 5, 9, 15, 19, and 21 of growth in each iron treatment and controls (f/2-Fe and f/2-Fe+DMSO).

After 21 days, the microalgae were centrifuged (375 ×*g*; 5 min), and the pellets were washed twice with iron-free HBS buffer (Hepes 20 mM, NaCl 150 mM, Chelex® 1 g/100 mL, pH 7.4) supplemented with 1 mM of the strong iron chelator DTPA to remove all iron bound to the outer cell walls of the symbionts. The pellet was dried at 60°C for 12 h, treated with 300 μL of double-distilled concentrated HNO_3 at 80°C, followed by 150 mL of H_2O_2 (30% w/w, Suprapur, Merck), and then dilution to 10 mL with ultrapure water. Blanks

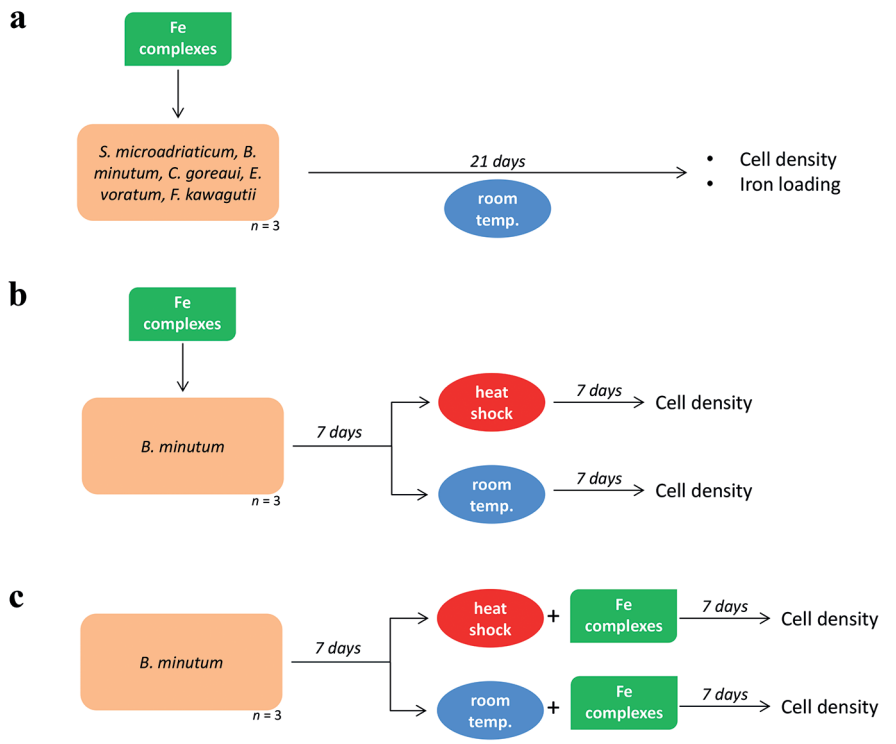

Figure 1. Outline of the experimental design for the study of the effect of iron compounds on the growth (a) or resistance to heat stress (b, c) of the Symbiodiniaceae species.

without microalgae were prepared in parallel, following the same procedure. Total iron concentration was determined in a model ZEEnit 60 atomic absorption spectrometer (Analytik Jena AG, Jena, Germany) equipped with a transverse heated graphite tube atomizer, an inverse and transverse two-field and three-field mode Zeeman effect background corrector, and a hollow cathode lamp of iron (wavelength = 248.3 nm, slit width = 0.8 nm, and lamp current = 4.0 mA). Pyrolytically coated transverse heated graphite tubes (Analytik Jena) were used throughout the measurements. All measurements were based on integrated absorbance values. Argon 99.998% (v/v) (Air Liquide Brazil, São Paulo, Brazil) was used as the purge gas. Calibration curves were obtained by using aqueous standard solutions. Samples were digested by adding 300 μL of HNO_3 (65 % v/v) and 100 μL H_2O_2 (30 % v/v) to 0.4 mg of samples, which were heated to 100°C for 5 min. The volume was adjusted to 2 mL using deionized water. The heating program used for iron measurement was [Step: Temperature, °C/Ramp (°C/s)/Hold (s)]: (Drying 1: 100, 10, 15); (Drying 2: 130, 10, 20); (Pyrolysis: 1000, 100, 20); (Atomization: 2200, 2600, 5), and (Cleaning: 2500, 1200, 5).

Effect of iron on recovery from heat shock

An outline of this experiment is displayed in Figure 1(b,c). These experiments were conducted using *Breviolum minutum* as a test organism. Aliquots of 1 mL (n=3) of the mother culture growing under iron limitation for several generations (*ca*. 10^4 cells/mL) were diluted to 30 mL with f/2-Fe. The samples were subjected to two different heat shock protocols. In the first, microalgae were treated with the iron complexes, DMSO, or blanks as indicated above, and incubated during 7 days. Then, a control group was kept at 21±1°C (room temperature) while part of the samples was subjected to a heat shock (34±1°C for 4 h). This temperature and time were chosen to abruptly induce stress by photosynthesis impairment (Iglesias-Prieto et al., 1992) and ROS formation (McGinty et al., 2012), without allowing time for organisms to

adapt by means of expression of heat shock proteins or antioxidant enzymes (Rosic et al., 2011). The two groups of microalgae were incubated for another 7 days, then counted. The second protocol was identical to the first, except that the iron complexes, DMSO, or blanks were added just *after* the heat shock. Final growth was also checked after 7 days.

Statistical Analyses

Variations in the final cell yields and iron concentrations were evaluated using one- or two-way ANOVA tests followed by Tukey's HSD ($p < 0.05$) in Origin®2016 software. Principal component analysis (PCA) was used to identify the iron compounds responsible for the main differences between species responses caused by the addition of different iron compounds in both culture growth and final iron concentration using MetaboAnalyst 5.0.

RESULTS AND DISCUSSION

Synthesis and characterization of the complexes

Fingerprint spectral signatures were used to confirm the formation of the desired iron complexes (Figure 2 and Table 1). Different solvents and concentrations were selected to obtain the clearest spectra. HBED is an oxygen-rich hexadentade chelator that forms a very stable complex with iron in a 1:1 stoichiometric proportion (Eplattenier et al., 1967). The maximum absorption at 480 nm probably corresponds to a ligand-to-metal charge transfer (LMCT; (Bannochie and Martell, 1989)). DFP presents a bidentate α-cetohydroxy function that completes the coordination environment of iron in a 3:1 (chelator:metal) stoichiometric proportion, with the transition at 514 nm corresponding to a LMCT (Karpishin et al., 1991;Nurchi et al., 2014).

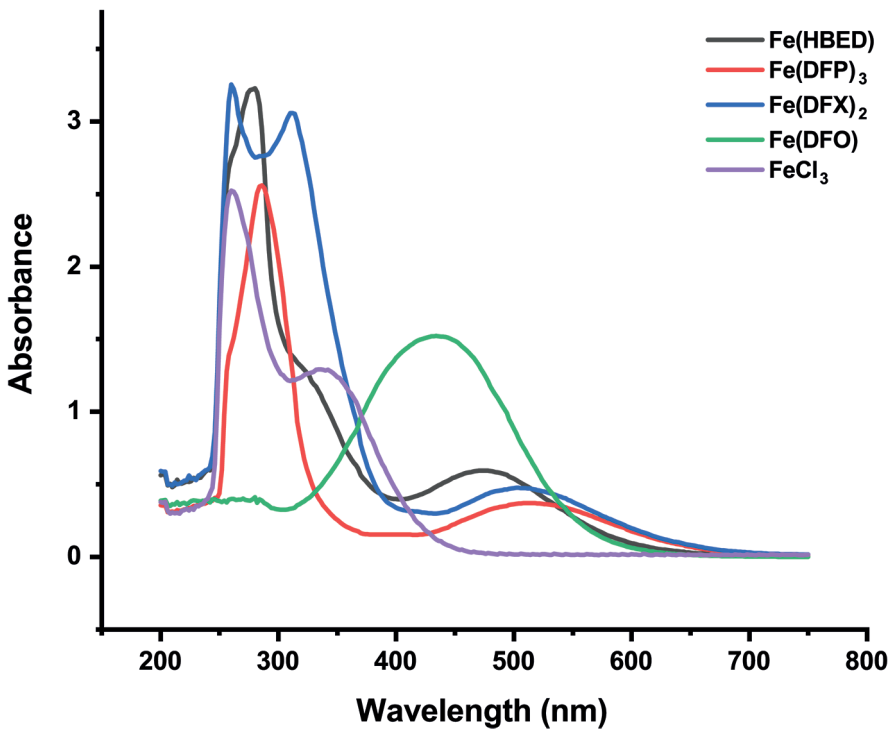

Figure 2. UV-visible electronic absorption spectra in either DMSO (Fe(HBED) 0.5 mM; Fe(DFP)3 0.1 mM, Fe(DFX)2 0.2 mM), or water (Fe(DFO) 2.5 mM). Fingerprint maximum absorbance wavelengths (λ_{max}) of all the complexes in the visible region (350–750 nm) are displayed.

Table 1. Physicochemical properties of the iron complexes. Maximum absorbance wavelenghts (λ_{max}) in the visible region (350–750 nm) are used for their identification, and the logarithm of their octanol-water partition coefficients (P_{OW}) are determined as a surrogate of their lipophilicities.

Complex	λ_{max} (nm)	LogP_{OW}
$Fe(DFX)_2$	510	1.060 ± 0.047
Fe(HBED)	480	0.608 ± 0.013
$Fe(DFP)_3$	514	0.536 ± 0.016
Fe(DFO)	430	0.339 ± 0.042

DFX displays a 2:1 stoichiometric proportion with a characteristic and non-attributed band at 510 nm (Heinz et al., 1999; Steinhauser et al., 2004). DFO is a bacterial siderophore of the hydroxamate family, binding iron in a 1:1 stoichiometric proportion (Anderegg et al., 1963a; Evers et al., 1989), whose red color arises from a spin-allowed transition centered at 430 nm (Monzyk and Crumbliss, 1982).

Since lipophilicity, assessed as P_{OW}, might be relevant to the ability of the complexes to diffuse through biological membranes, we generated complexes with a range thereof (Table 1). Chelators that display rings in their structure were usually less water soluble. As such, they are usually obtained in DMSO solution. DFX is a very hydrophobic molecule (Novartis, 2020), and $Fe(DFX)_2$ was also highly lipophilic.

Initial iron status

Iron is highly demanded by coastal photosynthetic dinoflagellates due to their mixotrophic lifestyle in the water column (Nodwell and Price, 2001). In the case of Symbiodiniaceae living in symbiosis with cnidarian hosts, current knowledge of symbiont iron demand is still limited. Iron demand generally increases during episodes of heat shock due to the increased metabolic rates needed for fast acclimation (Reich et al., 2020; Reich et al., 2021). In this case, the role of enhanced iron can be ambiguous (both integrating antioxidant defenses and promoting oxidative stress when “free” in the cytosolic medium), and the symbiont may have had selective pressure to keep the excess metal from harming the host (Reich et al., 2020). Indeed, sudden increases in free iron levels may be harmful to the calcification of coral and may lead to bleaching (Biscere et al., 2018), showing that it is important to control and explore specific mechanisms of iron supply rather than pouring simple iron salts into the oceans.

The use of iron chelates is not uncommon for microalgae. As observed for a number of terrestrial microorganisms (Mattos et al., 2013), marine microorganisms also display a promiscuous ability to internalize iron carried by bacterial siderophores (Hopkinson and Morel, 2009; Reich et al., 2020) such as *Marinobacter* (Amin et al., 2012). Iron internalization can be also achieved by reduction at the cell membrane (Hopkinson and Morel, 2009). Bound by stable organic molecules, iron complexes release iron in a more controlled manner and, with the correct choice of organic chelators (e.g. targeting moieties, electrical charge, or controlled hydrophobicities), may redistribute the metal to very specific cell compartments.

Cultivated in Guillard f/2-Fe medium (without iron supplementation) after 21 days, *B. minutum* and *E. voratum* displayed the highest cell density, followed by *C. goreaui* (Figure 3a). Intracelular iron concentration in control cultures are presented in Figure 3b. *C. goreaui* showed high Fe concentrations, followed by *E. voratum*, indicating that there is no direct correlation ($p > 0.05$) between growth ratios and initial concentration of iron for all the species. *B. minutum* and *E. voratum* appear to make the most of initial iron reserves, while *C. goreaui* show a less efficient investment of its stocks, possibly as a mechanism to inhabit regions where iron deprivation is important, confining the metal to specialized cell structures.

The *Symbiodinium* genus is globally distributed, with the highest diversity found in the Red Sea and, along with *B. minutum*, in the Caribbean (LaJeunesse et al., 2018). Both also occur in the southeastern coast of Brazil (Picciani et al., 2016). *E. voratum* is present in the Pacific region, while *F. kawagutii* appears to be circumscribed to the Great Barrier Reef and Hawaiian Islands (LaJeunesse et al., 2018). In contrast, *C. goreaui* is the zooxanthella with the most widespread geographical occurrence (LaJeunesse et al., 2018). This might be related to a greater ability to store Fe among other

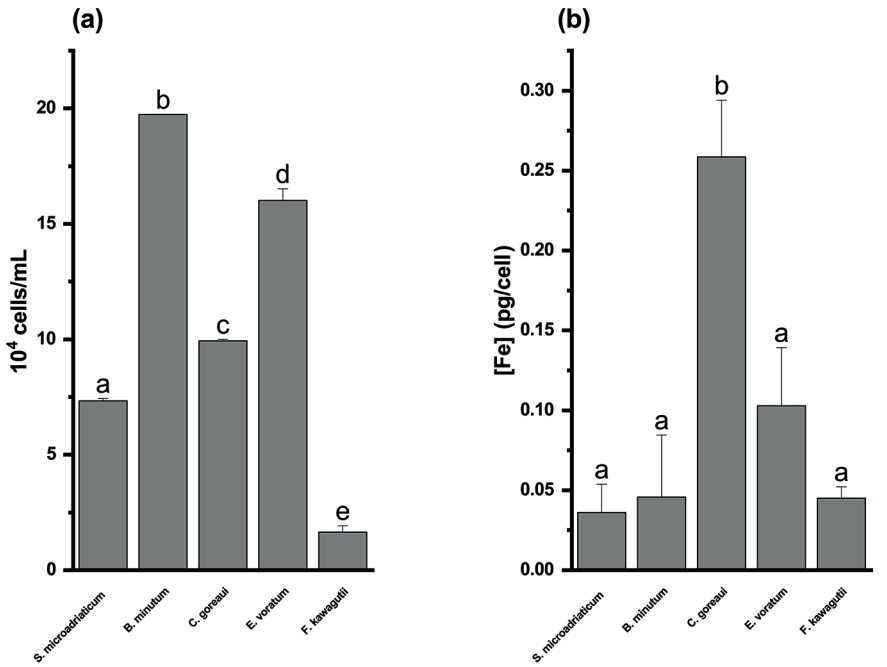

Figure 3. a) Maximum cell density attained in 21 days by each Symbiodiniaceae species growing in f/2-Fe (without supplemented iron); b) intracellular Fe concentrations after 21 days growing in f/2-Fe (without supplemented iron). Measurements not significantly different ($p > 0.05$) are represented with the same letter symbols. Differences were evaluated by one-way ANOVA and p values estimated with Tukey's post-hoc test.

nutrients compared to other studied species. The Caribbean region is not iron-limited due to the contribution of aeolian dust from the Sahara (Roff and Mumby, 2012). Also, the Red Sea, between two deserts, undergoes significant iron deployment. Therefore, *S. microadriaticum* and *B. minutum* might have evolved without the selective pressure of intracellularly stockpiling the metal. On the other hand, *C. goreaui*, as a global colonist of cnidarians, probably evolved under selective pressure to stock the metal, which may explain it having the highest initial stock of iron in our study.

Effect of iron compounds on the density of Symbiodiniaceae after 21 days

The variations on cell population of *S. microadriaticum*, *B. minutum*, *C. goreaui*, *E. voratum,* and *F. kawagutii* after the treatment with different iron compounds were plotted as growth curves (Figure 4). PCA of final symbiont density after treatment with different iron compounds is displayed in Figure 5.

PCA separates in Axis 1 the species that benefited from Fe supplementation from those that displayed slight growth (Figure 5). Axis 2 separates the species whose controls (control and DMSO) grew less than the treatments with the addition of Fe, especially in the case of *F. kawagutii*, whose controls did not grow at all, indicating that Fe was a limiting factor. Therefore, *F. kawagutii* might always depend on external iron sources. Such findings are in agreement with the results of the initial iron intracellular concentrations (Figure 3b), which revealed that this species has the lowest Fe stocks. *Cladocopium goreaui* growth was slight, with little difference between control and treatments with Fe supplementation, except for the case of Fe(II). This may be due to the existence of internal iron reserves in this symbiont (Figure 3b). Such a response may be related to its persistence in the wild, especially in ocean areas with less iron. Both *B. minutum* and *E. voratum* grew much better than their controls under Fe supplementation, and even the lag phases were smaller than those of other

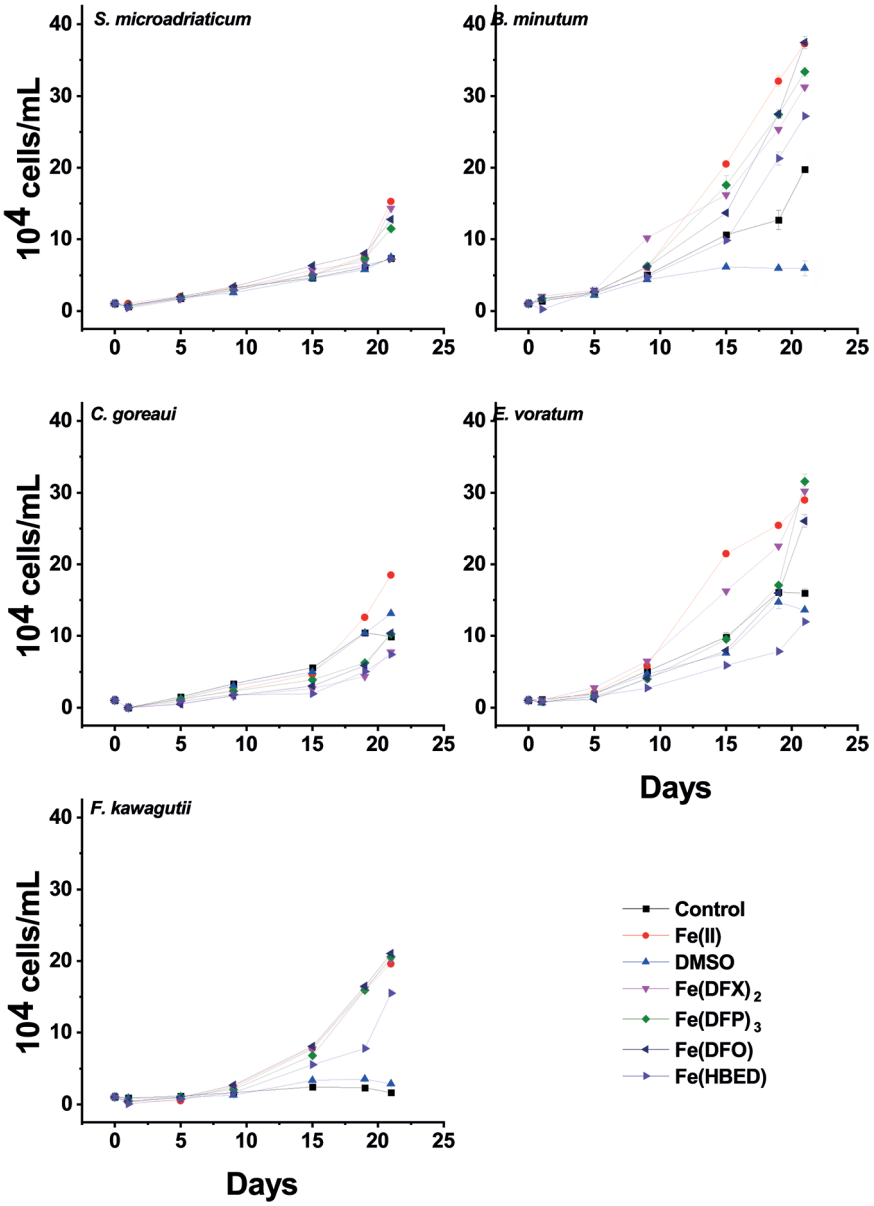

Figure 4. Growth of Symbiodiniaceae (0 - 21 days) in control cultures (Guillard-f/2-Fe and Guillard-f/2-Fe+DMSO) and with the addition of iron complexes (iron concentration = 11.7 µM).

species (Figure 4). *Fugacium kawagutii* densities were not affected after 21 days, and an exponential phase was never attained (Figure 4). This species is likely only transiently associated with the cnidarian host (LaJeunesse et al., 2018), and its difficulty to assimilate iron compounds might be one of the reasons for its somewhat less common occurrence.

Except for Fe(HBED), all the iron complexes were similar or better than free Fe(II) in promoting growth after 21 days. $Fe(DFX)_2$, the most lipophilic, was particularly effective in this regard, along with free iron. This is interesting for the development of seeding strategies with more stable and persistent forms of iron. Control without iron showed no stimulation. Growth promotion by the

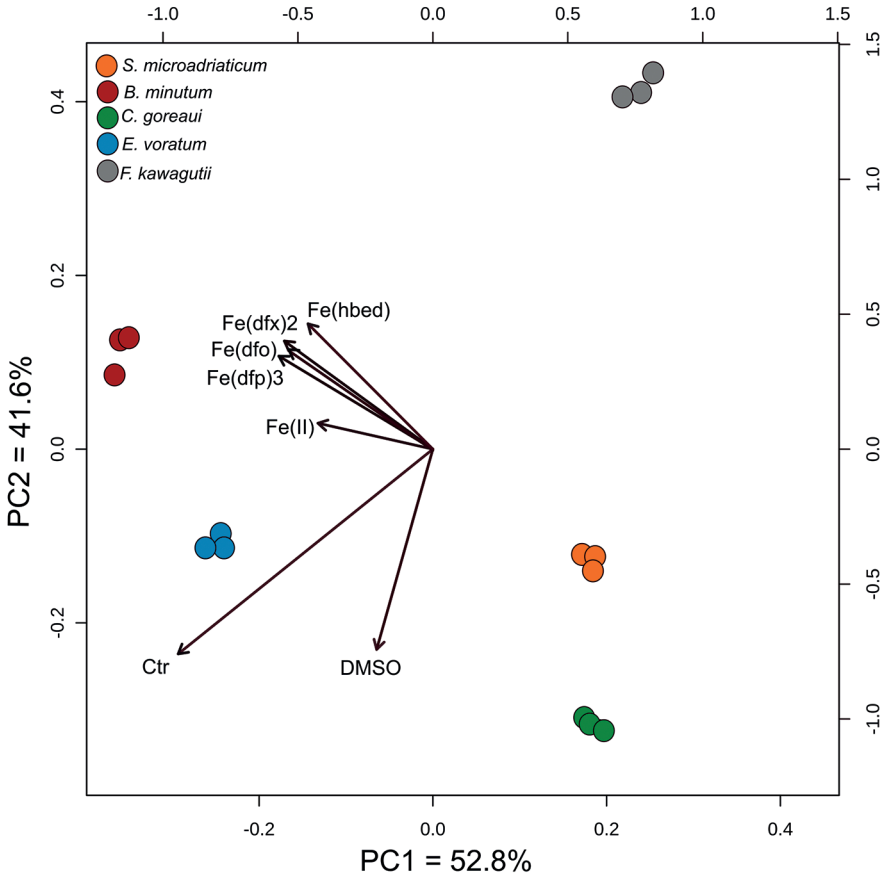

Figure 5. Differences in the cell densities of Symbiodiniaceae species after 21 days given by the addition of distinct iron compounds into their growing media represented by principal component analysis (PCA). Controls (Ctr, DMSO) and iron compounds were represented by the single vectors.

iron derivative of the bacterial siderophore DFO possibly mimics the natural mutualism found between some bacteria and microalgae for iron acquisition (Naito et al., 2008; Hopkinson and Morel, 2009). Interestingly, *C. goreaui* and *E. voratum* were the only species in which the presence of DMSO did not hamper growth and cell density at 21 days. DMSO elicited a positive growth response in *C. goreaui* (Figure 4). Indeed, DMSO, a highly cell permeant, well-tolerated molecule (up to 1 M in *Symbiodinium* spp (Lin et al., 2019)), is one of the biogenic precursors of dimethylsulfide, a potential scavenger of free radicals in algae (Hatton and Wilson, 2007; Deschaseaux et al., 2016). This could explain its promotion of growth in species with the highest initial content of iron, as they would have an advantage if able to adsorb an antioxidant precursor.

Effect of iron compounds on final iron load

Cellular iron concentrations after 21 days under different iron treatments in the five symbionts are displayed in Figure 6. PCA analysis of the iron concentration in the symbionts after 21 days under different treatments is displayed in Figure 7.

PCA separated by Axis 1 the species with high and low Fe intracellular concentrations (Figure 7). *Cladocopium goreaui* was able to stock up to ten times the Fe concentration of the control group. *F. kawagutii* was also able to stock Fe, but to a lesser extent. In both cases, $Fe(DFX)_2$ and Fe(II) were the main sources (Figure 6). Axis 2 separates *E. voratum* from *B. minutum* and *S. microadriaticum,* indicating that the latter two had the lower Fe content, whereas *E. voratum* significantly increased its stocks with $Fe(DFX)_2$ and Fe(II) supplementation

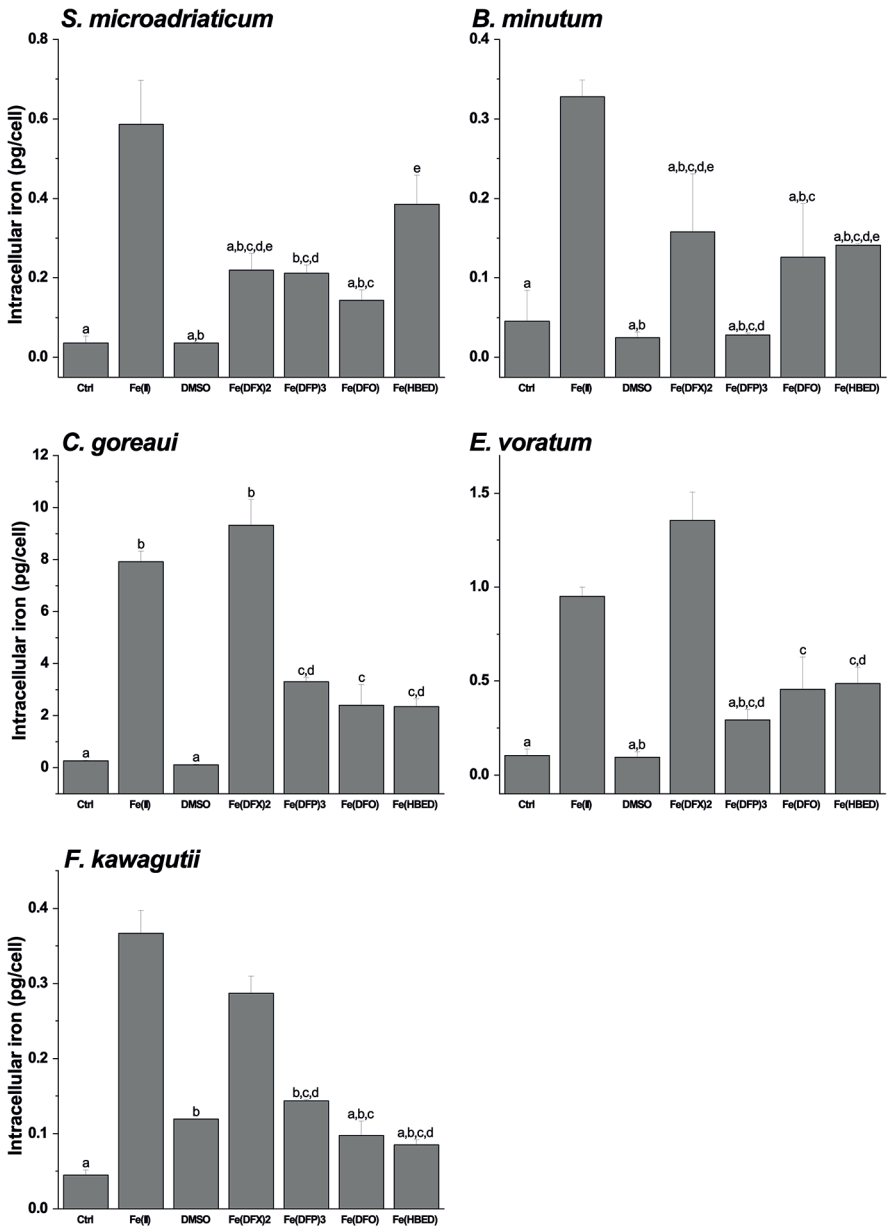

Figure 6. Intracellular iron concentrations in the five Symbiodiniaceae species after 21 days of growth. Measurements not significantly different ($p > 0.05$) are represented with the same letter symbols. Differences were evaluated by One-way ANOVA and p values estimated with Tukey's post-hoc test.

(Figure 7). These results emphasize the ability of *C. goreaui* to accumulate intracelular Fe.

The effects of the iron compounds in the final iron load of Symbiodiniaceae appeared to be related to their partition coefficients (a measure of the compound lipophilicity; Table 1), showing that this physicochemical property was relevant in providing the chemicals access to the organisms. However, the possibility of an active transport (not the focus herein) cannot be ignored. Free iron and the more lipophilic $Fe(DFX)_2$ constituted very effective iron loading substances (Figure 6). However, Fe(DFO), which is hydrophilic, generally performed well (Figure 6), which suggests the possibility active transportation. The relative high contribution of hydrophobic Fe(HBED) to the final iron content

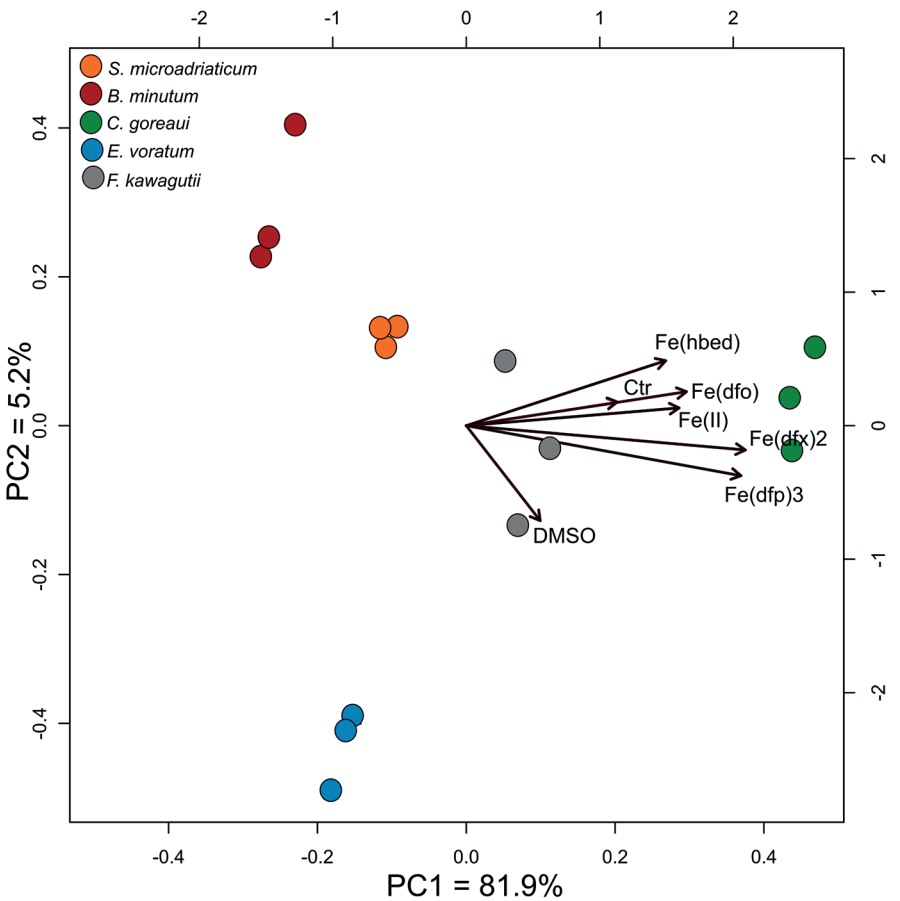

Figure 7. Differences in the final intracellular iron concentration of Symbiodiniaceae species given by the addition of distinct iron compounds into their growing media represented by principal component analysis (PCA). Iron compounds were represented in single vectors.

(Figure 6) generally does not match its contribution to increased cell growth (Figure 5), which could be explained by its very high stability, especially when compared to Fe(DFO) (log K_{ML} = 36.6 and 28.3, respectively (Anderegg et al., 1963b;Choudhary et al., 2021)). This poses an obvious thermodynamic hindrance for the release of the metal in biological media. Therefore, lipophilicity and stability should be considered together when designing iron stimulation strategies to Symbiodiniaceae.

The finding that several metal complexes can promote both growth and iron load in Symbiodiniaceae is interesting for new supplementation strategies. In particular, the natural product Fe(DFO) is ecologically less harmful than the usual iron complex used in Guillard f/2 medium, Fe(EDTA). Much interest has been placed into finding substitutes for the environmentally unfit EDTA (which displays poor biodegradation and ability to mobilize non-target metals from the substrate due to its lack of specificity). A siderophore such as DFO could potentially perform better when in live algal feed production (Sauvage et al., 2021), even though its loading ability is generally lower than that of $Fe(DFX)_2$.

Effect of iron on recovery from heat shock

The thermosensitive symbiont, *B. minutum* (Reich et al., 2021) was treated with iron compounds, both before and after the induced heat shock, as a way to determine whether iron could assist in the recovery of zooxanthella and if the order of the events would make any difference. The results of cell density after 7 days of heat shock are displayed in Figure 8.

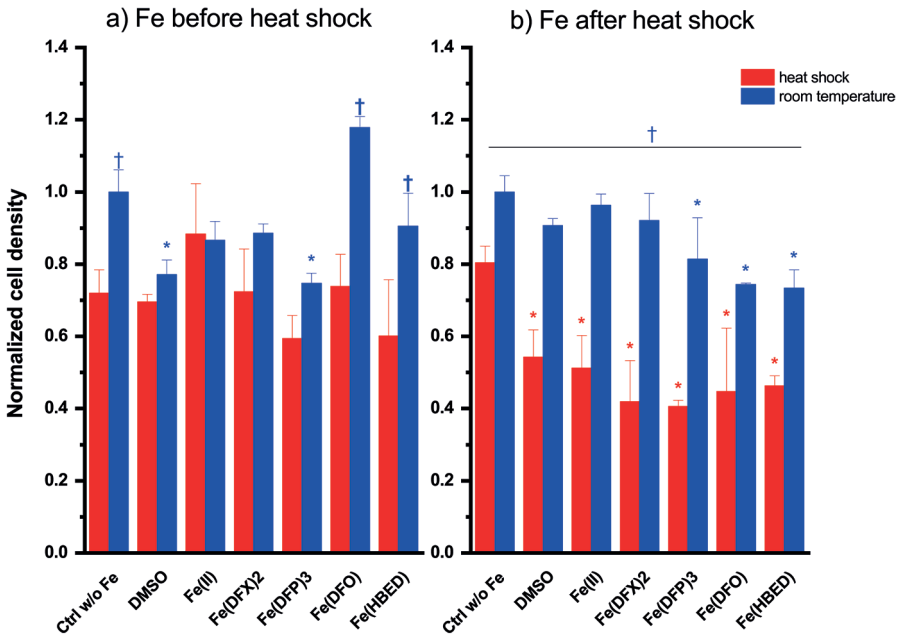

Figure 8. Effect of the order and type of Fe supplementation over the cell density of *B. minutum* undergoing heat shock (34o C) or kept at room temperature (21o C). (a) Cells were treated with iron compounds, kept for seven days, subjected to the thermal treatment, kept for another seven days, and then counted. (b) Cells were kept for seven days, subjected to the thermal treatment (followed immediately by the treatment with iron compounds), kept for another seven days, and then counted. Cell densities were normalized against their respective controls without iron and heat shock. Differences were evaluated by two-way ANOVA, and p values were estimated with Tukey's post-hoc test. †: $p < 0.05$ after comparing temperatures within the same treatment. *: $p < 0.05$ in comparison with the respective control without iron, at the same temperature.

The administration of iron compounds 7 days before the heat shock (34±1° C for 4 h), and just after the heat shock event (respectively, Figures 8a and 8b), resulted in different cell recoveries. In both treatments, the heat shock had deleterious effect on the cultures, but this was *ca*. 30% more intense in the case of Fe supplementation after the shock (Figure 8b). When the iron supply *preceded* the shock, it did not significantly impact the treatments ($p < 0.05$) compared to the controls, in most cases. Within seven days, Fe supplementation did not hamper the ability of the dinoflagellate to recover, suggesting that in some measure, iron can better prepare it for the stress.

Fe addition *after* the heat shock seemed to be deleterious compared to the control without iron (Figure 8b), as all treatments led to significantly reduced ($p < 0.05$) cell densities. This could be an indication that, during its recovery from the heat shock, *B. minutum* is more sensitive to the deleterious effects of abrupt iron loading, since it may generate transient pools of redox active, labile iron. The response to this injury may involve increased ferritin expression as a strategy to store the metal under mineralized, inert forms, as observed in Symbiodiniaceae in symbiosis with *Acropora millepora* (Csaszar et al., 2009) and other algae (Merchant et al., 2006); expression of metal-binding peptides, such as metallothioneins and phytochelatins (Perales-Vela et al., 2006; Balzano et al., 2020); induction of other stress proteins; or even undergoing metabolic changes (Bozhkov et al., 2010). This could be also a strategy for the protection of the host by the symbiont to preventing its expulsion.

Considering that high temperature inhibits the assimilation of iron from transferrin in *B. minutum*, leading to iron deficiency (Song et al., 2015), previous supplementation of iron in regions prone to episodes of excess heat could increase the resiliency of this thermosensitive organism.

CONCLUSION

The response of the five Symbiodiniaceae species to the treatment with well-defined iron(III) complexes was grouped as follows. *C. goreaui* and *E. voratum* had the highest natural amounts of iron stocks, but this physiological ability did not necessarily translate into growth. In contrast, *B. minutum* was able to convert most iron sources into growth. In *S. microadriaticum* and *F. kawagutii*, the action of the iron compounds was inconclusive. Enhanced thermal tolerance in *B. minutum* was only acquired by iron supplementation before heat shock. Both the synthetic $Fe(DFX)_2$ and, to a lesser extent, natural Fe(DFO) promoted growth and iron loading while forming stable metal complexes that control metal availability. Thus, they may be interesting iron complexes for future study with iron supplementation *in vitro* and in the environment.

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AUTHOR CONTRIBUTIONS

J.M.D.R.: Conceptualization; Investigation; Methodology; Software; Formal Analysis; Writing - review & editing;

M.T.B.: Software; Formal analysis; Writing - review & editing;

A.C.E.: Formal analysis; Writing - review & editing;

C.S.N.: Resources; Formal analysis; Writing - review & editing;

F.S.C.: Resources; Conceptualization; Software; Formal analysis; Writing - review & editing;

B.P.E.: Conceptualization; Supervision; Project administration; Funding acquisition; Writing – original draft; Writing - review & editing.

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