REPORT

Endosymbiotic dinoflagellates pump iron: differences in iron and other trace metal needs among the Symbiodiniaceae

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Abstract Iron (Fe) is essential to the physiology and growth of marine phytoplankton. It remains unclear how important iron is to the functional ecology of symbiotic dinoflagellates in the family Symbiodiniaceae, and whether limitations in iron ultimately affect the health and productivity of coral hosts, especially during episodes of ocean warming. Five Symbiodiniaceae species (spanning three genera) were used to investigate the effects of reduced iron availability on cell growth and the acquisition of other trace metals. When grown under iron replete conditions, intracellular iron quotas (content) reflected a large biochemical demand and ranged from 7.8 to 23.1 mmol Fe mol Phosphorus^{-1}. *Symbiodinium necroappetens* was the only species that acclimated and maintained high growth rates while subjected to the lowest iron treatment (250 pM Fe'). Cultures surviving under low iron concentrations experienced changes in cellular concentrations (and presumably their use as cofactors) of other trace metals (e.g., zinc, copper, cobalt, manganese, nickel, molybdenum, vanadium), in ways that were species-specific, and possibly related to the natural ecology of each species. These changes in trace metal contents may have cascading effects on vital biochemical functions such as metalloenzyme activities, photosynthetic performance, and macronutrient assimilation. Furthermore, these species-specific responses to iron limitation provide a basis for investigations on how iron availability effects cellular processes among species and genera of Symbiodiniaceae, and ultimately how metal shortages modulate the response of coral–algal mutualisms to physiological stressors.

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Introduction

Iron and other trace metals are required for the growth of macro- and microalgae. As an essential trace metal, iron is needed for many biochemical pathways and cellular processes, especially in photosynthetic organisms (Fig. 1; Martin et al. 1991; Raven et al. 1999; Merchant 2007). Numerous culture and field studies have demonstrated that iron's relatively low availability and supply limit phytoplankton growth and abundance (Brand et al. 1983); and why iron availability directly influences primary productivity in the food webs of various marine ecosystems (Martin et al. 1991; Behrenfeld and Milligan 2013).

Many biochemical pathways and cellular processes require trace metals, especially iron, for normal functioning (Fig. 1). As a cofactor for photosynthesis, iron is bound to

Fig. 1 Schematic representing some of the numerous biochemical pathways and molecular processes where trace metals are necessary for fundamental cell function and cellular responses to physiological stress. For each example, the elemental symbol is presented for metals that are or may be used as a cofactor in proteins or protein complexes, which perform a specific enzymatic process or electron transfer
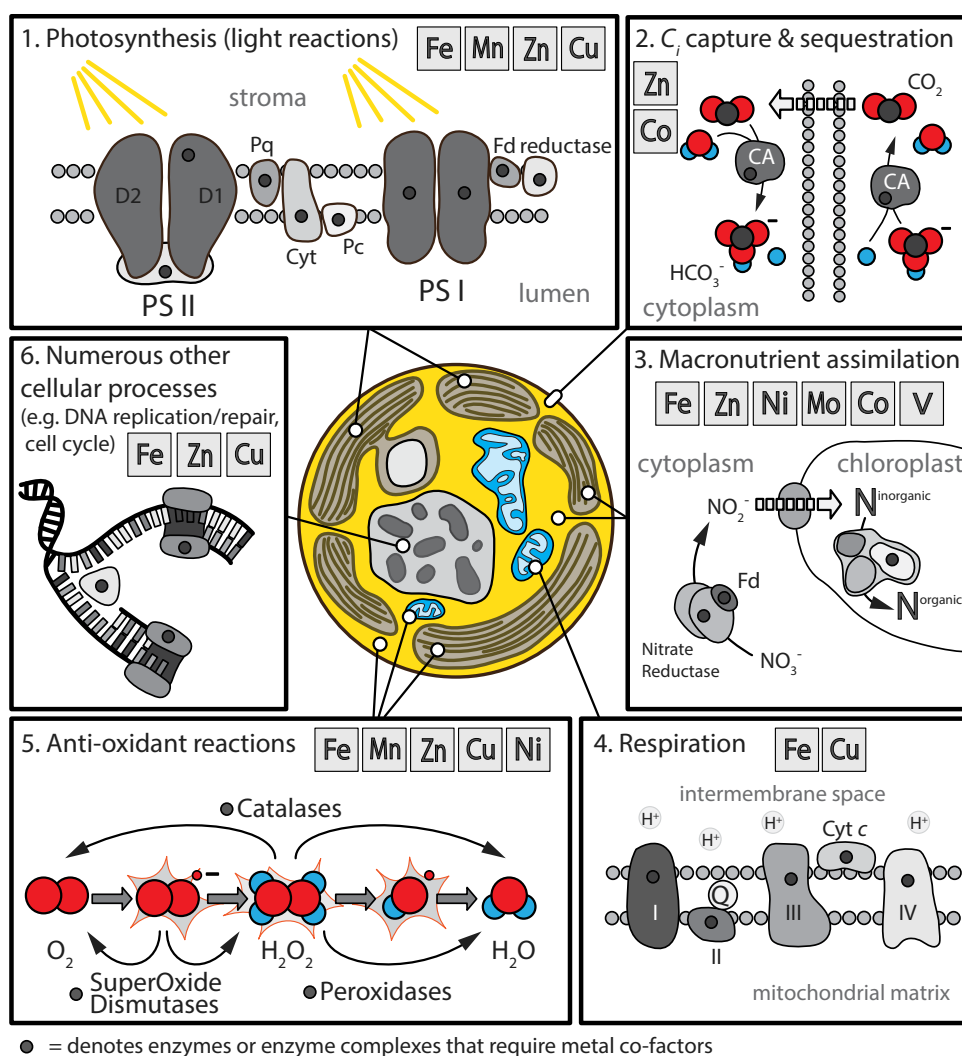
proteins needed in photosystem I, photosystem II, the electron transport chain (plastoquinone, cytochromes), and ferredoxin activity (Raven et al. 1999; Crichton and Pierre 2001). Its use by scavenging enzymes helps prevent cellular damage by neutralizing reactive oxygen species (Wolfe-Simon et al. 2005). Furthermore, iron is utilized by enzymes important for macronutrient assimilation such as nitrate and nitrite reductase (Raven 1988; Morel et al. 1991). Thus, iron is especially important to the healthy functioning of all photosynthetic organisms (Behrenfeld and Milligan 2013).

Increasing evidence supports the importance of iron and other trace metals for the stability of the mutualisms between reef-building corals and their endosymbiotic dinoflagellates (Shick et al. 2011; Ferrier-Pagès et al. 2018). The emergence of Symbiodiniaceae in the Mesozoic coincides with major transitions in oceanic trace metal availability and biogeochemistry (Falkowski et al. 2004; Katz et al. 2004; LaJeunesse et al. 2018). While iron is

often deficient in coral reef ecosystems (Entsch et al. 1983), adequate iron availability, including other trace metals, has been deemed imperative to the physiology and health of reef-building corals (Biscéré et al. 2018; Ferrier-Pagès et al. 2018). Low iron availability increases a colony's sensitivity to thermal stress, which can cause a breakdown of the mutualism (i.e., "coral bleaching"; Shick et al. 2011). Further, trace metals are required by most enzymes used for antioxidant protection and are therefore important to boosting thermal stress tolerance (Lesser 1996; McGinty et al. 2012; Krueger et al. 2015; Levin et al. 2016). Collectively, these observations highlight the potential importance of iron and other trace metals in the physiological response of reef corals to climate change-induced stressors.

Despite the apparent importance of iron and other trace metals to the physiology of symbiotic dinoflagellates in the family Symbiodiniaceae, our understanding of how much is required to meet the metabolic needs of various species

remains limited. The free-living *Fugacium kawagutii* is the only species of Symbiodiniaceae for which iron requirements are known in detail (Rodriguez et al. 2016; Rodriguez and Ho 2017, 2018). As with many other dinoflagellates, the high iron requirement of *F. kawagutii* suggests that large quantities of iron are also necessary for the maintenance of mutualistic symbiosis involving dinoflagellates (Ho et al. 2003; Rodriguez et al. 2016; Rodriguez and Ho 2017, 2018).

To assess this possibility, a comparative study was endeavored to measure the physiology and cell proliferation of various Symbiodiniaceae growing at different iron concentrations. Here, we examined how exposure to different iron concentrations altered growth kinetics of five species of Symbiodiniaceae representative of three genera. By comparing strains with free-living, necrotrophic, and symbiotic lifestyles, we assessed how ecologically differentiated species responded to iron stress through changes in cell proliferation and adjustments to internal concentrations of other trace metals. The influence of low iron availability on the concentrations of other trace metals can have cascading effects on numerous vital biochemical pathways. By assessing the range of iron requirements among different species, we begin to consider the relative importance of iron availability and acquisition for the health and growth of coral colonies depending on the symbiont species they harbor.

Methods

Culture conditions

Five species of Symbiodiniaceae (*Symbiodinium microadriaticum*, *Symbiodinium necroappetens*, *Breviolum minutum*, *Breviolum psygmophilum*, and *Effrenium voratum*) were acclimatized to a modified L1 medium in 250-mL acid-washed polycarbonate bottles (Table 1; Guillard and Hargraves 1993). Cultures had been maintained in the LaJeunesse Lab culture collection since 2004. Strain identity was confirmed by Sanger sequencing of the ribosomal ITS2 marker at the Penn State Genomics Core Facility. All culture preparation work occurred in a class-100 trace metal clean laboratory at Academia Sinica, and all plastic ware was prepared with the appropriate protocols (Rodriguez et al. 2016; Rodriguez and Ho 2017, 2018). Symbiodiniaceae were grown in the surface seawater collected from the South China Sea and initially filtered using Whatman Polycap filters. To remove background trace metals, seawater was passed through a Chelex® 100 resin at a rate of 2.5 mL min^{-1}. Subsequently, the purified seawater was filter-sterilized using 0.22-μm Millipore filters and transferred into sterilized 1-L polycarbonate bottles. Background trace metal concentrations were routinely measured at Academia Sinica, and background iron concentrations were around 0.10 nM (Ho et al. 2010; Ho 2013; Wang et al. 2014).

Culture conditions were derived from previous work with *Fugacium kawagutii* (Rodriguez et al. 2016; Rodriguez and Ho 2017, 2018). Concentrated standards of trace metal grade purified major nutrients, trace metals, and vitamins were added to the culture medium to reach the initial concentrations of nitrate (800 μM), phosphate (50 μM), trace metals (Mn, Zn, Co, Cu, Ni, and Mo, 10 nM each), cyanocobalamin (0.40 nM), biotin (2.1 nM), and thiamine (300 nM). For experimentation, the five species were exposed to four different iron concentrations, including iron starvation (0 nM total dissolved Fe), low iron (50 nM), intermediate iron (100 nM), and iron replete (250 nM) treatments. This led to 20 total treatments and 60 culture bottles ($n = 3$). Iron enrichment was done using FeCl$_3$·6H$_2$O (Fe^{3+}) following Morel et al. (1979) and further described in the studies (Morel et al. 1979; Ho et al. 2003; Rodriguez et al. 2016). To regulate metal bioavailability, trace metal grade ethylenediaminetetraacetic acid (EDTA) was added to each bottle to reach a final EDTA concentration of 20 μM. Based on the Le Chatelier principle, EDTA serves as a buffer to keep the inorganic or bioavailable fraction (Fe′) relatively constant. The EDTA ligand-chelated fraction (organic fraction) is not bioavailable to microalgae (Anderson and Morel 1982). With the addition of EDTA, estimated inorganic concentrations of 0, 250, 500, and 1250 pM Fe′ represented the bioavailable fractions, corresponding to the total dissolved iron concentrations 0, 50, 100, and 250 nM Fe, respectively (Rodriguez et al. 2016; Rodriguez and Ho 2017, 2018). Hereafter, the iron treatments will be referred to as

Table 1 Species, strain, and (former) clade identification of Symbiodiniaceae used for experimentation

Species	Strain	Former clade
Symbiodinium microadriaticum	CCMP 2464/rt362(1)	A
Symbiodinium necroappetens	MAC-225	A
Breviolum minutum	CCMP 2460/rt002(1)	B
Breviolum psygmophilum	PurPFlex	B
Effrenium voratum	rt 383(2)	E

starvation (0 pM Fe′), low iron (250 pM Fe′), intermediate iron (500 pM Fe′), and replete iron (1250 pM Fe′).

Prior to and during experimentation, cultures were maintained on a 12:12-h light:dark cycle with light intensity at 650 ± 50 µmol photons m^{-2} s^{-1} and at ambient temperature (26 °C). Symbiodiniaceae cultures were maintained at saturating light levels to prevent low-light acclimation and to enable comparison with other bodies of work (Ragni et al. 2010; Rodriguez et al. 2016; Rodriguez and Ho 2017, 2018). For low, intermediate, and replete iron treatments, Symbiodiniaceae were acclimatized to experimental iron concentrations for 1 month prior to experimentation. Iron starvation treatments were inoculated from Symbiodiniaceae reared at the low iron treatment. All treatments were started from stock Symbiodiniaceae (at the appropriate iron concentrations) cultures at maximum cell density. All treatments were run with triplicate replicates with starting cell densities between 1000 and 2000 cells mL^{-1}. After inoculation, Symbiodiniaceae growth was measured every other day using a Beckman Coulter Counter Multisizer 3 with a 100-µm aperture tube until cultures reached stationary phase. All growth monitoring occurred during the light phase of the light:dark cycle.

Determination of intracellular metal quotas (content)

During mid-exponential phase, ten million cells of Symbiodiniaceae were harvested for determination of intracellular elemental quota (iron, zinc, manganese, copper, nickel, cobalt, molybdenum, vanadium, and phosphorus) using high-resolution inductively coupled plasma mass spectrometry (HR-ICPMS, Element XR, Thermo Scientific). For each triplicate replicate, the appropriate volume of cells was harvested onto acid-washed 25-mm (2 µm pore size) Millipore TTTP polycarbonate filters during the light phase of the light:dark cycle. Harvested cells were washed with ultrapure Milli-Q water and digested in 50% nitric acid in preparation for HR-ICPMS. The details of the analytical precision, accuracy, and detection limits of the ICPMS method for microalgae are described in the studies (Ho et al. 2003; Ho 2013). Trace metal content was normalized to mol phosphorus (P) following Redfield ratios and because P is analyzed simultaneously with trace metals by HR-ICPMS (Redfield 1934).

Statistical analyses

Specific growth rate was calculated as $\frac{\Delta \ln(\text{cell density})}{\Delta \text{time (days)}}$. Metal uptake rates were calculated by multiplying specific growth rate and intracellular iron quota. Metal net use efficiency was calculated by dividing specific growth rate with intracellular iron quota. All statistical analyses were run in R v3.3.2 (R Core Team 2016). Since data did not meet the assumptions of parametric statistics, a Kruskal–Wallis (KW) test was used to determine differences in mean cell density, intracellular metal quotas, uptake rates, and net use efficiencies using the PMCMR package (Pohlert 2014). Subsequently, a pairwise test for multiple comparisons of mean rank sums (Dunn's test) was used as a post hoc test to determine differences between treatment groups using a Benjamini–Hochberg p adjustment to account for false discovery rate (Benjamini and Hochberg 1995). Data were visualized using ggplot2 v2.2.1, ggpubr v0.1.6, and cowplot v0.8.0 (Wickham 2009; Wilke 2016).

A principal component analysis (PCA) was used to determine multivariate clustering of Symbiodiniaceae trace metal content. Trace metal content data were log-transformed, and individuals with missing values were omitted. The FactoMineR package was used to generate and visualize PCA statistics (contribution and correlation of variables in the principal components). A PCA biplot was generated using the FactoMineR fviz_pca_biplot function with points overlaid using the ggplot2 geom_point function so multiple factor levels (iron concentration and species) could be visualized by shape/color. The ggplot2 stat_ellipse function was used to display a confidence (0.95) ellipse for each species. All data and code are available on GitHub https://github.com/hgreich/Symbiodiniaceae_Pump_Iron.

Results

Symbiodiniaceae growth increases with iron concentration

Low iron concentrations resulted in decreased specific growth rates and maximum cell density (Figs. 2, 3). Both Symbiodiniaceae species ($F = 5.1$, $p < 0.01$) and iron treatment ($F = 45.3$, $p < 0.01$) had statistically significant effects on cell densities (Fig. 2). However, the interaction between species and iron concentrations was only marginally significant ($F = 2.2$, $p = 0.07$). Under iron starvation conditions, all Symbiodiniaceae exhibited diminished growth curves similar to one another and were unable to reach exponential growth phase (Fig. 2). However, growth under low, intermediate, and replete iron concentrations reached exponential growth phase and was statistically similar across all isolates (Figs. 2, 3). Despite the similarity of growth curves, the individual treatments reached exponential growth phases at different time points and exhibited nominal increases in specific growth with increased iron concentration above 250 pM Fe′ (Figs. 2, 3).

Fig. 2 Cell culture densities over time for five Symbiodiniaceae exposed to different iron concentrations (spanning starvation–replete conditions). Iron concentration is denoted by plot symbol. Points represent the average of triplicate replicates. Error bars represent ± standard deviation
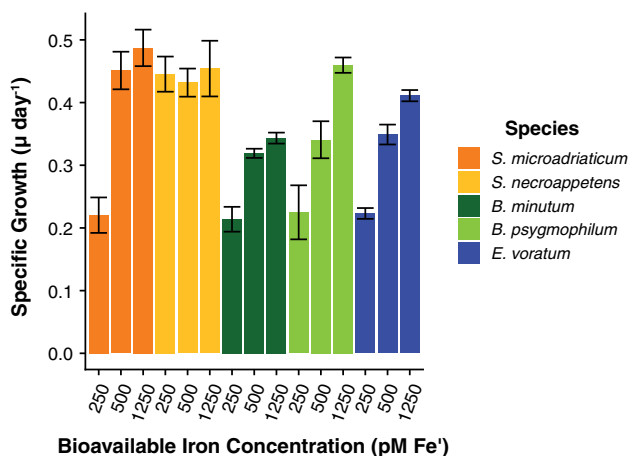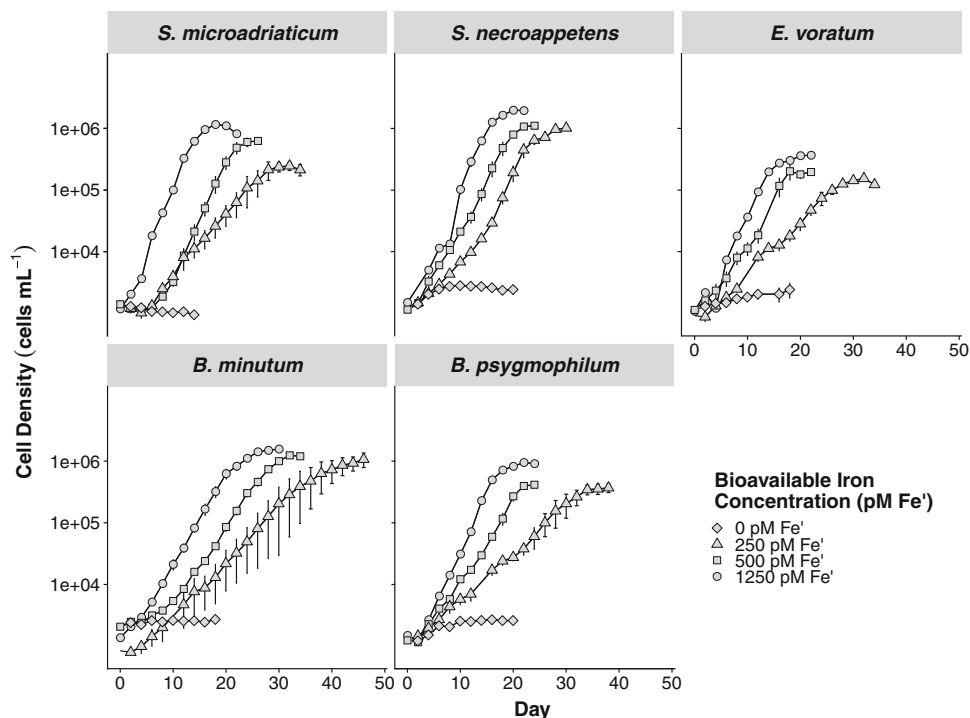

Fig. 3 Specific growth rate (μ day^{-1}) of five species exposed to iron concentrations spanning low (250 pM Fe′) to replete (1250 pM Fe′) iron concentrations. Specific growth was not calculated for treatments exposed to iron starvation conditions (0 pM Fe′) because growth did not occur. Bars represent the average of triplicate replicates. Error bars represent ± standard deviation

Symbiodiniaceae iron content (quota) increases with iron concentration

Growth under decreased iron concentrations corresponded to reduced intracellular iron content for all species (Fig. 4a). Because iron starvation prohibited exponential growth, there was not enough cell biomass in the iron starvation treatment to obtain HR-ICPMS data. Thus, all trace metal content comparisons were made between the low, intermediate, and replete iron treatments. The output of all pairwise statistical comparisons is available on GitHub, and several comparisons are highlighted below. There was a significant decrease in intracellular iron content with decreases in iron supply when normalized to phosphorus (P) content (mmol Fe mol P^{-1}; KW chi-squared = 81.2, df = 14, p value = 1.7e−11) and cell volume (μm^3 mL^{-1}; KW chi-squared = 65.85, df = 12, p value = 1.9e−09, Fig. 4a, S1). Cellular phosphorus concentrations varied by species but were consistent for all within-species comparisons at the different iron concentrations ($p < 0.05$ for each within-species comparison; Fig. S1).

The extent of iron content decline between different treatments was species-specific. *Symbiodinium microadriaticum*, *B. psygmophilum*, and *E. voratum* displayed significant decreases in iron content when exposed to low and replete iron concentrations ($p = 0.02$, $p < 0.01$, and $p = 0.01$ respectively; Fig. 4a). Their iron content at intermediate iron concentrations displayed nominal decreases when compared to those grown at low and replete conditions. However, the iron content of *S. necroappetens* and *B. minutum* at iron replete conditions was significantly larger than counterparts at low ($p < 0.01$) and intermediate iron treatments ($p = 0.01$ and $p = 0.03$, respectively; Fig. 4a). At all treatments, *E. voratum* and *B. psygmophilum* iron contents were more than double the size of the iron content of *S. microadriaticum*, *B. minutum*, and *S. necroappetens* (Fig. 4a).
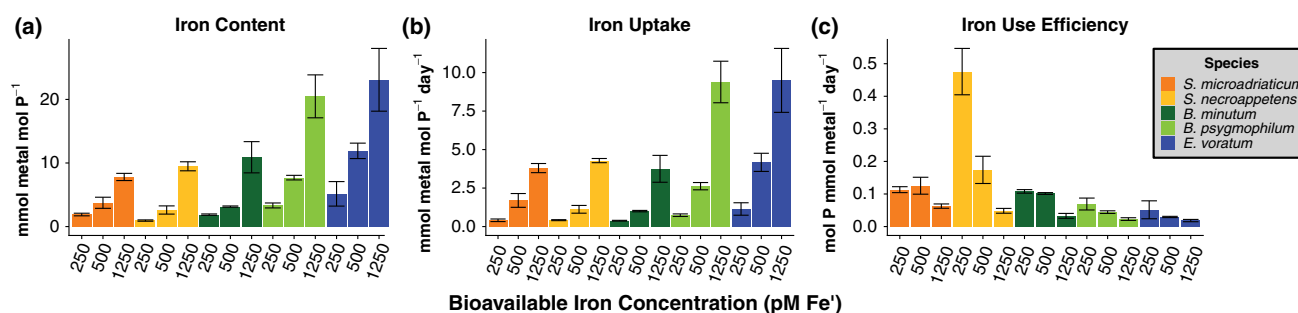
Fig. 4 a intracellular iron content (quotas), **b** iron uptake rates, **c** iron net use efficiency for five species of Symbiodiniaceae exposed to different iron concentrations. Bars represent averages of $N = 6$ samples. Error bars represent $\pm$ standard deviation

Iron uptake rates were significantly higher at elevated iron concentrations for all five species (KW chi-squared = 81.32, df = 14, p value = 1.61e−11, Fig. 4b). Conversely, iron use efficiency decreased inversely with iron concentration (KW chi-squared = 79.87, df = 14, p value = 2.99e−11, Fig. 4c). Iron net use efficiency was significantly higher at low iron concentration treatments relative to iron replete counterparts for *S. necroappetens*, *B. psygmophilum*, and *B. minutum* ($p = 0.01$, $p = 0.03$, and $p = 0.03$, respectively; Fig. 4c).

Variation of Symbiodiniaceae trace metal content (quotas)

In addition to iron, intracellular zinc, manganese, copper, nickel, cobalt, molybdenum, and vanadium were detected in the five Symbiodiniaceae species (Fig. 4). Symbiodiniaceae demonstrate substantial variation in compensation for low iron exposure with each species displaying a unique set of iron–metal interactions. Here, an iron–metal interaction occurs when a given Symbiodiniaceae species' trace metal quota, uptake rate, or net use efficiency changes significantly between different iron treatments (Fig. 6a). Briefly, substantial between- and within-genus variation was found and changes in trace metal use strategies in response to low iron exposure did not follow phylogenetic patterns (Figs. 5, 6, S2, S3).

Within the genus *Symbiodinium*, iron had little effect on other trace metal usage for *S. necroappetens*, but clear interactions between iron–zinc, iron–nickel, iron–copper, iron–cobalt, iron–molybdenum, and iron–vanadium were measured for *S. microadriaticum* (Figs. 5, 6a, S2, S3). Under intermediate iron concentrations, *S. microadriaticum* nickel content was significantly higher than at low iron concentrations ($p < 0.01$) and replete conditions ($p = 0.03$, Fig. 5). Additionally, nickel uptake rate was significantly faster at intermediate iron conditions relative to low iron ($p < 0.01$) and replete ($p = 0.02$, Fig. S2) concentrations. Conversely, nickel net use efficiency was significantly lower at intermediate iron concentrations

relative to low ($p < 0.01$) and replete ($p = 0.01$) treatments (Fig. S3). *Symbiodinium microadriaticum* zinc content was significantly higher at iron replete conditions relative to intermediate ($p = 0.03$) and low ($p = 0.02$) treatments, whereas replete *S. microadriaticum* zinc uptake rate was only significantly higher than the low iron treatment ($p < 0.01$; Figs. 5, S2). Cobalt and molybdenum uptake rates were significantly faster at intermediate iron treatments relative to low iron concentrations ($p = 0.01$ and $p = 0.03$, respectively; Fig. S2). Copper net use efficiency was significantly larger at iron replete conditions relative to low iron conditions ($p = 0.03$, Fig. S3). Lastly, *S. microadriaticum* vanadium uptake rate was significantly faster at replete iron concentrations relative to low iron conditions ($p = 0.01$; Fig. S2).

Similarly, within the genus *Breviolum*, no iron–metal interactions were detected in *B. minutum*, whereas *B. psygmophilum* demonstrated iron–manganese, iron–cobalt, and iron–vanadium interactions (Figs. 5, 6a, S2, S3). *Breviolum psygmophilum* manganese content was significantly larger at intermediate iron conditions than at replete conditions ($p = 0.04$), whereas manganese net use efficiency was significantly larger at iron replete conditions relative to intermediate iron ($p = 0.02$) and low iron ($p = 0.03$) treatments (Figs. 5, S3). Additionally, at iron replete concentrations, cobalt net use efficiency was larger than at low and intermediate iron treatments ($p = 0.04$ and $p = 0.05$, respectively; Fig. S3). *Breviolum psygmophilum* vanadium uptake rate significantly increased between low iron and replete iron treatments ($p < 0.01$, Fig. S2).

Effrenium voratum displayed iron–nickel, iron–molybdenum, and iron–vanadium interactions (Figs. 5, 6a, S2, S3). Nickel content and uptake rate were significantly lowest at low iron treatments relative to intermediate ($p < 0.01$ for both comparisons) and replete treatments ($p = 0.02$ for both comparisons; Figs. 5, S2). Conversely, nickel net use efficiency was significantly larger at the low iron treatment relative to the intermediate treatment ($p < 0.01$, Fig. S3). Molybdenum content ($p < 0.01$) and uptake rate ($p < 0.01$) were significantly smaller at the low
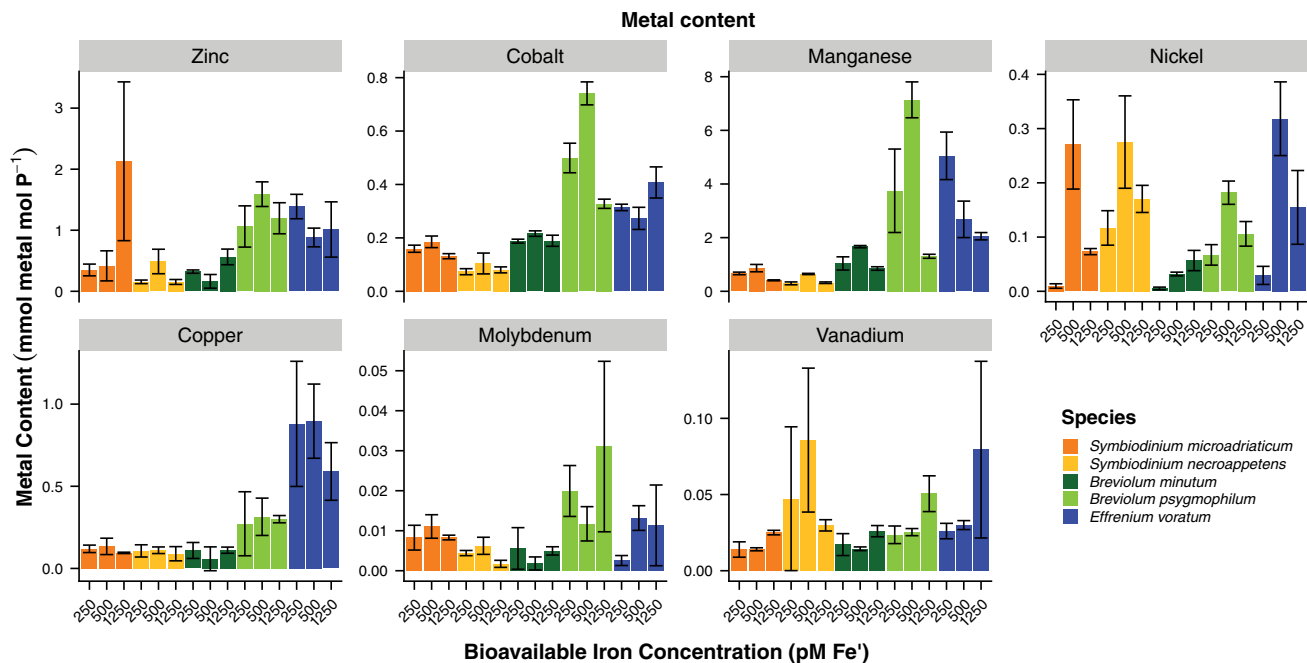
Fig. 5 Changes in intracellular trace metal content at low to high iron concentrations. Bars represent averages of $N = 6$ samples. Error bars represent $\pm$ standard deviation

iron concentration treatment relative to intermediate counterparts (Figs. 5, S2). Additionally, molybdenum uptake rate also significantly decreased between the replete and low iron treatments ($p = 0.03$, Fig. 5). Contrastingly, molybdenum net use efficiency decreased significantly between the intermediate and low iron treatments ($p = 0.03$, Fig. S3). Like molybdenum, vanadium uptake rate significantly decreased between the replete and low iron treatments ($p < 0.01$, Fig. S2).

The PCA of Symbiodiniaceae trace metal content yielded three main clustering domains by species: *B. psygmophilum* and *E. voratum*, *B. minutum* and *S. microadriaticum*, and *S. necroappetens* only (Fig. 6b). Further clustering by iron concentration can be observed within some species clusters (Fig. 6b). The first two dimensions/principal components (PCs) of the PCA explained 65.9% of the variance (PC1—45.2% variance, and PC2—20.8% variance; Fig. 6b). Cobalt (20.7%), zinc (18.8%), manganese (18.4%), copper (15.7%), and iron (11.7%) contributed the most variation to PC1, and all had positive correlations (Fig. 6b). In PC2, vanadium (38.3%), nickel (33.6%), and cobalt (10.6%) contributed the most variation to PC2 (Fig. 6b). In PC2, nickel and vanadium had positive correlations where cobalt was negative (Fig. 6b). Since many of the loadings are in the positive (right) side of PC1, the individuals (primarily *B. psygmophilum* and *E. voratum*) further to the right of the biplot had higher trace metal content (Fig. 6b). Pairs of loadings

(arrows) that are close together (e.g., manganese and cobalt) had high positive correlation (Fig. 6b).

Discussion

The relatively high quantities of iron required for cell division and growth underscore the importance of micronutrient (i.e., trace metals) availability for species of Symbiodiniaceae (Figs. 2, 3, 4, S4). These needs appear to have important physiological consequences, which deserve further consideration when characterizing the health and stable functioning of coral–dinoflagellate mutualisms. Their demand for iron may relate to the maintenance of numerous biochemical pathways and enzymes needing metal cofactors (Fig. 1). As dinoflagellates, these demands may stem from having evolved in environments where metal concentrations were relatively high (e.g., shelf or coastal waters) in addition to having the cellular machinery for carrying out photosynthesis and heterotrophy. Differences in iron requirements and trace metal profiles between species appear to correspond with ecological guilds, which suggest that metal availability might influence the processes of natural selection and niche diversification (Figs. 5, 6).

Fig. 6 Comparison between phylogenic relationships and species ecology with changes in trace metal content resulting from physiological responses to iron limitation. While phylogenetic relationships do not explain trace metal requirements, there appears to be a strong correspondence with ecological guild. **a** Phylogeny showing relationships among Symbiodiniaceae species used in this research (highlighted in color). Additional species were included to provide an evolutionary context with unexamined genera. Elemental symbols designate species that exhibited altered use of trace metals when at different iron concentrations. **b** Principal component analysis (PCA) of trace metal content among species grown at different iron concentrations. Vector length is correlated with contribution in the first two principal components. Ellipses represent confidence (0.95) intervals for the multivariate distribution of each species
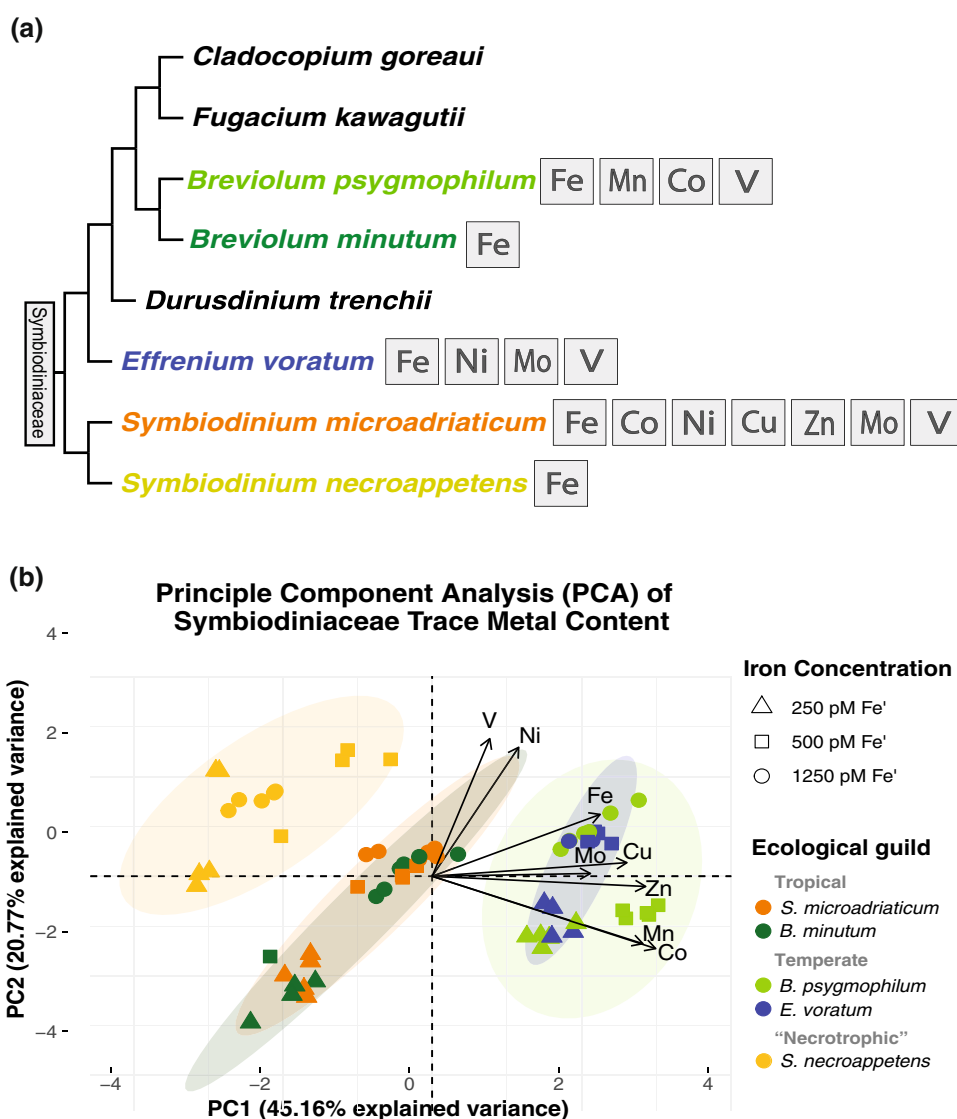
High iron requirements

Symbiodiniaceae generally required large amounts of iron to reach maximum growth relative to other microalgae and dinoflagellates grown in culture (Figs. 2, 3, 4, S4; Sunda and Huntsman 1995; Rodriguez et al. 2016; Rodriguez and Ho 2017, 2018). Several explanations as to why Symbiodiniaceae maintain large iron reserves and require high iron supply for cell proliferation in culture are discussed below.

The capacity for mixotrophic nutrient acquisition (photosynthesis and consuming prey) by most dinoflagellates, including Symbiodiniaceae, may contribute to a greater iron demand relative to other microalgae (Jeong et al. 2012). When free-living, even temporarily, symbiotic species of Symbiodiniaceae may possess the ability to feed on prey to compensate for nutrient limitation and elevate internal nutrient reserves (Stoecker et al. 1997; Smalley et al. 2003). Additionally, the iron cofactors required for prey assimilation cellular machinery (i.e., digestion, phagocytosis, ion trafficking) may demand increased iron reserves (Stossel 1974).

There is a tendency for microalgae in coastal environments to have higher iron requirements relative to oceanic counterparts (Sunda and Huntsman 1995). Oceanic species of diatoms and coccolithophores are able to subsist and continue to proliferate at far lower iron concentrations relative to coastal diatoms and dinoflagellates (including the Symbiodiniaceae; Sunda and Huntsman 1995; Rodriguez et al. 2016; Fig. S4). The lower iron requirements of open-water species likely have resulted from selection pressures that demand economizing the use of iron and other trace metals. However, because most photosynthetic dinoflagellates live in coastal surface waters with high iron concentrations, they are rarely iron-limited and therefore appear to lack the necessary adaptations for living in low iron environments (Strzepek and Harrison 2004).

As a necessary cofactor for enzymes involved in many metabolic pathways (Raven et al. 1999), iron is likely important to the healthy functioning of coral–dinoflagellate mutualisms (Fig. 1). Because of their extensive use in the process of photosynthesis, it is not surprising that trace metals in reef-building corals are most concentrated in the symbiont relative to host tissue and skeleton (Mitchelmore et al. 2003; Reichelt-Brushett and McOrist 2003; Horwitz et al. 2014; Ferrier-Pagès et al. 2018). In addition to maintaining iron required for its own photo-physiological purposes, the symbiont's ability to effectively store/retain iron from the host could prevent damage to the host from the accumulation of reactive oxygen species during stress events (Lesser 1996; Shick et al. 2011; Parkinson et al. 2015; Levin et al. 2016). Thus, the efficient iron sequestration may aid the normal function of symbiont cells when residing beneath layers of plasma membranes and host cell cytoplasm.

The consequence of insufficient iron availability on other trace metals

While iron is vital to most biochemical functions, many other trace metals are also important to cell physiology and function (Fig. 1). Changes in trace metal compositions were different for each species during exposure to low iron concentrations (e.g., shifts in intracellular zinc, manganese, copper, nickel, cobalt, molybdenum, vanadium; Fig. 4a). Certain shifts in metal concentrations among the five species presumably compensated for iron starvation (Fig. 1). The replacement of iron by other trace metal cofactors constitute an acclimatory process that helps to maintain enzymatic activities and normal biochemical functions in microalgae (reviewed in Blaby-Haas and Merchant 2012; Twining and Baines 2013) and in plants (reviewed in Puig et al. 2007). Presumably, different shifts in metal contents observed in the Symbiodiniaceae may compensate for iron limitation in ways proposed below.

Photo-physiological demand may underlie shifts in trace metal usage following exposure to low iron supply. The utility of manganese and copper in photosynthesis can compensate for insufficient iron access (Wood 1978; Raven et al. 1999; Maldonado et al. 2006). Each PSII reaction center requires four manganese atoms (Raven et al. 1999; Fig. 1). Copper electron donors can assist thylakoid maintenance when iron supply is sparse (Maldonado et al. 2006; Fig. 1). The ability of *E. voratum* and *B. psygmophilum* to increase manganese content during exposure to lower iron concentration may aid in the maintenance of PSII (Figs. 1, 5). Similarly, *S. microadriaticum's* ability to alter copper efficiency could assist thylakoid maintenance via iron–copper replacement during iron limitation (Wood 1978; Maldonado et al. 2006; Figs. 1, 5). Ultimately, the

ability to utilize manganese and copper while iron is limited may improve photosynthetic efficiency and explain photo-physiological differences among these species when subjected to heat stress (Figs. 5, S2, S3; Goyen et al. 2017; Biscéré et al. 2018; Mansour et al. 2018).

A decrease in micronutrients has important implications for numerous enzymatic processes important to cell growth. Several pathways involved in macronutrient assimilation rely on intracellular metals that can become depleted during exposure to low iron conditions. Metalloenzyme activity including, carbonic anhydrase zinc-cobalt needs (carbon cycle), alkaline phosphatase zinc requirements (phosphorus cycle), and nitrate reductase molybdenum-vanadium cofactors (nitrogen cycle) may be impaired by changes in trace metal content caused by iron limitation (Morel et al. 1994; Shaked et al. 2006; Twining and Baines 2013; Fig. 1). Zinc content decreased significantly in *Symbiodinium microadriaticum* when grown at low iron, which would further reduce cell proliferation by compromising the production of photosynthate (via drop in carbonic anhydrase activity) and its ability to assimilate phosphate (via decline in alkaline phosphatase activity; Figs. 1, S2). Similarly, iron-limited *E. voratum*, *B. psygmophilum*, and *S. microadriaticum* all showed decreases in molybdenum and vanadium, which would slow or halt cell division in these species by impairing nitrogen assimilation (observed in Figs. 1, S2). When iron-limited, the compensatory losses of other essential trace metals and disruption of nutrient assimilation could compromise the persistence of symbiotic species *in hospite*.

Additional considerations of factors influencing trace metal uptake

Ultimately, it remains unknown why some trace metals increased in cells grown at low iron, and whether changes in their concentrations were because of active or passive transport. While typically specific to one element, flexibility for other metals by some membrane transporters may result in non-specific uptake (Masmoudi et al. 2013). Thus, low specificity in metal transporters could contribute to the rise in some trace metals for certain Symbiodiniaceae under iron limitation (Fig. 1). Moreover, changes in metal composition resulting from non-specific transportation may not necessarily fulfill a particular biochemical purpose (Kosman 2003).

In nature, microalgal assimilation of bacterial siderophores can aid in their acclimation to low iron concentrations (Soria-Dengg and Horstmann 1995; Wilhelm et al. 1996). When secreted, these compounds allow bacteria to persist at low iron conditions by scavenging iron (preferentially Fe^{3+}). Genomic studies of Symbiodiniaceae have found evidence for siderophore genes (Shoguchi et al.

2013; Aranda et al. 2016; Parkinson et al. 2016), although their presence probably originated from the genomes of co-occurring bacteria in these cultures. Putative interspecific variation in siderophore abundance and activity in cultures may explain some differences in trace metal compositions observed (Figs. 4, 5). However, EDTA was included in the growth medium of these experiments to minimize iron bioavailability and reduce the influence of background bacterial communities (and their siderophores) present in each culture (Hopkinson and Morel 2009).

Differential nitrogen and phosphorus requirements between dinoflagellate species may contribute to their trace metal needs (Muggli and Harrison 1996; Wiedenmann et al. 2013; D'Angelo and Wiedenmann 2014). Changes in available nitrogen and phosphorus sources can also affect trace metal contents via active or passive transport. Moreover, for species capable of living as intracellular symbionts, access to nutrients changes dramatically when surrounded by a host plasma membrane. Further experimentation on interactions between macro- and micronutrient demands on isolated and *in hospite* cells may lead to a better understanding of nutrient exchange dynamics among coral–dinoflagellate symbioses.

The potential role of trace metals in niche diversification

Members of the family Symbiodiniaceae embody a broad range of physiological and ecological diversity, including free-living, necrotrophic, and symbiotic species with geographic distributions spanning tropical to temperate zones. Moreover, symbiotic species associate with many invertebrate and protist hosts, which present numerous challenges of living in different intracellular environments among these evolutionarily divergent hosts. The availability of trace metals probably varies considerably across all of these abiotic and biotic environments. Therefore, it is not surprising that differently adapted Symbiodiniaceae have different trace metal quotas and that their physiologies differ in the presence of different iron concentrations (Fig. 6).

Slight differences in trace metal requirements may ultimately explain certain physiological differences between (and within) species and may regulate to some extent their geographic and ecological distributions (Strzepek and Harrison 2004; Aichelman et al. 2019; Hoadley et al. 2019; Fig. 6). On a global scale, regional differences in metal chemistries may explain why certain symbiont genera are more common and ecologically dominant than others between ocean basins (Baker 2003; LaJeunesse 2005; Moore et al. 2013). Moreover, long-term, or persistent, changes in standing metal concentrations may favor the shift, or rise in dominance, of certain species, or

genera, over others. As discussed earlier, it remains unknown to what extent intracellular environments also impose selection pressures related to trace metal availability and acquisition. In combination with external environmental pools, it is likely that host species biochemistries further dictate metal availability to resident symbionts.

For example, the genus *Breviolum* appears to have undergone an adaptive radiation mostly in the Caribbean (LaJeunesse 2005). The separation between the Atlantic Ocean and Pacific Ocean coincides with a period of global cooling and increased input of iron dust to the Caribbean Sea (Martínez-Garcia et al. 2011). *Breviolum minutum* probably evolved as a species during a Pleistocene radiation in the western tropical Atlantic, whereas *B. psygmophilum* appears to have emerged during a Pliocene adaptive radiation (LaJeunesse 2005; LaJeunesse et al. 2012; Lewis et al. 2019). It is conceivable that the increased iron input from the desertification of North Africa during these independent radiations contributed to the regional ecological dominance of this genus (LaJeunesse 2005; Martínez-Garcia et al. 2011; Lewis et al. 2019). Ultimately, determining whether major shifts in trace metal availability influences diversification among and competitive dynamics between Symbiodiniaceae must be further explored through additional comparative research.

While preliminary, multivariate analysis of trace metal profiles from the species examined in this study corresponded to their geographic distributions and/or ecological guilds. Three distinctive clusters were characterized when trace metal contents for all five species were analyzed together (Fig. 6). One of these groupings corresponded to temperate species (i.e., *B. psygmophilum* and *E. voratum*), a second grouping related to tropical species found in mutualisms with cnidarian hosts (*Breviolum minutum* and *Symbiodinium microadriaticum*), while the third corresponded to an opportunistic and potentially necrotrophic species (*S. necroappetens;* Fig. 6). The temperate, "cold-water" species possessed relatively high trace metal concentrations, and the multivariate analysis separated them from the others (Jeong et al. 2014; Thornhill et al. 2008; Fig. 6). Their high metal content may relate to their existence in temperate and coastal environments where iron and other trace metals are in greater supply (Moore et al. 2001; Fig. 5). The relationships between cellular contents and requirements of trace metals, and whether these values correspond consistently to ecological attributes and geographic distributions should be considered as more species are analyzed in this manner.

Symbiodinium necroappetens grew well under low iron concentrations and exhibited a unique trace metal composition (Figs. 3, 6). This species is sometimes detected in

bleached or diseased coral colonies in the Caribbean Sea (LaJeunesse et al. 2015). The bioavailability of iron decreases in warmer waters (Liu and Millero 2002). Episodes of warming anomalies associated with mass coral bleaching could exacerbate the effects of thermal stress on normal symbionts, by limiting access to iron important for acclimation (Ferrier-Pagès et al. 2018). Because of its low iron requirements, *S. necroappetens* may simply better endure severe warming events, while the normal symbionts found in various hosts often decline precipitously (Hoffmann et al. 2012). Therefore, distinct metal profiles, like those of *S. necroappetens*, hint at how micronutrient concentrations may influence niche partitioning among species of Symbiodiniaceae.

Toward a better understanding of trace metals on the biology of Symbiodiniaceae

To what extent iron availability influences physiological differences among Symbiodiniaceae, and their associations with particular cnidarian and protistan hosts requires considerably more investigation. The differences in iron requirements described for the small number of Symbiodiniaceae species investigated here indicate a potentially large breadth in specific nutrient requirements within and between species across this dinoflagellate family and its genera. Furthermore, there may be an unrealized role of trace metals in the response of these mutualisms to continued climate change.

Compliance with ethical standards

Conflict of interest On behalf of all authors, the co-corresponding authors state that there is no conflict of interest.

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