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The physiological and ecological responses of marine zooplankton to iron

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Abstract of the Dissertation

The physiological and ecological responses of marine zooplankton to iron

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Crustacean zooplankton provide the energetic link between phytoplankton and higher trophic levels in the ocean, including economically important fish species. Their activities also influence biogeochemical cycling through production of fast sinking fecal pellets and the regeneration of nutrients. Previous studies have indicated that zooplankton productivity can be limited by energy (carbon) and by a deficiency of the macronutrients nitrogen and phosphorus in their diets. However, the influence of dietary supply of trace elements on zooplankton productivity has been largely unstudied. Broad limitation of phytoplankton productivity by dissolved iron in the ocean results in larger variability in Fe:C ratios in phytoplankton than in their zooplankton grazers. In my dissertation I tested the general hypothesis that low Fe contents in Fe-limited phytoplankton can cause physiological stress in crustacean zooplankton. I addressed this problem using laboratory experiments and field studies in a region with low trace element concentrations.

First the egg production rates and naupliar survivorship of a common temperate copepod *Acartia tonsa* were assessed with feeding three species of algae *Thalassiosira oceanica,*

Rhodomonas salina, and *Isochrysis galbana* grown under Fe-replete and Fe-depleted conditions. Reduced egg production rates and naupliar survivorship of the copepods fed Fe-depleted algae clearly indicated that secondary production can indeed be limited by Fe content of food; egg production rates of *A. tonsa* were positively correlated with the rate of Fe assimilated from food.

Fatty acids and sterol concentrations in Fe-replete and Fe-depleted algae and in the copepods consuming these algae were further analyzed to determine whether the effect of Fe on copepod reproduction can be explained by alteration of lipid content and composition in algae and copepods in response to Fe limitation. Gas chromatography coupled with mass spectrometry analysis revealed a negative effect of Fe deficiency on the total fatty acid concentration, total polyunsaturated FA concentration and combined concentration of eicosapentaenoic acid and docosahexaenoic acid in algal food. However, the differences between treatments in total and single fatty acid(s) as well as sterols of algae were not proportional to copepod egg production rates, nor were the copepod lipids consistently affected.

Radiotracer techniques were used to determine whether zooplankton are able to adjust their assimilation and retention of Fe and C appropriately in face of Fe deficiency in food. Pulse-chase experiments were conducted on *A. tonsa* fed Fe-replete and Fe-depleted algae radio-labeled with ^{59}Fe and ^{14}C. No clear indication of stoichiometric regulation on Fe by *A. tonsa* was seen from the results; instead, this copepod had a tendency of releasing Fe at faster rates when fed food of low Fe:C.

As *A. tonsa* is a coastal and estuarine copepod, which probably never experiences Fe limitation anywhere in its natural range, I further tested whether copepods which possibly live in Fe-deficient water periodically also display an Fe-limited effect by conducting a reproductive study with the copepod *Calanus pacificus* collected from water off La Jolla, California, which belongs to a seasonally Fe limiting current system (California Current System). Results clearly show that the reproduction of *C. pacificus* can also be limited by Fe-deficiency in their diets, although the biovolume of dietary algal cells is another factor affecting reproduction in addition to the dietary Fe content.

For the field portion of this dissertation study, I participated in a cruise to the Costa Rica Upwelling Dome – a low Fe and presumbably low Zinc area – to determine if size fractionated zooplankton samples displayed different C, N, P, and trace metal content as well as RNA:DNA ratios from the samples collected from areas of different Fe availability. Analyzed data revealed that P, Fe, Zn, and RNA:DNA ratios of zooplankton samples were all significantly lower than the analysis performed for zooplankton from other waters, presumably enriched in trace metal Fe and Zn. Furthermore, the elemental ratios varied with zooplankton size, suggesting a growth tradeoff for dealing with trace nutrient deficiency.

Results from this dissertation study indicate that marine mesozooplankton can be affected by Fe deficiency in food. Given the role that zooplankton play in the cycling of Fe and C, these results could have implications for biogeochemical cycles. In addition, as copepods are an important food source of many economically important fish species, the effect of dietary Fe on the growth and reproduction of copepods could eventually help us to better understand variability in fisheries production. Overall, this research has the potential to transform our understanding of spatial and temporal patterns in marine zooplankton productivity and community structure.

Table of Contents

List of Tables

List of Figures

Chapter 6

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Chapter 1

Introduction

ZOOPLANKTON IN THE OCEAN

Zooplankton are lynchpin organisms in the ocean by serving as conduits for energy and organic matter between phytoplankton and higher trophic levels. Zooplankton comprise a key portion of the food chain that supports many economically important fish. A top-down control on the abundance of copepods by fish has been widely observed in many different marine systems including the Southern Benguela System, South Africa, Ghana, Japan, the Black Sea, the North Sea, the central Baltic Sea and the North Pacific (Shiomoto et al. 1997, Verheye and Richardson 1998, Cury et al. 2000, Reid et al. 2000, Mollmann and Koster 2002), indicating a tight predatory relationship between fish and copepods.

In addition to their role in marine food webs, zooplankton also influence the fate of carbon and other nutrients in marine ecosystems. As primary grazers of phytoplankton, mesozooplankton contribute to global carbon export by converting a large fraction of organic carbon they derive from algal food into their fecal pellets, which can sink out of the euphotic zone at a relatively fast rate (on the order of magnitude of 10^2 m day^{-1}, Angel 1984). This sinking carbon helps support benthic food webs. Mesozooplankton are also important producers of dissolved organic carbon (DOC) and regenerators of essential metals and trace elements that are accessible to phytoplankton and bacteria through respiration, excretion, sloppy feeding and leakage from newly produced fecal pellets (Storm et al. 1997, Moller and Nielsen 2001, Moller et al. 2003, Moller 2007). Respiration and excretion are major routes of metabolic carbon loss for animals and can be responsible for up to 56% C loss in copepods (Xu and Wang 2003). In addition to their role in C cycling, zooplankton can also facilitate the regeneration and sequestration of other nutrient elements in marine systems, including the macronutrients nitrogen and phosphorous as well as the trace elements iron, manganese and zinc (Fisher et al. 1991, Lee and Fisher 1992, Hutchins et al. 1993, Hutchins and Bruland 1994, Wang et al. 1996, Hassett et al. 1997). These nutrient cycling processes can affect the nutrient bioavailability in seawater and further influence phytoplankton growth and community composition (Elser et al. 1988).

The physiological activities of mesozooplankton including copepods are tightly linked to food availability. A large number of studies have addressed the impact of food limitation on marine copepod feeding behavior, somatic growth, reproduction, and metabolic rates. For instance, both lab and *in situ* measurement of egg production rates and body size of particle-feeding copepods *Paracalanus parvus* and *Acartia tonsa* showed a clear exponential correlation with chlorophyll a and particulate C concentration in the surrounding water (Checkley 1980, Durbin et al. 1983, Kiørboe et al. 1985). Similarly, juvenile specific growth rates, developmental rates and final body weight of *Calanus pacificus*, *Pseudocalanus* sp. and *A. tonsa* also increased hyperbolically with food concentration (Vidal 1980, Berggreen et al. 1988). Food limitation can also be reflected by lower respiration and excretion rate (Kiørboe et al. 1985). These observed effects of food limitation were considered as partly resulting from declined ingestion rate and thus insufficient energy supply at low food concentration (Frost 1972, Kiørboe et al. 1985).

Aside from food quantity, the nutritional value of diets such as elemental composition can also play a significant role in affecting zooplankton physiological activities. Nitrogen is a major limiting factor in marine ecosystems, and thus received much attention in food quality studies. Feeding on food of low N:C could increase the N-gross growth efficiency of the copepod *Acartia tonsa*, indicative of a N limitation of this copepod (Jones et al. 2002). Additionally, both ingestion and egg production rates of *A. tonsa* have been shown to be negatively correlated with C:N ratio of the algal food, and positively correlated with particulate N concentration in their food (Kiørboe 1989, Jones et al. 2002). The survivorship, somatic growth and developmental rates of the copepod *Pseudocalanus elongatus* also declined when fed N-limited *Thalassiosira weissflogii* (Koski et al. 1998). Low N food could also result in increased production of resting eggs by *A. tonsa* (Augustin and Boersma 2006). Similarly, in the freshwater system dominated by P limitation, *Daphnia* fed food of low P concentration had reduced P excretion, final body mass, reproductive rate, clutch sizes and growth rate, and increased mortality rates (Olsen and Ostgaard 1985, Hessen 1992, Sterner et al. 1993, Sterner

and Hessen 1994, Urabe et al. 1997, Elser et al. 2001).

It has been argued that dietary elemental stoichiometry alone may not be an adequate indicator of food quality. Rather, the content of biochemical molecules such as lipids and proteins in food should also be considered. The lack and imbalance of some essential fatty acids in diatoms has been suggested to be responsible for the reduced reproductive success in zooplankton during spring diatom blooms (Jónasdóttir et al. 1998). Indeed, a large number of studies have reported that copepod reproduction is strongly correlated with the content of some polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), as well as cholesterol in diets (Jónasdóttir 1994, Pond et al. 1996, Kleppel et al. 1998, Hassett 2004). Moreover, some sterols and essential PUFAs required for zooplankton development and reproduction are synthesized *de novo* exclusively by phytoplankton and can only be obtained by zooplankton from food (Goulden and Place 1990, Pond et al. 1996). The lipid content of organisms can be affected by the supply of some essential elements. Macronutrient elements N, P and Si have all been reported to affect plankton lipids (Shifrin and Chisholm 1981, Mortensen et al. 1988, Jónasdóttir 1994, Klein Breteler et al. 2005). In particular, limitation of N and P has been observed to reduce the composition of PUFAs and dominant sterols in algae, which further reduced the growth and developmental rate of zooplankton predators (Jónasdóttir 1994, Breteler et al. 2005). Trace metals such as zinc, copper, cadmium and mercury have also been reported to affect phytoplankton fatty acid and sterol composition due to their toxic effects (Gillan et al. 1983, Jones et al. 1987, McLarnon-Riches et al. 1998). However, little is known about the effect of insufficient supply of trace metals on fatty acid or sterol contents in phytoplankton, which could possibly indirectly affect zooplankton growth and reproduction. As lipids are energy rich compounds, while Fe affects respiratory activity and energy yields of organisms, some effects of Fe limitation on plankton lipid concentration and composition may be expected.

AN ARGUMENT FOR FE LIMITATION OF MARINE ZOOPLANKTON

Despite the fact that little information is available, Fe limitation of mesozooplankton in marine systems is of great potential. The issue of Fe limitation of primary production and phytoplankton biomass has received attention for over two decades. Iron limits the primary production in over 30% of the world's ocean (Martin 1991, de Baar et al. 2005, Hutchins and Bruland 1998, Hutchins et al. 1998). This limitation prevents phytoplankton from completely using phosphate and nitrate, resulting in High-Nutrient Low-Chlorophyll (HNLC) conditions (Minas and Minas 1992). Despite being the fourth richest element in the Earth's crust, Fe is depleted in seawater because of its low solubility with the thermodynamically stable 3+ oxidation state in oxygenated seawater (Morel and Hering 1993, Stumm and Morgan 1996). Complexation of Fe by organic ligands allows some Fe to be retained in surface waters, but the availability of this fraction to phytoplankton is not so clear.

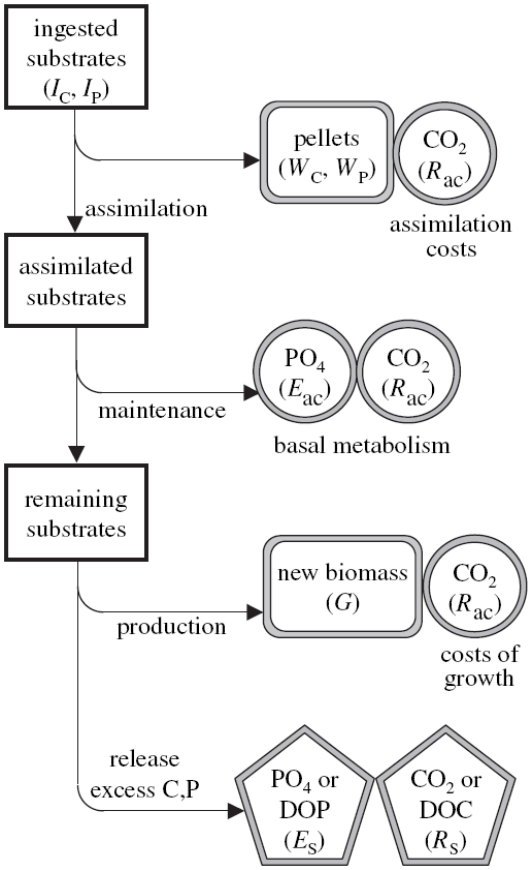
While most studies of Fe limitation have only focused on phytoplankton, it is conceivable that zooplankton may also be Fe limited by consuming phytoplankton food of low Fe content. Iron is essential for zooplankton due to its role in mitochondria of catalyzing redox reactions during respiration. When normalized to cytochrome *c*, respiratory electron transport systems have a 60% higher requirement of Fe than do photosynthetic electron transport systems (Raven 1988). Because of the enrichment of Fe in the respiratory cytochromes, Fe concentrations in zooplankton are at least 10-fold higher than the concentrations of other trace elements (Schonebeck 1992). In fact, protozoa fed Fe deficient bacteria had a reduced growth rate, suggesting an Fe limitation of microzooplankton (Chase and Price 1997). It is important to study whether the growth and reproduction of marine mesozooplankton can also be affected by Fe-limited phytoplankton.

Stoichiometric model analysis

In and of itself, an investigation of Fe concentrations in phytoplankton and zooplankton alone is not enough to tell whether Fe limitation in zooplankton is likely or not. Because Fe and C may be assimilated differently from food, and excreted at different rates once assimilated,

the Fe:C ratio in food that can lead to Fe limitation of zooplankton may differ from the Fe:C ratio of zooplankton tissues. To determine the Fe:C ratio in phytoplankton prey that should cause Fe limitation of zooplankton given what we know about zooplankton physiology, I have adopted a conceptual model that quantitatively relates the fate of C and P with the change of both food quantity (C availability) and quality (P:C ratio) (Figure 1, Anderson and Hessen 2005). In this model, zooplankton are assumed to have a fixed stoichiometry. The nutrient elements C and P ingested by zooplankton can be either assimilated or egested as fecal pellets. The assimilated nutrients are then sequentially allocated to three compartments. The first compartment is related to "maintenance", which reflects the replacement of biochemical components lost in the process of normal metabolic activity (i.e., metabolic turnover). The remaining nutrients after allocation to maintenance will then contribute to the second compartment, production, which includes growth as well as the production of gametes. Finally, the nutrients in excess of need for growth and reproduction can be excreted back into the environment in the form of dissolved compounds. Allocation of C or P to this final pool is called post-absorptive stoichiometric regulation because it allows the organism to maintain its fixed stiochiometry in the face of elemental imbalances in supply. The proportion of nutrients allocated to each compartment varies with many factors such as the amount of ingested C and the ratio of dietary C to other nutrient elements.

Fig. 1 C and P allocation after ingestion (from Anderson and Hessen 2005)



This conceptual model has been specifically used to quantify the critical P:C ratio in phytoplankton food where the production of zooplankton switches from being determined by the rate of C assimilation (C limitation) to being determined by the rate of P assimilation (P limitation), here defined as threshold elemental ratio (TER) (Anderson and Hessen 2005). Alternatively, the TER can be defined as the ratio of P:C in food that causes the amount of C available for growth to match the C required for production given 1) the amount of P available for growth and 2) the C:P ratio in zooplankton tissues. When thought of this way, the TER is the P:C ratio in food that causes the following identity to be true

$$\beta_P (I_C \theta_f^* - P_0) = \Phi_C (\beta_C - \zeta_I) (I_C - C_0) \theta z \quad (1),$$

where I_C is the carbon specific C ingestion rate (day^{-1}), θ_f* is the TER (mol P mol C^{-1}), which is equivalent to the ratio between the carbon specific P and C ingestion rates (I_P/I_C) when equation 1 is true, Φ_C is a factor accounting for the energetic cost of biosynthesis (dimensionless), β_C and

β_P are the assimilation efficiencies of C and P, respectively (dimensionless), ζ_I is a factor accounting for the cost of assimilating and absorbing ingested material in the gut (dimensionless), C_0 is the amount of ingested C needed to offset basal respiration (d^{-1}), P_0 is the amount of ingested P needed to offset losses due to "basal" excretion (mol P mol C^{-1} day^{-1}), and θz is zooplankton P:C (mol P mol C^{-1}). Equation 1 can be solved for θ_f^*

$$\theta_f^* = [\Phi_C (\beta_C - \zeta_I) (I_C - C_0) \theta z / \beta_P + P_0] / I_C \quad (2).$$

Copepod production is phosphorous limited if food P:C is below θ_f^*, and C limitation will occur if food P:C is above θ_f^*.

By assuming that ingestion rate and assimilation efficiency do not vary with Fe content in food and that zooplankton maintain a fixed Fe:C ratio in their bodies, a rough TER Fe:C can be estimated by using equation (2) with parameter values adopted from the literature. As in the case with P, ingested Fe is allocated to fecal pellets, maintenance, production and the dissolved pool. The fate of ingested Fe is linked to Fe distribution within phytoplankton cells (Hutchins et al. 1995). For example, Fe associated with membrane proteins in phytoplankton, such as NADH dehydrogenase, many Fe-S proteins, cytochrome *c* oxidase and cytochrome *f/b6* complexes, is more likely to be incorporated into fecal pellets than it is to be assimilated. Because much of the Fe in phytoplankton cells is associated with these fractions, copepods tend to assimilate Fe less efficiently than P, for which the assimilation efficiency is typically 0.8 (Andersen et al. 2005). Assimilation efficiencies of C and Fe (β_C and β_{Fe}) have been measured as 0.42 and 0.21, respectively, for copepods fed algae grown under Fe deplete conditions, while under Fe-replete conditions, the measured values for β_C and β_{Fe} were 0.65 and 0.11 (Schmidt et al. 1999). The same study also estimated Fe excretion to be 2.5×10^{-5} mol Fe mol C^{-1} d^{-1} (~0.28 d^{-1} in Fe-specific terms) under the Fe-deplete condition and 1.8×10^{-5} mol Fe mol C^{-1} d^{-1} (~0.20 d^{-1} in Fe-specific terms) under the Fe-replete condition. The ingested Fe allocated to maintenance, Fe_0, can be estimated by dividing estimates of excretion by the corresponding assimilation efficiencies for Fe. Martin and Knauer (1973) found that zooplankton Fe:C, θ_Z ranged between 26 to 131 μmol Fe mol C^{-1} with an average of 88 μmol Fe mol C^{-1}. To

complete the calculation, I assumed a C ingestion rate of 0.59 day^{-1} (Paffenhofer and Koster 2005), an efficiency of C utilization for biosynthesis of 0.75 (Masuzawa et al. 1988), and a cost of assimilation of 0.06 (Kuijper et al. 2004). Ingested C allocated to maintenance, C_0, is assumed equivalent to the basal respiration rate, calculated from the Kiørboe et al. (1985) study as 0.2 day^{-1}, divided by the term.

Different from expectation, this calculation suggests that algal food is always deficient in Fe relative to C requirements for growth in copepods. Substituting the parameters in Equation 1 with the above values, I calculated a threshold Fe:C of 171 μmol Fe mol C^{-1} for Fe-deplete condition and 340 μmol Fe mol C^{-1} for the Fe-replete scenario. Thus, copepods would be Fe-limited if the Fe:C in their algal food is below 171 μmol Fe mol C^{-1} in Fe-deplete phytoplankton and below 340 μmol Fe mol C^{-1} in the Fe-replete diet. By comparison, the Fe:C ratios in phytoplankton and protozoans are lower than the TER even under Fe-replete conditions: 17 μmol Fe mol C^{-1} for Fe-deplete diatoms and 35 μmol Fe mol C^{-1} for Fe-replete diatoms. These results suggest that variations in Fe:C ratios in copepod food should affect copepod productivity in regions of the ocean experiencing severe to moderate Fe stress, which at the very least comprise 40% of the ocean.

To better understand the implications of these calculations, it is critical to go back to the model and review the assumptions behind the TER calculation. One of the parameters that is most likely to change with food condition is ingestion rate, I_C, although it was assumed to be unchanged in the model calculation. Many studies have shown that ingestion rate increases with food quantity (Kiørboe et al. 1985, Thompson et al. 1994, Besiktepe and Dam 2002). One hypothesis might be that copepods can compensate for low Fe food by ingesting more phytoplankton cells over a certain period of time. With the change of ingestion rate, some other parameters are expected to change as well. For example, if more C is ingested during increased ingestion of phytoplankton cells, respiration rate or organic carbon excretion rate would have to increase to excrete more excess C.

Another assumption is that copepods are able to regulate internal Fe to maintain strict

stoichiometric homeostasis (Anderson et al. 2005). Zooplankton are known for their ability to maintain homeostasis of macronutrients (Sterner and Hessen 1994, Anderson and Hessen 2005, Sterner 1997). When confronting food with low N or P content, zooplankton increased their assimilation efficiency for these nutrient elements, and allocated a larger fraction of the assimilated N and P to protein and RNA, respectively (Hessen et al. 2007). At the same time, excess C was released by post-absorptive stoichiometric regulation such as by enhancing respiration rate, or through elevating excretion of dissolved organic compounds (Anderson et al. 2005). Alternatively, if nutrients like N and P were in excess, the excretion of N and P in the form of ammonia and soluble reactive P increased (Scavia and Gardner 1982). So far it is unknown whether similar homeostatic stoichiometric regulation exists for trace elements like Fe when C is excess. Although the ingested Fe can be assimilated, egested or excreted like N and P (Hutchins et al. 1995, 1999), its assimilation is believed to be passively governed by the cytological distribution of Fe in the phytoplankton diet. Little study has been on whether the fate of Fe is instead stoichiometrically regulated by either increasing assimilation or decreasing excretion.

A JUSTIFICATION FOR A FIELD STUDY

As has been mentioned above, Fe limitation of primary production widely takes place in the open ocean, and thus ecological and physiological conditions of organisms inhabiting *in situ* Fe-limited regions are expected to be different from those of lab cultured organisms. For this reason, a field study on the effect of Fe deficiency on zooplankton is warranted.

One remarkable difference between laboratory and field environment is that the plankton community in the field has a much higher diversity, and may assist in mitigating nutrient imbalance. While phytoplankton are usually considered as the major food source for zooplankton due to their abundance and nutrient content, other microplankton such as cyanobacteria and protozoa can also serve as food supplies. In fact, microorganisms other than eukaryotic phytoplankton contain different nutrient content and can help compensate for the

deficiency of certain nutrients. One example is that adding the cyanobacterium *Synechococcus elongates*, which is commonly recognized as low quality food, to a food suspension of low P concentration improved the growth and reproduction of five species of daphnids, due to the enrichment of P in S. *elongates* (DeMott 1998). Additionally, protists such as ciliates can supplement copepods with PUFAs including EPA and DHA by elongating and desaturating the PUFA precursors derived from their bacterial diets (Veloza et al. 2006). Regarding the phytoplankton community itself, the community composition also varies with season in the field, and provides zooplankton with food of different abundance and nutrition.

Many zooplankton living in the field have evolved special strategies to overcome food shortage, either of food quantity or quality. The adaptation of zooplankton to food availability is perhaps most evident in polar areas, where primary production shows strong seasonal variation due to light availability. A number of the strategies involve storing lipids as energy reserves for overwintering. For example, the Arctic copepods *Calanus hyperboreus* and *C. glacialis* as well as the Antarctic species *Calanoides acutus* and *Rhincalanus gigas* synthesize and store large amounts of high-energy wax esters with long-chain monounsaturated fatty acids and alcohols (20:1 and 22:1 isomers) in their oil sacs for overwintering (Hagen and Auel 2001). Another Antarctic species *Calanus propinquus* on the other hand, synthesizes primarily triacylglycerols, dominated by long-chain monounsaturated fatty acids with 22 carbon atoms (22:1ω9 and 22:1ω11) to accumulate energy reserves more efficiently (Hagen and Auel 2001). In addition to lipid storage, zooplankton can also change their life cycle and feeding behavior to adapt to food shortage. One typical strategy adopted by copepods is diapause, meaning that the animals can reduce their metabolic rates to extremely low levels at late copepodite stages to minimize their food demand. This strategy has been seen for the Arctic/North Atlantic species *Calanus hyperboreus*, *C. glacialis* and *C. finmarchicus*, the Antarctic species *Calanoides acutus,* and other species (Hagen and Auel 2001). Other strategies include producing buoyant, slowly developing and ascending eggs at depth before the start of the spring bloom, (exhibited by *Calanus hyperboreus* and *Neocalanus sp.*), feeding on ice algae by the Antarctic copepod (such

as by *Paralabidocera antarctica* and adult krill, Conover and Huntley 1991), and switching food from algae to other resources (as seen with *R. gigas,*Graeve et al. 1994).

The adaptation of zooplankton to low food quality is less studied, yet some hints have been shown. For example, a field study of *Daphnia galeata* showed that this freshwater zooplankton can increase its clearance rate to compensate for the low P:C ratio in its food (Darchambeau and Thys 2005). Another strategy that *Daphnia* use to compensate for P deficiency is luxury uptake of P from food and P storage (Sterner and Schwalbach 2001). Zooplankton may also alter their life-histories by investing different amounts of energy in reproduction and somatic growth when facing nutrient imbalance. For instance, *Daphnia* fed a large amount of N and P-deficient food has been reported to reduce its somatic growth rate until reaching an older age, as well as to produce smaller, aborted eggs, indicating a sacrifice of fast growth and reproduction for basal maintenance (Urabe and Sterner 2001, Færøvig and Hessen 2003). Currently no information is available on whether zooplankton inhabiting HNLC regions of the oceans show adaptations to the deficiency of trace metals.

DISSERTATION STRUCTURE

In this dissertation, I implemented both laboratory and field studies to test the hypothesis that trace metal iron can have limiting effects on the growth and reproduction of marine mesozooplankton. I also explored the possible reasons behind the observed effects and the responses of zooplankton to dietary Fe availability in their physiological activities.

In Chapter 2 of this dissertation, I cultured three species of marine microalgae: the diatom *Thalassiosira oceanica*, the cryptophyte *Rhodomonas salina* and the prymnesiophyte *Isochysis galbana* under Fe-replete and an Fe-depleted conditions to differentiate the Fe content in these algae, and then studied the egg production rate and naupliar survivorship of the copepod *Acartia tonsa* fed these algae. Here *A. tonsa* was chosen as a model copepod species because of its ubiquity in temperate waters and ready availability on Long Island. It is also primarily a herbivore with relatively small body size (average cephalothorax length of female = 0.82 mm,

Kiørboe et al. 1985), which allows it to respond to the variation in food conditions in a short period of time.

To determine whether the effect of Fe on copepod reproduction can be explained by the alteration of lipid content in response to Fe limitation, in Chapter 3 I analyzed fatty acids and sterol concentrations in Fe-replete and Fe-depleted algae as well as in *A. tonsa* consuming these algae. Gas chromatography coupled with mass spectrometry (G C-MS) was used to analyze the lipid concentration in the organisms. The lipid composition was then compared with the reproduction results in Chapter 2 to discuss the possible indirect influence of Fe on copepod reproduction through affecting algal biochemical molecules.

The experiments described in Chapter 4 were conducted using radiotracer techniques to investigate whether copepods are able to adjust their assimilation and retention of Fe and C appropriately when fed Fe-depleted food. In addition, the cytosolic distribution of Fe in microalgae cultured under different Fe conditions was examined, along with a measurement of the respiratory rate of the copepods. *A. tonsa* was still the model copepod used in the experiments. All these analyses were then integrated together to provide a broad picture on the ability of this copepod to regulate Fe in the face of dietary Fe deficiency.

As *A. tonsa* primarily lives in estuaries and coastal waters, and thereby probably rarely experiences Fe limitation anywhere in its natural range, in Chapter 5 I further tested whether copepods which possibly experience Fe deficiency periodically can also display a similar response to Fe-limitation as observed with *A. tonsa*. To achieve this goal, I conducted a reproductive study with the copepod *Calanus pacificus* collected from the water off La Jolla, California. Due to its larger body size (female prosome length = 2.57 mm, Runge 1984), the correlation of *C. pacificus* grazing and reproductive activities with food particle size was also discussed.

Finally, Chapter 6 describes the elemental stoichiometry and fitness of zooplankton collected from an *in situ* low Fe region. This field study was conducted during a cruise in the Costa Rica Dome, a shallow, upwelling area with its center at 8° N, 90° W. The zooplankton

samples were fractionated into four size categories: 0.2-0.5, 0.5-1, 1-2, and 2-5 mm. The concentrations and ratios of the elements C, N, P, Fe, and Zn in zooplankton of each size group were reported. The fitness of the animals was assessed using RNA:DNA ratios, and was compared to the results reported in previous studies.

Chapter 2

Can copepods be limited by the iron content of their food?

Abstract

In laboratory experiments, we show that naupliar survival and egg production by the copepod *Acartia tonsa* was significantly lower when they were fed iron-deficient algal cells than when they were fed a diet of Fe-replete cells which had two orders of magnitude higher Fe levels. Naupliar survival after 1 week was reduced from 60% for copepods feeding on Fe-replete diatoms (*Thalassiosira oceanica*) to 0% for those feeding on Fe-deficient cells. The decline of egg production rate was greatest (83%) when *T. oceanica* was used as prey, and smaller (20-30%) when the cryptophyte *Rhodomonas salina* and the prymnesiophyte *Isochrysis galbana* were used as food. The assimilation rates of Fe and C from Fe-enriched and Fe-deficient algae were determined using radioisotopes. Egg production was hyperbolically dependent on the Fe assimilation rate ($r^2 = 0.71$). The effect of Fe on copepod reproduction rates could not be explained by changes in ingestion rate and the rate of carbon assimilated, as there was no significant difference of ingestion rate and C assimilation between Fe-replete and Fe-depleted treatments. Iron might limit zooplankton productivity in High-Nutrient, Low-Chlorophyll regions.

Introduction

Copepods are lynchpin organisms whose activities influence the fate of energy and nutrients in the surface waters of the ocean. They partly comprise the base of the food chain that supports many economically important fish (Miller 2004) and also influence the fate of carbon and other nutrients in marine ecosystems. While food availability clearly affects marine copepod production (Kiørboe et al. 1985, Richardson and Verheye 1998, Saiz et al. 1999), the effect that elemental composition of food has on marine copepods is less well understood. In freshwater ecosystems, low concentrations of phosphorus in the food of *Daphnia* reduce P excretion, final body mass, reproductive rate, clutch size, and growth rate, while increasing mortality rate (Sterner et al. 1993, Sterner and Hessen 1994, Elser et al. 2001). Likewise, marine copepods fed a variety of algal prey with low cellular nitrogen:C ratios grow and develop slower, and produce fewer eggs (Jones et al. 2002). Low N diatom food can also result in increased production of resting eggs by *Acartia tonsa* (Augustin and Boersma 2006). However, N limitation in marine systems may not be as pronounced as P limitation in freshwater systems. N recycling and N-fixation compensate to some degree for ecosystem level imbalances in N, and cellular N content in phytoplankton varies less than does cellular P content (Elser and Hassett 1994, Hassett et al. 1997). We are unaware of any published study documenting limitation of marine zooplankton by metals.

The potential for significant widespread limitation of marine copepod productivity by low Fe in food is large and yet to be explored. Phytoplankton production is limited by Fe in 30-40% of the ocean (Hutchins and Bruland 1998, Hutchins et al. 1998, de Baar et al. 2005). Phytoplankton vary in their Fe:C ratios in direct relation to ambient dissolved Fe concentrations (Sunda and Huntsman 1995, 1997). In cultured phytoplankton, Fe:C ratios can be as high as 2000 μmol mol^{-1} under Fe-sufficient conditions and as low as 2 μmol mol^{-1} under Fe-limited conditions (Sunda and Huntsman 1995, 1997; Fig. 1). *In situ* measurements of phytoplankton and seston in High-Nutrient, Low-Chlorophyll (HNLC) regions after Fe addition indicate that Fe:C ratios of potential prey for copepods typically range between 20 and 40 μmol mol^{-1} under

Fe-replete conditions (Boyd et al. 2004, Twining et al. 2004). While certain diatoms in Fe-limited regions may exhibit Fe:C ratios in this range (Twining et al. 2010), typical values for Fe:C ratios under low Fe conditions range from 1.5 - 14.7 μmol mol^{-1} (Twining et al. 2004).

Dietary Fe can become so depleted under Fe-limited conditions that growth and reproduction of zooplankton grazers may be determined by the Fe:C ratios in their food rather than by food availability. The few existing measurements of Fe:C ratios in zooplankton from open ocean areas are much higher than typically observed in Fe-limited phytoplankton, suggesting that Fe limitation of zooplankton production is clearly possible, especially in low Fe waters (Fig. 1). In the waters of Monterrey Bay and off Hawaii, Martin and Knauer (1973) measured average Fe:C ratios of nearly 90 μmol mol^{-1} for zooplankton samples dominated by copepods, with values ranging from 17-132 μmol mol^{-1}. Working in the northwestern Mediterranean, Fowler (1977) measured average Fe:C ratios of 30 μmol mol^{-1} in euphausiids, while Martin and Knauer (1973) and Masuzawa et al. (1988) measured Fe:C ratios of 51 and 52 μmol mol^{-1} in Monterrey Bay and the Japan Sea, respectively. All of these ratios are greater than the highest Fe:C ratio (14.1 μmol mol^{-1}) measured for Fe-limited phytoplankton (Fig. 1).

As with phytoplankton, Fe is an essential element for zooplankton. Respiratory cytochromes are presumably the most important internal reservoir of Fe in heterotrophic plankton. When normalized to cytochrome *c,* respiratory electron transport systems have a 60% higher requirement of Fe than do photosynthetic electron transport systems (Raven 1988). Because much of their biochemical machinery is dedicated to enzymes involved in photosynthesis and related processes, phytoplankton Fe quotas are likely to be lower than those for copepods. Indeed, protozoa fed Fe deficient bacteria exhibit reduced growth rates, suggesting that Fe can limit heterotrophic protozoa (Chase and Price 1997). However, there have not been similar studies on Fe limitation of marine metazoans feeding on Fe-limited phytoplankton.

Here we present the first study on the Fe limitation of mesozooplankton. To investigate the potential Fe limitation of mesozooplankton, experiments were conducted with a model

copepod *Acartia tonsa* under different dietary Fe conditions. Egg production and naupliar survival were evaluated while *A. tonsa* were fed on phytoplankton of either high or low Fe contents. The rates of Fe assimilated by *A. tonsa* after ingestion were also measured and related to rates of egg production.

Materials and methods

Both cultured and freshly collected copepods of the species *A. tonsa* were used in experiments. *A. tonsa* was chosen because it is readily available in local waters and easy to manipulate and is a widely used model copepod. To start cultures, individuals were collected at night from Stony Brook Harbor on the incoming tide using a 200 μm mesh Nitex net. Adults and copepodites of *A. tonsa* were carefully separated from other species under a dissecting microscope. Individuals (ind.) were then transferred to 2 L flasks filled with 0.22 μm filtered Stony Brook Harbor water, and maintained with bubbling at 21°C on a 14:10 hour light:dark cycle. These copepods weigh approximately 7 μg ind.$^{-1}$, calculated from the length-weight relation $W=13.4L^3$, where W is the dry weight (μg) and L is the cephalothorax length (mm) and C was assumed to account for 45% of the dry weight (Kiørboe et al. 1985). Copepods were fed a mixed diet of the cryptophyte, *Rhodomonas salina,* and the prymnesiophyte, *Isochrysis galbana,* on a daily basis.

Three phytoplankton species were used as food sources in the experiments. The diatom *Thalassiosira oceanica* was selected because it is an oceanic species capable of maintaining moderate growth rate under Fe-depleted conditions (Sunda et al. 1991). However, diatoms are not always considered an optimal food for copepods (Jónasdóttir 1994, Hasset 2004). Thus, *R. salina* and *I. galbana* were selected because they are commonly used in copepod cultures as high quality diet (Table1). All three phytoplankton species were cultured under either Fe-replete and Fe-depleted conditions at 17°C on a 14:10 hour light:dark cycle at a light intensity of 120 μmol quanta m^{-2} s^{-1}. The culture media were made with chelexed seawater (Price et al. 1988/1989) amended with f/2 (Guillard and Ryther 1962) levels of chelexed sodium nitrate and

potassium phosphate and, for the diatom, sodium silicate. Trace metals except Fe were added into an ethylenediaminetetraacetic acid (EDTA) buffer system as described by Sunda and Huntsman (1995), which includes: 0.1 mmol L^{-1} EDTA, 50 nmol L^{-1} $MnCl_2$, 40 nmol L^{-1} $CuCl_2$, 100 nmol L^{-1} $ZnCl_2$, 40 nmol L^{-1} $CoCl_2$, and 100 nmol L^{-1} $NiCl_2$ in the media.

The Fe-replete treatments were produced by adding dissolved Fe as $FeCl_3$ to a concentration of 100 nmol L^{-1}. No Fe was added to the Fe-depleted treatments; only dissolved Fe that remained after chelexing the seawater was present in the media. The cell densities and sizes were determined daily by a Multisizer® III Coulter Counter. The growth rates of algae (d^{-1}) were calculated after every 24 h of growth, as $(\ln N_{t+1} - \ln N_t)/t$, in which t (d) is elapsed time in days, and N_{t+1} and N_t are the cell densities on days t and t+1. To assess the degree of Fe stress experienced by the cells, the quantum yield of fluorescence, F_v/F_m, was also measured as described in Geider (1993). Briefly, 5 mL phytoplankton samples were left in the dark for at least 15 min to purge photosystem II of electrons, thereby maximizing photosynthetic efficiency and minimizing fluorescence. The minimal fluorescence (F_0) was then measured as in vivo fluorescence by a Turner Design® 10-AU fluorometer. The maximal fluorescence (F_m) was measured after addition of 25 µL of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) solution, which resulted in a final DCMU concentration of 10 µmol L^{-1}, to block movement of excited electrons through the electron transport chain of photosystem II. F_v/F_m was calculated as $(F_m - F_0)/F_m$. The phytoplankton cultures were considered ready to be used as food when growth rate and photosynthetic efficiency of Fe-depleted cells remained at their lowest point for 3 continuous days.

To determine cellular C and N contents of the algal cells, the culture was passed at low vacuum pressure through precombusted Whatman GF/C filters that were then fumed over 12 mol L^{-1} HCl for 24 h to drive off inorganic carbon and dried for at least 24 h at 60 ºC. The remaining C and N were determined using a Flash EA1112 CN Analyzer (CE Elantech) and atropine as a standard; analytical precision was <1%. The resulting particulate C and N mass were divided by the total cell biovolume of samples to determine C and N per cell biovolume.

In addition, biogenic silica was also determined for diatoms under Fe-replete and Fe-depleted conditions. Diatoms cells were filtered onto rinsed 1 μm pore size polycarbonate membrane filters and frustules were dissolved in boiling 0.2 mol L^{-1} NaOH (Brzezinski and Nelson 1989). Silicate concentrations in the resulting solution were determined after reactions with acid molybdate by measuring absorbance at 810 nm using a Cary 50 Spectrophotometer fitted with a 1 cm cell (Varian). Finally, 8.26 - 56.4 $\times 10^7$ cells of each species were harvested on acid-washed 1 μm polycarbonate membrane filters for Fe analysis. Each filter was rinsed with 20 mL of oxalate reagent (Tovar-Sanchez et al. 2003) and frozen prior to analysis by Inductively Coupled Plasma Mass Spectroscopy (ICP-MS). Analyses were performed with an Agilent 7500cx ICP-MS with a lower limit of detection for Fe of 50 nmol L^{-1}; analytical precision was 2.4%. Blank filters through which seawater containing no cells was passed were also analyzed and were found to be below detection.

In the experiment involving copepod egg production rate, there were 6 treatments in total, with 3 diet species and 2 Fe conditions for each species. Before the measurement of egg production, copepods were pre-acclimated to either Fe-replete or Fe-depleted food conditions for 2 d. The algal cultures in all treatments were diluted by the addition of sterilized chelexed seawater to achieve a fixed concentration of 500 μg C L^{-1}. At this concentration, *A. tonsa* feeding rates on both *Thalassiosira* and *Rhodomonas* are maximal (Kiørboe et al. 1985, Besiktepe and Dam 2002). During the experiment, 3 replicate batches of female copepod adults, each with 20 individuals, were transferred to acid-washed 250-mL Erlenmeyer flasks containing 100 mL of the phytoplankton suspensions. Two control flasks for each treatment with no copepods were used to monitor the algal cell growth in the absence of copepods. Copepods were fed in the dark to minimize algal growth thereby keeping the same food concentrations as the beginning of feeding throughout the experiments.

Every 24 h copepods and eggs were collected by gravity filtration through 250 μm and 20 μm nitex meshes, respectively. The captured copepods that were living were counted and transferred into a new set of flasks with new algal food at a concentration of 500 μg C L^{-1}. The

dead copepods were discarded. The eggs collected were resuspended into Petri-dishes with filtered seawater and counted using a dissecting microscope. Ingestion rates were also monitored to determine if copepod grazing activity may have affected egg production rate. Ingestion rates (μg C ind.$^{-1}$ d^{-1}) were calculated by subtracting the final amount of carbon in algal cells from the initial value and dividing the result by the time interval (1 d) and the number of feeding individuals during that interval. To account for losses due to respiration, or gains due to dark carbon fixation, the daily rate of change in algal carbon within control flasks was subtracted from the change in the experimental flasks. This calculation provides the most direct estimate of the carbon flow into the animal during the experiment. Algal carbon was determined by measuring algal cell densities and volumes using a Multisizer® III Coulter Counter and multiplying the total algal cell volume by the C content (Table 1). This procedure was repeated for each of the 5 days of the experiment. At least one third of the algal cells remained in feeding flasks at the end of the 24 h feeding, so that algal food was not completely depleted during the incubation.

To determine the Fe content of copepods presented with Fe-replete and Fe-depleted diets, 3 replicate cultures, each containing 250 individual copepods, were fed either Fe-replete or Fe-depleted algal food in 1-L food suspensions held in acid-washed 2-L bottles containing 500 μg C L^{-1} algal cells. Animals were fed in the dark and the food was replenished every day for 3 d. Preliminary experiments showed that under these conditions incubations longer than 3 days resulted in excessive mortality of copepods which could reduce the accuracy of copepod Fe analyses. After 3 days of feeding, copepods from each feeding bottle were collected by gravity filtration through an acid-washed 10 μm polycarbonate membrane, oxalate-rinsed, then frozen and analyzed for Fe content as described for the phytoplankton. The Fe content of blank filters was below detection.

In the experiment that assessed responses of copepod naupliar survivorship to differences in Fe content of food, 100 newly hatched nauplii from eggs produced under each Fe condition were placed in acid-washed 6-well plates, with 2 nauplii in each well. Each well was

filled with 5 mL of either Fe-replete or Fe-depleted *T. oceanica* suspension (at 500 μg C L^{-1}). The number of surviving nauplii was counted each day and the nauplii were transferred to a set of new containers with renewed food. This experiment lasted as long as there were nauplii alive in both treatments.

Radioactive Fe and C were used to determine the assimilation of Fe and C in the copepods. Radioactivities of 68 kBq of $^{55}FeCl_3$ and 616 kBq of $NaH^{14}CO_3$ were added to 250 mL phytoplankton cultures, which corresponded to additions of 1.53 nmol L^{-1} $FeCl_3$ and 8.33 nmol L^{-1} $NaHCO_3$, respectively, to the solutions. These additions did not affect phytoplankton growth relative to the pre-labeled cultures. After 1 week of exposure to ^{55}Fe and ^{14}C, the phytoplankton cells were fed to copepods (at 500 μg C L^{-1}). Preliminary experiments demonstrated that animals fed Fe-depleted diets typically exhibited substantial mortality within 2-3 d, therefore the copepods were not acclimated to Fe-depleted diets prior the assimilation experiments. For each treatment, 3 replicate batches of copepod adults, each with 50 individuals, were placed in acid-washed containers with 50 mL labeled food suspension and allowed to feed for 30 min. The feeding time was chosen to be shorter than the gut passage time of calanoid copepods at 21°C on a variety of foods (Dam and Peterson 1988, Irigoien 1998) so as to avoid overestimating elemental assimilation. After feeding, copepods were carefully rinsed with 0.1 mmol L^{-1} EDTA solution and then chelexed seawater. They were then immediately transferred to unlabeled food suspensions with the same food concentration as the labeled food suspensions. The copepods were allowed to depurate the radiolabeled elements in this solution for 5 h, a period sufficient to allow the copepods to empty their guts of unassimilated food (Reinfelder and Fisher 1991). After depuration, copepods were separated from the food suspensions by gravity filtration through a 250 μm mesh and rinsed with oxalate reagent and then seawater to remove the surface-bound Fe from copepods. Copepods were then transferred to 20 mL scintillation vials each containing 1 mL Perkin Elmer® Solvable and the vials were placed in a 60°C oven for 1 h. After cooling, each vial received 200 μL H_2O_2 and was heated at 60°C for an additional 30 min. After cooling, 10 mL Ultima Gold liquid

scintillation cocktail was added to each vial. The ^{55}Fe and ^{14}C radioactivity of each sample was measured with a Tri-Carb® Liquid Scintillation Analyzer. Radioactivity counts were converted to moles of Fe and C using the specific activity of the radioisotope in each sample. The rate of Fe and C assimilated during the radiotracer experiments was determined by first estimating the specific activities of ^{55}Fe and ^{14}C in algal food and then dividing the radioactivity of ^{55}Fe and ^{14}C retained by copepods after 5 h of depuration by these specific activities. The specific activity in food was determined by dividing the radioactivity per cell prior to feeding by the total C or Fe per cell.

Data on algal conditions including growth rate, cell volume, photosynthetic efficiency, and elemental content were compared using analysis of variance (ANOVA). Data on egg production rates, ingestion rates, as well as C and Fe assimilation were analyzed using full factorial ANOVA that included level of Fe limitation of food (TREAT), algal food species (ALGA) and, in the case of egg production rate, day (DAY) as factors. Box-Cox transformations were used to meet the assumptions of normality and constant variance (Box and Cox 1964). Differences among group means were tested using Tukey's Honestly Significant Difference test assuming a significance level of 0.05. All ANOVA analyses were conducted using JMP® 7.0 (SAS Corp). Relationships between egg production and C and Fe assimilation were assessed by ordinary least squares regression of egg production on the logarithm of assimilation. As the replicate observations of assimilation and egg production were not paired, mean values for each combination of food species and Fe treatment were used in these regressions. Because egg production declined over time for the experiments that used *T. oceanica* as a food source, the value for egg production rate used in these regressions was the mean of replicates at the final time point. For the data from experiments on naupliar survivorship, instantaneous mortality rate (m) was estimated by regressing the number of surviving nauplii (N_t) on time (t), using the exponential equation $N_t = 100e^{-mt}$, where 100 is the initial number of nauplii. Averages and standard errors for m were estimated using non-linear regression via the Levenberg-Marquardt search algorithm in SigmaPlot 11.2 (Systat Software,

Marquardt 1963).

Results

Algal Fe, C, Fe:C, growth rate, cell volume, and photosynthetic efficiency all significantly decreased ($p < 0.05$) after being cultured in Fe-depleted media for 3 weeks (Table 1). Growth rates of Fe-depleted algae decreased to < 50% of those of Fe-replete algae, whereas cell volumes of Fe-depleted cells decreased to about 60% of Fe-replete cells and photosynthetic efficiency decreased to < 60% of those in Fe-replete cells. The largest difference in photosynthetic efficiency between Fe-replete and Fe-depleted cultures was seen in the diatom *T. oceanica*. Fe-depleted diatoms also exhibited lower cellular concentrations of C and N, and 3.75 times higher cellular Si:C ratios relative to Fe-replete cells. Fe concentrations in Fe-depleted cells (amol μm^{-3}) decreased to only 0.9%, 0.5%, and 0.1% in *T. oceanica, R. salina, and I. galbana*, respectively, of concentrations in Fe-replete cells (Table 1). Because cell volumes also decreased in Fe-depleted cells, the Fe content $cell^{-1}$ decreased from 55.04 to 0.31 amol (0.6%) for *T. oceanica*, from 259.84 to 0.85 amol (0.3%) for *R. salina*, and from 357.88 to 0.28 amol (0.08%) for *I. galbana*. With increasing exposure times (> 2 weeks), Fe-depleted *T. oceanica* cells became elongated and agglomeration increased, while the Fe-depleted flagellate cells maintained their normal shape but had smaller cell volumes. The cellular N concentration in Fe-depleted *T. oceanica* cells was 52% of that in Fe-replete cells, but 1.45 times of that in *R. salina* cells; N content in *I. galbana* cells was unaffected by Fe treatments (Table 1). There was no effect of Fe on the C:N ratios of *T. oceanica* or *I. galbana* s, but C:N of *R. salina* declined significantly from 6.0 in Fe-replete cells to 4.7 in Fe-limited cells, suggesting that the cytoplasmic protein content may have been elevated in Fe-depleted algae.

Egg production (Fig. 2) was always lower for copepods fed an Fe-depleted diet, regardless of the algal species that was used as food ($p < 0.0001$ for TREAT). When *T. oceanica* was used as a food source, the rate of egg production decreased significantly over the 5-d feeding period in the low Fe treatment ($p < 0.003$), but in all other cases egg production did not

vary systematically over the course of the experiment ($p > 0.07$). The use of different food species resulted in sizeable differences in egg production rate ($p < 0.0001$ for ALGA). The ANOVA results also indicate that the size of the effect of the Fe treatments on egg production also varied among the food species ($p < 0.0001$ for interaction between TREAT and ALGA). After 5 d of feeding on *T. oceanica*, copepods produced eggs at a rate of 7.4 eggs ind.$^{-1}$ d^{-1} under Fe-replete conditions, and 0.6 eggs ind.$^{-1}$ d^{-1} in the Fe-depleted treatment. When fed on Fe-replete *R. salina* and *I. galbana*, the egg production rate after 5 d of feeding was 15.4 eggs ind.$^{-1}$ d^{-1} and 8.5 eggs ind.$^{-1}$ d^{-1}, respectively, while Fe-depleted *R. salina* and *I. galbana* diet at the same time resulted in egg production rates of 10.4 and 6.2 eggs ind.$^{-1}$ d^{-1}, respectively (Fig. 2).

All nauplii in the Fe-depleted treatment died within a week after hatching, whereas 60% of the nauplii fed on Fe-replete diatoms survived during this period, although development to the copepodite stage was not observed by the end of the experiment in either case. The daily mortality rate of nauplii fed on Fe-depleted *T. oceanica* was higher than that for nauplii fed Fe-replete food for every 1 d interval during the experiment (Fig. 3). Average instantaneous mortality estimated by regression was 30±4% d^{-1} when nauplii were fed Fe-depleted food and 8±0.4% d^{-1} when fed Fe-replete food.

Carbon ingestion rates of algae ranged between 1.31 and 3.01 µg C ind.$^{-1}$ d^{-1} under all food conditions during the 5-d feeding period. C ingestion rates were not affected by Fe status of algal food for any algal species (Fig. 4; p = 0.831 for TREAT). C ingestion rates of copepods ingesting *T. oceanica* were significantly higher than those feeding on *R. salina* and *I. galbana* ($p < 0.001$ for ALGA), but no significant difference was observed between *R. salina* and *I. galbana* treatments ($p = 0.455$ for ALGA).

Carbon assimilation was unaffected by the Fe content of the diet ($p = 0.7$, Table 2), but Fe assimilation was significantly greater (21-28 times) when copepods fed on Fe-replete cells than when fed Fe-depleted cells ($p < 0.001$). The Fe:C ratios in copepods that fed on Fe-replete cells (47.8 ± 7.9 µmol mol^{-1}) were not significantly different from Fe:C ratios in copepods that

fed on Fe-depleted algae (54.8 ± 8.7 μmol mol^{-1}). No significant differences were noted for Fe concentrations in copepods among the 3 algal diets. The Fe assimilation data indicated that the daily assimilated Fe per individual copepod feeding on Fe-replete algae was only about 1% of the total body Fe content of copepods feeding on *T. oceanica* diet, 2.9% of those feeding on *I. galbana* diet, and 6.5% of those feeding on *R. salina* diet, therefore should no change copepod body Fe content.

The average egg production rate after 5 days of exposure was significantly related to the rate of Fe assimilated after ingestion across all treatments (Fig. 5A; $r^2 = 0.70$, $p = 0.003$). The regression of egg production against the ratio of Fe and C assimilation provided a slightly better fit ($r^2 = 0.74$, $p = 0.001$), although the improvement is not statistically significant. In contrast, there was a negative relationship between copepod egg production rate and the rate of C assimilated in the species ($r^2 = 0.52$, $p = 0.1$) (Fig. 5B). As C assimilation did not differ with Fe status of the food algae, none of the within species variation in egg production rate could be explained by C assimilation.

Discussion

Results of the experiments clearly show that egg production and naupliar survival can be suppressed when copepods are presented with an Fe-depleted diet. As with other animals that do not contain hemoglobin, Fe is primarily used in zooplankton in the respiratory electron transport chain (Chase and Price 1997). Specifically Fe is involved in Fe-S reaction centers within many enzymes involved in redox reactions with the cell, including reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase, the cytochrome bc_1 complex and cytochrome *c* oxidase. Therefore Fe is tightly related to energy production and use by copepods. For *A. tonsa*, the cost of biosynthesis, including the formation of new material for juvenile growth and adult egg production, accounts for 50-74% of the increase in respiration related to feeding (the specific dynamic action, SDA) under high food conditions and for 79-118% of the SDA under food deficient conditions (Kiørboe et al. 1985). Therefore limited access to Fe leading to lower

energy availability is likely to have immediate repercussions for the ability of *A. tonsa* to grow and reproduce. Ingestion and assimilation were both less sensitive to Fe limitation in our experiments. Respiratory costs associated with these processes account for far less of the total respiratory demand in *A. tonsa* (18-28% of SDA for the cost of C absorption, 0.9-14% of SDA for the cost of feeding, digestion and excretion, Kiørboe et al. 1985). As both processes are also more critical to the immediate survival of individuals than growth and reproduction, maintaining these functions in the face of low dietary Fe may be both cost-effective and adaptive.

The availability of Fe can affect other factors that may influence copepod growth, so it is important to determine whether our results can be explained as an indirect consequence of such changes. For instance, increases in copepod reproduction and gonad maturation rate after in situ Fe additions have been attributed to increased food availability rather than food quality (Rollwagen Bollens and Landry 2000, Jansen et al. 2006). In our experiments, however, the ingestion rates and carbon assimilation rates were not correlated to egg production rates. Our ingestion rates, 1.31 to 3.01 μg C ind.$^{-1}$ d^{-1}, were lower than those in some studies (Besiktepe and Dam 2002), but within the range of other studies using the same C concentrations of *Thalassiosira* and *Rhodomonas* as food (Roman 1984; Kiørboe et al. 1985). As no change in phytoplankton abundance occurred in either Fe-replete or Fe-depleted control flasks, differential algal growth among treatment flasks also could not have caused food levels to differ between treatments over the course of the experiments. Cell size did change markedly in response to Fe availability, with Fe-depleted cells displaying smaller size and, in the case of diatoms, lower cellular C and N concentration. However, such cell size and C effects were considered when adjusting food levels for feeding the copepods. As a consequence, ingestion rate did not differ between Fe levels.

The assimilation of C and N from food by copepods could potentially also vary in response to Fe deficiency of algae. The efficiency of assimilation of organic C and N from food can be affected by the level of silicification in diatoms, which can increase in response to Fe

limitation (Hutchins and Bruland 1998, Table 1). However, the rate of C assimilated by copepods from diatoms did not differ between Fe-replete and Fe-depleted diets, nor did it differ between Fe-depleted and Fe-replete cells of the other two species or between diatoms and non-diatoms. This is not unexpected given the design of the experiment. Because the initial amount of C available in food was the same for all treatments and high enough to saturate ingestion, ingestion rates were similar among treatments. With similar ingestion rates copepods from the low and high Fe treatments should have assimilated similar amounts of C. It is conceivable that Fe deficiency can also affect the N content of algal food. Assimilatory nitrate reduction has high Fe and energy requirements and nitrate was the sole form of nitrogen available to algae in our cultures (Guillard and Ryther 1962). Although the phytoplankton cultured under different Fe conditions did differ in their cellular N content, Fe-depleted algae did not display higher C:N. Moreover, copepods assimilated the same amount of C from Fe-replete and Fe-depleted algae. Therefore, it seems unlikely that less N was assimilated from Fe-depleted than from Fe-replete algae, and that N limitation explains reduced egg production in copepods fed low Fe food.

Iron deficiency may also contribute to changes in the cellular composition of essential dietary components in algae, such as proteins and essential lipids, some of which have been shown to affect egg production in copepods (Kleppel et al. 1998, Hassett 2004). As Fe limitation may affect the energy available for biosynthesis of these energy rich compounds and the abundance of Fe dependent enzymes is important in their synthesis, Fe limitation may affect lipid content and composition of algal food. A separate analysis of fatty acids and sterol concentration and composition of Fe-replete and Fe-depleted algal species used as food in this study is described elsewhere (Chen et al. 2011b). Whereas significant effects of Fe were noted on lipid concentration and composition of plankton, fatty acid content was not strongly correlated to egg production rate across the data as a whole. Nevertheless, the lipid content of phytoplankton varies across species, and this could partly explain the difference in egg production rate of *A. tonsa* fed different algal species, for example, the higher egg production rate with *R. salina* as food than the other two species. Such biochemical differences between

algae may account for the higher egg production supported by Fe-depleted *R. salina* than by the other two species of Fe-replete algae, and further verifies the greater suitability of *R. salina* as a food resource.

Our results show a tight correlation between the rate of Fe assimilated by copepods and egg production rate across all experiments, clearly indicating the requirement of Fe for maximizing egg production. However, the response of egg production to Fe assimilation rate was not linear, suggesting a threshold Fe assimilation rate which leads to intense Fe limitation. For example, the egg production rate exhibited by copepods fed Fe-deleted *T. oceanica* was much lower than all the other treatments, which may indicate that Fe assimilation rate by copepods in this experiment was well below the threshold rate. By comparison, Fe assimilation rate of copepods fed either *I. galbana* or *R salina* was not as low, so the response of egg production to Fe limitation was more modest when these species were used as food.

In addition to the lower rate of assimilation, faster loss of assimilated Fe under Fe depleted conditions may also contribute to lower reproduction rate. Fe recycling by zooplankton has been observed to be more efficient in an oceanic HNLC area than coastal Fe-enriched water (Hutchins et al. 1993). A shorter biological half life of Fe in copepods (1.0 d) under an Fe-limited condition compared to the biological half life of 1.3 d under Fe-replete conditions was also seen in a laboratory study (Schmidt et al. 1999). Results from these studies suggest that once Fe is assimilated from food, it is first allocated to maintenance-related activities, such as feeding, digestion and excretion, before it is used for biosynthesis-related activities such as growth and reproduction. Therefore, the observed reduction in egg production when copepods were fed Fe-depleted food could be due to a combination of insufficient Fe assimilation and to the preferential allocation of assimilated Fe for metabolic activities other than reproduction.

The existence of Fe limitation could improve our understanding of zooplankton productivity. In marine food-web models, marine zooplankton productivity is presumed to be limited primarily by food availability. However, in many Fe limited regions the low Fe:C ratios

in phytoplankton may result in lower zooplankton production than might be expected based on the algal biomass present. While this issue has not been addressed directly in the field, studies that compare plankton responses to variations in Fe supply often report a mismatch between primary productivity and secondary productivity. For example, during the Kerguelen Ocean and Plateau comparison study (KEOPS), diatom biomass in Fe-rich water was 3.5-35 times higher than that in the adjacent HNLC water during the seasonal bloom, whereas zooplankton biomass was only 2 times higher (Armand et al. 2008, Carlotti et al. 2008). In the Subarctic Pacific Iron Experiment for Ecosystem Dynamics Study II (SEEDS II), when primary production increased 3 fold after Fe fertilization, micro- and mesozooplankton did not respond to Fe fertilization distinctly (Kudo et al. 2009; Tsuda et al. 2009). Future efforts need to determine if mismatches between phytoplankton and zooplankton productivity are predictable based on the Fe status of phytoplankton in a region.

Our study provides clear evidence that copepods can be negatively affected by a reduction in dietary Fe. In addition, the comparison of available data of the in situ Fe:C ratio of phytoplankton and zooplankton suggests that Fe limitation of zooplankton may be a widespread phenomenon (Fig. 1). The experimental conditions resulted in gradients of Fe supply and demand that were similar to those observed in seston and zooplankton in the field. Algal growth rates were similar to those reported for phytoplankton in culture (Sunda and Huntsman 1995) and in situ (Landry et al. 2000). Fe quotas of copepods in our study were comparable to the field measurement of Martin and Knauer (1973, Fig. 1). While these are the only direct comparisons we can make at this time, it suggests that *A. tonsa* does not have an unusual requirement for Fe compared to species collected from open ocean waters. However, we acknowledge that the species used in these experiments typically lives in coastal areas and may never experience Fe limitation. Consequently, *A. tonsa* may be more sensitive to dietary Fe deficiency than species that typically inhabit low Fe regions. Our work leaves open the question of whether copepods in the open ocean can adapt to low Fe conditions by adjusting their assimilation, retention or requirement for dietary Fe. Obviously, this work indicates that

similar studies need to be conducted on zooplankton species that regularly experience low Fe conditions before the extent of limitation by Fe can be determined. Such studies need to take into consideration variations in the elemental requirements of species and the potential for physiological trade-offs. It is also needed to try to determine the critical Fe:C threshold in food at which mineral limitation of zooplankton production occurs.

Table 1. Comparison of cell volume, cellular C, N, and Fe concentration, C:N, Fe:C, and Si:C ratios, growth rate, and photosynthetic efficiency (F_v/F_m) between Fe-replete and Fe-depleted phytoplankton cells; brackets contain % of Fe-replete values for Fe-limited cells. All data are presented as means from replicate cultures ± 1 standard deviation (SD), where *n* values are shown in the table. For each species and treatment, mean daily growth rates ± 1 SD were calculated for the final 3 consecutive days after 2 to 3 weeks of culturing. nd: not determined.

Algal species	Fe treatment	Cell volume (μm^3) *n* = 3	Cellular C (fmol μm^{-3}) *n* = 5	Cellular N (fmol μm^{-3}) *n* = 5	Cellular Fe (amol μm^{-3}) *n* = 3-4	C:N (mol mol^{-1}) *n* = 5	Fe:C (μmol mol^{-1}) *n* = 3-4	Si:C (mol mol^{-1}) *n* = 3	Growth rate (d^{-1}) *n* = 3	F_v/F_m *n* = 3
T. oceanica	replete	128±7	13.5±1.63	2.3±0.03	0.43±0.05	6.0±0.1	31.5±3.6	0.08±0.05	1.46±0.10	0.55±0.03
	depleted	78±4(61%)	7.1±0.45	1.2±0.08	0.004±0.003	6.0±0.2	0.6±0.5	0.3±0.02	0.74±0.06	0.20±0.004(36%)
R. salina	replete	128±10	17.4±1.74	2.9±0.33	2.03±1.84	6.0±0.1	23.5±3.5	nd	1.27±0.09	0.72±0.04
	depleted	85±4(66%)	19.8±0.88	4.2±0.08	0.01±0.004	4.7±0.1	0.5±0.2	nd	0.43±0.01	0.39±0.01(54%)
I. galbana	replete	46±3	21.2±0.51	2.5±0.07	7.78±1.08	8.4±0.1	36.7±5.1	nd	1.21±0.06	0.65±0.03
	depleted	28±2(61%)	18.2±0.44	2.2±0.03	0.01±0.001	8.4±0.1	0.7±0.1	nd	0.49±0.01	0.39±0.01(60%)

Table 2. Rates of Fe and C assimilation in copepods (pmol ind.$^{-1}$ d^{-1}, µmol ind.$^{-1}$ d^{-1}, respectively) fed on three species of algae. Values shown are means from 3 replicate cultures ± 1 SD.

		T. oceanica	*R. salina*	*I. galbana*
Fe	Fe-replete	0.138±0.022	0.881±0.143	0.397±0.073
	Fe-depleted	0.005±0.0007	0.038±0.006	0.019±0.003
C	Fe-replete	0.186±0.034	0.118±0.014	0.134±0.052
	Fe-depleted	0.174±0.046	0.128±0.027	0.133±0.048

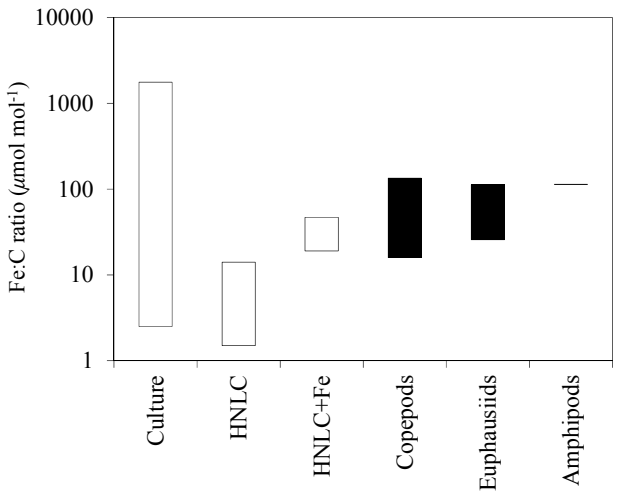


Fig. 1. Comparison of published Fe:C ratios in phytoplankton (open boxes) and crustacean zooplankton (filled boxes). Data for each category are from the following sources: phytoplankton cultures: Sunda and Huntsman (1995, 1997); High-Nutrient, Low-Chlorophyll (HNLC) regions before Fe addition: Schmidt and Hutchins (1999), Maldonado and Price (1999), Abraham et al. (2000), and Twining et al. (2004); HNLC regions after Fe addition: Twining et al. (2004), Boyd et al. (2004); copepods: Martin and Knauer (1973); euphausiids: Martin and Knauer (1973), Fowler (1977), Masuzawa et al. (1988); amphipods from Musazawa et al. (1988).

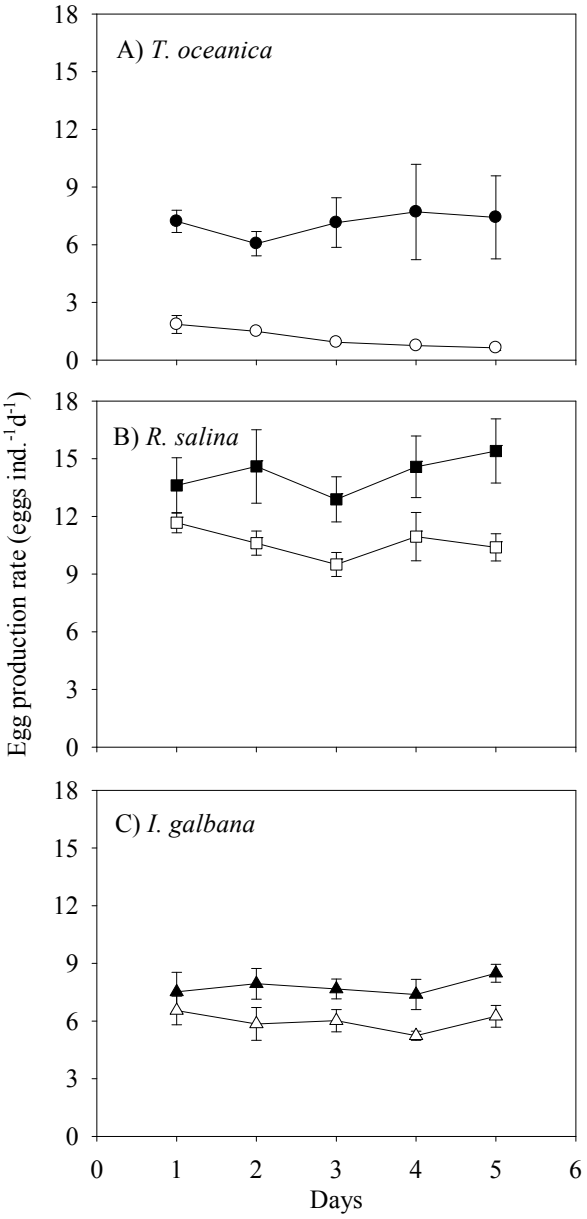


Fig. 2. Egg production rates of *A. tonsa* feeding on Fe-replete (filled symbols) and Fe-depleted (open symbols) cells of (A) *T. oceanica*, (B) *R. salina*, and (C) *I. galbana* over a 5-d period. Data points are means of 3 replicates ± 1 SD.

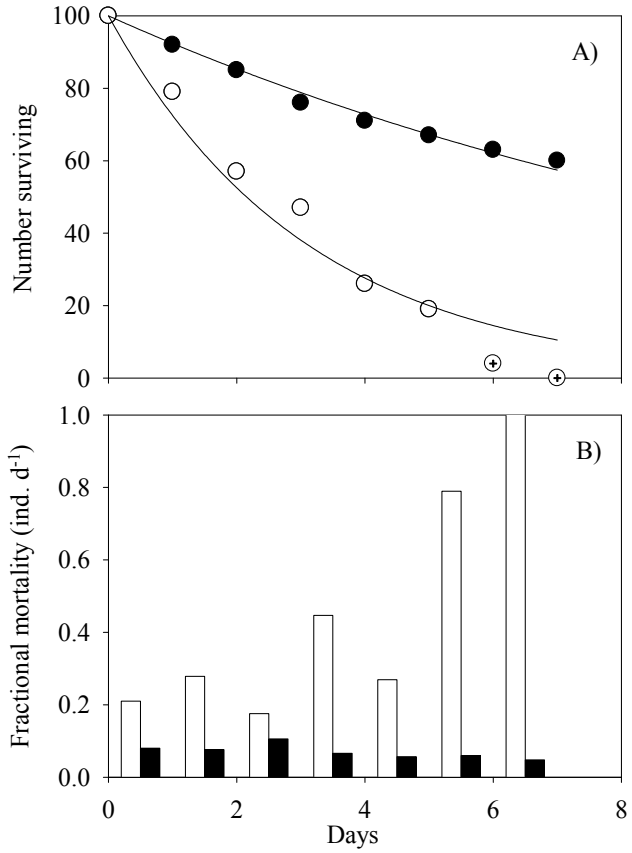


Fig. 3. Naupliar survival of *A. tonsa* feeding on Fe-replete or Fe-depleted *T. oceanica*. (A) Time series of number of surviving individuals for the Fe-depleted (open circles) and Fe-replete (filled circles) treatments. The lines represent best fit regressions of the form $N_t = 100\ e^{-mt}$, where N_t is the number of individuals alive at time *t*, and *m* is the instantaneous mortality rate. The last two points for the Fe-depleted treatment (open circles with crosses) were not used to fit the lines because of elevated mortality rates during these periods. (B) Time series of mortality rate for the Fe-depleted (open bars) and Fe-replete (filled bars). Mortality calculated as the fraction of individuals that died during that interval divided by the number alive at the start of the interval.

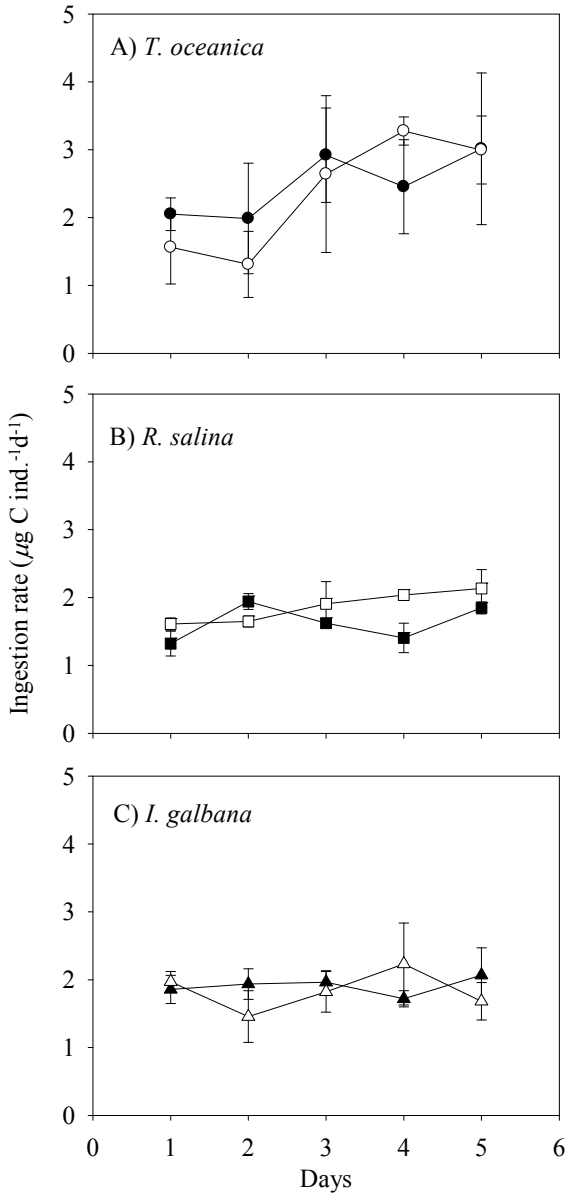


Fig. 4. Ingestion rates of *A. tonsa* feeding on Fe-replete (filled symbols) and Fe-depleted (open symbols) cells of (A) *T. oceanica*, (B) *R. salina*, and (C) *I. galbana* over a 5-d period. Data points are means of 3 replicates ± 1 SD.

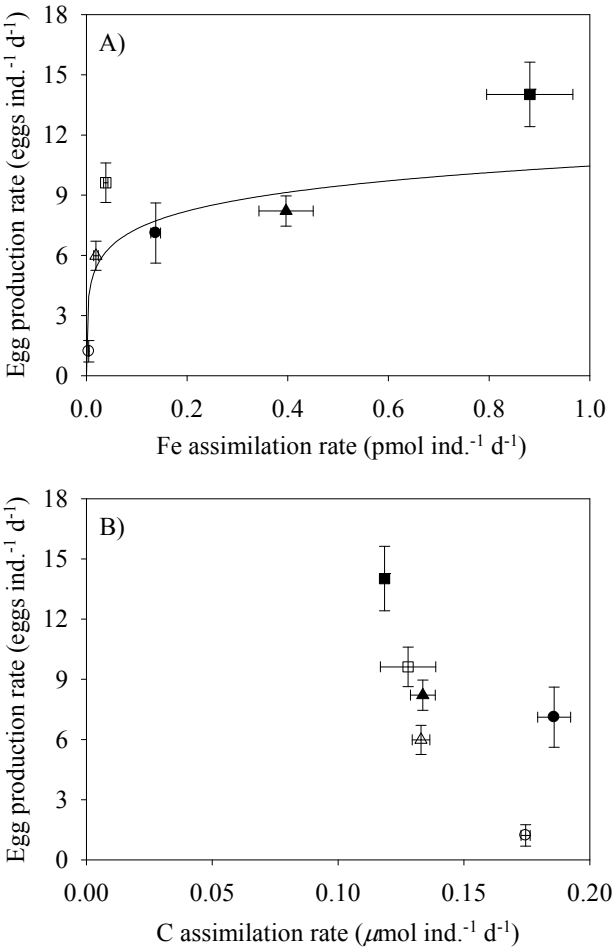


Fig. 5. Egg production rate of *A. tonsa* as a function of (A) Fe, and (B) C assimilated daily from *T. oceanica* (circle), *R. salina* (square), and *I. galbana* (triangle) diets. Filled symbols and open symbols represent Fe-replete and Fe-depleted treatments, respectively. The line in (A) represents the best fit of the data to the equation $y = 12.45+1.83 \ln x$ ($r^2 = 0.71$). No consistent or significant correlation of egg production and daily C assimilation was found. The *R. salina* and *T. oceanica* diets showed an inverse relationship between egg production rates and assimilated C, whereas egg production rates of copepods fed *I. galbana* were unaffected by the rate of C assimilated.

Chapter 3

Influence of iron on fatty acid and sterol composition of marine phytoplankton and copepod consumers

Abstract

We conducted laboratory experiments to determine the influence of iron on the fatty acid (FA) and sterol composition of the diatom *Thalassiosira oceanica*, the cryptophyte *Rhodomonas salina*, the prymnesiophyte *Isochrysis galbana*, and the copepod *Acartia tonsa* fed these algae. Algal cells were cultured with no added Fe or with Fe added at 100 nmol L^{-1}. Fe-deficient culture medium resulted in lower growth rates, cell volumes, and photosynthetic efficiencies for all algal species. The total mass of FA $cell^{-1}$ was 2.2 to 3.1 times as high in Fe-replete cells compared to Fe-deficient cells; on a carbon normalized basis the FA concentrations of Fe-replete algal cells were 1.4-1.5 times as high. Differences were noted between Fe treatments for specific saturated, monounsaturated, and polyunsaturated FAs within each algal species. Across all species, total C-normalized 20:5ω3 + 22:6ω3 concentrations declined 18% in Fe-deficient *T. oceanica*, 25% in Fe-deficient *R. salina*, and 35% in Fe-deficient *I. galbana*. Another consistent difference in FA composition between Fe treatments was for 18:4ω3 which had a C-normalized concentration about two-fold higher in Fe-replete algae. No consistent pattern for all algal species was evident for Fe effects on algal sterol concentration and composition, and in response to Fe limitation sterols were conserved more than C and FAs in these cells. Copepods feeding on Fe-replete and Fe-deficient algae displayed smaller differences in FA and sterol concentrations than their algal diets. Differences in lipid concentration and composition between Fe-replete and Fe-deficient algae may partly explain the lower egg production rate of copepods feeding on Fe-deficient algae.

Introduction

Iron is a limiting nutrient for phytoplankton growth in over 30% of the world's oceans including the Equatorial Pacific Ocean, the Southern Ocean, the Subarctic Pacific, and some coastal areas (Martin et al. 1991, Hutchins and Bruland 1998, Hutchins et al. 2002). Fe-deficient phytoplankton cells have lower nitrogen, phosphorus and sulfur concentrations and higher silicon (Hutchins and Bruland 1998, Twining et al. 2004), but only a few studies have examined the influence of Fe on the concentrations of other cellular components such as lipids (Van Leeuwe et al. 1997, Liu et al. 2008), even though lipids may greatly affect the physiology of the plankton and their nutritional value for herbivores. Further, Fe is an essential component of fatty acid desaturases (Los and Murata 1998, Shanklin and Cahoon 1998) and may therefore influence the degree of FA saturation in marine plankton.

We have recently shown that the copepod *Acartia tonsa* displays different survivorship and reproductive rates when fed Fe-replete *vs* Fe-deficient algae (Chen et al. 2011a). The decline in egg production was particularly evident for copepods fed Fe-deficient diatoms (an 86% decline, compared to declines of 25-30% for flagellate diets), but across all three algal diets tested, *Thalassiosira oceanica*, *Rhodomonas salina*, and *Isochrysis galbana*, egg production rates correlated well with Fe assimilation in copepods. Because egg production could be influenced by dietary lipids, notably sterols with regulatory function, and essential FAs (Jónasdóttir 1994), we conducted a study to evaluate the influence of Fe on the lipid composition of phytoplankton and copepods. Sterols and essential polyunsaturated fatty acids (PUFAs) are synthesized exclusively by phytoplankton de novo and are required for somatic growth and reproduction in zooplankton (Goulden and Place 1990, Pond et al. 1996). Certain FAs are also precursors of eicosanoids, the latter being involved in various physiological functions including osmo-regulation and immune responses (George et al. 2008). Previous studies have shown that plankton lipids can be affected by macronutrients (Shifrin and Chisholm 1981, Jónasdóttir 1994, Klein Breteler et al. 2005), Si (Mortensen et al. 1988), light conditions (Shifrin and Chisholm 1981, Renaud et al. 1991), temperature (Mortensen et al. 1988), and organic contaminants (Fisher and Schwarzenbach 1978). There are very few studies which have evaluated the influence of metal nutrients on the lipid composition of marine organisms (Nechev et al. 2006). Liu et al. (2008) found that Fe enrichment can increase the weight-normalized extractable lipid produced by cultures of *Chlorella vulgaris*, but they did not examine the composition of the lipids in their cultures. Van Leeuwe et al. (1997) found that additions of 2 nmol L^{-1} Fe had a marked effect on the FA composition of certain Southern Ocean plankton assemblages but not others.

We tested the hypothesis that Fe deficiency can affect the composition, particularly degree of unsaturation, of algal FAs and sterols, and consequently affect the lipid content and egg production of copepods fed these Fe-deficient phytoplankton. In this study, we determined the FA and sterol composition of Fe-deficient and Fe-replete cells of three algal species held in culture. We then determined the FA and sterol content of copepods fed Fe-replete and Fe-deficient phytoplankton. Particular attention was given to the polyunsaturated FAs 20:5ω3 and 22:6ω3 since they have been shown to be important nutrients for egg production and hatching success in copepods (Jónasdóttir 199, Kleppel et al. 1998, Arendt et al. 2004). Among the sterols, cholesterol enrichment in food can enhance copepod egg production (Hassett 2004) and is required for animal growth due to its role in plasma membrane health; it cannot be synthesized de novo by marine crustaceans (Goad 1981, Hassett and Crockett 2009).

Materials and methods

The ubiquitous calanoid copepod *A. tonsa* was used in experiments due to its availability and dominance in coastal waters. Individual copepods were collected from Stony Brook Harbor and were carefully picked out of the zooplankton mixture under a dissecting microscope. They were then maintained in filtered Stony Brook Harbor water with bubbling at 21°C on a 14:10 hour light:dark cycle. For copepod maintenance, the cryptophyte *R. salina* and the prymnesiophyte *I. galbana* were cultured in f/2 medium (Guillard and Ryther 1962) prepared with 0.2 μm filtered surface seawater collected 8 km off Southampton, New York (salinity: 34) at 17°C and a 14:10 hour light:dark cycle, and were used to feed *A. tonsa* on a daily basis.

We used the same three species of phytoplankton as diets as in our previous study that addressed Fe limitation effects on copepod egg production (Chen et al. 2011a). All species were held in clonal unialgal cultures that were maintained axenically, and all species are readily eaten by *A. tonsa*. Further, the oceanic diatom *T. oceanica* can maintain a moderate growth rate under Fe-deficient conditions (Sunda et al. 1991). All three phytoplankton species were cultured in Southampton seawater that had then been passed through chelex resins to remove trace metals (Price et al. 1988/1989). The water was then enriched with f/2 levels of macronutrients including N and P, and for the diatom, Si, at 17°C on a 14:10 hour light:dark cycle (120 μmol quanta m^{-2} s^{-1}). Metal additions included 50 nmol L^{-1} $MnCl_2$, 40 nmol L^{-1} $CuCl_2$, 100 nmol L^{-1} $ZnCl_2$, 40 nmol L^{-1} $CoCl_2$, and 100 nmol L^{-1} $NiCl_2$ with 0.1 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA) (Sunda and Huntsman 1995). Different

amounts of Fe, as $FeCl_3$, were added into the media; in Fe-replete media, the total concentration of dissolved Fe was 100 nmol L^{-1} and for Fe-deficient media, no Fe was added. We used 100 nmol L^{-1} in Fe-replete media because at this concentration intracellular Fe:C is saturated in various species of algae (Sunda and Huntsman 1995); this concentration is still < 1% of total Fe in standard growth media like f/2. The phytoplankton cell densities and sizes were determined by a Multisizer™ III Coulter Counter to trace growth response to Fe conditions. To assess the degree of Fe stress experienced by the cells, the quantum yield of fluorescence, F_v/F_m, was also measured (Geider 1993). Cell size, C and Fe content of phytoplankton cells were determined as in Chen et al. (2011a). The phytoplankton cultures were considered ready to be used as food when limited growth and photosynthetic efficiency of Fe-deficient cells were observed and held stable for 3 d. Fe limitation of phytoplankton in the low Fe media was indicated by decreased cell volume and photosynthetic efficiency (F_v/F_m) (Table 1). The Fe:C ratios in Fe-replete cells were well within the range of Fe:C measured in algal cultures (Sunda and Hunstman 1995) and field-collected phytoplankton from Fe-enriched waters (Boyd et al. 2004, Twining et al. 2004), 3-20 times higher than Fe:C in phytoplankton from high nutrient, low chlorophyll (HNLC) waters (Chen et al. 2011a), and about 50 times higher than in cells grown under the same conditions without added Fe (Table 1).

For the preparation of phytoplankton samples for lipid analysis, cell suspensions were filtered at low vacuum pressure onto precombusted Whatman™ GF/C filters such that between 1.9×10^7 and 1.6×10^8 cells were collected on each filter. Four replicate samples were prepared for each treatment. Filters were then placed in precombusted glass scintillation vials and stored at -20°C until analysis.

Fe-replete and Fe-deficient phytoplankton cells were used to feed *A. tonsa*. These cultures were diluted by addition of sterilized chelexed seawater to achieve a fixed concentration of 500 μg C L^{-1}. The cellular C contents of each algal species caught on precombusted glass fiber filters (samples containing 0.71-1.84 mg dry wt) were measured with a Flash EA1112 carbon nitrogen analyzer (CE Elantech). Duplicate batches of 250 copepod adults were placed in acid-washed 250-mL Erlenmeyer flasks containing 200 mL of phytoplankton suspension that were held in the dark to minimize algal growth during the feeding. Copepods were allowed to feed for 24 h, during which they consumed all of the Fe-replete and Fe-deficient food; food was replenished every 24 h and animals were allowed to feed for 3 d (longer feeding of Fe-deficient algae resulted in excessive mortality of *A. tonsa*). Dead animals were picked out and discarded. Copepods were then captured on precombusted

Whatman GF/C filters, 170-240 copepods per filter. There were duplicate samples for each treatment. Filters were then placed in precombusted scintillation vials and stored at -20°C until analysis at Skidaway Institute of Oceanography.

Lipids were extracted from filtered phytoplankton and *A. tonsa* using methylene chloride:methanol (2:1, v:v) and ultrasonication. After each 20 s ultrasonic treatment, solvents were removed and extractions were repeated two additional times. Combined methylene chloride:methanol extracts were washed with 5% NaCl solution, centrifuged, and the methylene chloride layer was pipetted off and dried over anhydrous Na_2SO_4. Lipid extracts were saponified with aqueous 0.5 mol L^{-1} KOH for 2 h at 100°C. Nonsaponifiable lipids (alcohols and sterols) were extracted out of the alkaline solution with hexane (3×, 5 mL), after which FAs were extracted with hexane (3×, 5 mL) following acidification to pH 2 with 6 mol L^{-1} HCl. The non-saponifiable lipids were silylated with N,O-bis(trimethylsilyl)trifluoroacetamide/pyridine (2 h, 60°C) and analyzed by gas chromatography-mass spectrometry (GC-MS) using a non-polar capillary column (Agilent JandW DB-5™, 30 m × 0.25 mm inner diameter (i.d.), 0.1 μm film thickness; temperature program 80-230°C at 3° min^{-1}; helium carrier gas) installed in an Agilent 6890 G C and coupled to an Agilent 5793 mass spectrometer operating in electron ionization (EI) mode. Cholestane was added as an internal standard immediately prior to GC-MS analysis. FAs were methylated with diazomethane and analyzed using a polar capillary column (Restek Rtx-Wax™, 30 m × 0.25 mm i.d., 0.1 μm film thickness; temperature program 80-320°C at 3° min^{-1}; helium carrier) in the same instrument. Methylnonadecanoate was used as an internal standard. Abundances of the major sterol-trimethylsilyl ethers and FA methyl esters were determined from full scan (50-650 da) EI total ion currents relative to the respective internal standards assuming constant detector responses. FA and sterol identifications were verified with GC-MS.

The lipid concentration of phytoplankton is presented as mg g^{-1} C. The unsaturation index (UI) of the FAs was calculated as: UI = (Σ mol% of unsaturated FA × number of double bonds of each FA) / Σ mol% of saturated FA. Mean copepod dry weight of 7 μg $ind.^{-1}$ (Kiørboe et al. 1985) were used; we did not observe any difference in copepod size fed Fe-replete and Fe-deficient diets. C was assumed to account for 46% of total copepod dry weight (Kiørboe et al. 1985).

Differences of individual and total phytoplankton FAs and sterols were analyzed by 1-way analysis of variance (ANOVA) using Sigmaplot 11.0 (Systat Software).

Results

Phytoplankton fatty acids

The C normalized FA concentrations (mg g^{-1} C) are shown in Table 2. Significantly higher concentrations of total cellular FAs and polyunsaturated FAs (PUFAs) were present in all three species of Fe-replete phytoplankton cells ($p<0.01$). Differences in total FA concentrations between Fe treatments were comparable for all species, with C-normalized mass of FAs in Fe-deficient cells ranging between 65% and 74% of that in Fe-replete cells (126 mg g^{-1} C in Fe-deficient *T. oceanica vs* 176 mg g^{-1} C Fe-replete *T. oceanica*, 95 mg g^{-1} C in Fe-deficient *R. salina vs* 146 mg g^{-1} C in Fe-replete *R. salina*, and 129 mg g^{-1} C in Fe-deficient *I. galbana vs* 174 mg g^{-1} C in Fe-replete *I. galbana*). More strikingly, given that Fe-replete cells had significantly higher cell volumes (Table 1, $p < 0.001$), the total FA content of Fe-deficient cells (pg $cell^{-1}$) was 32% of that in Fe-replete cells of *T. oceanica* (cellular C was 46% in Fe-deficient *T. oceanica*), 45% of that in Fe-replete *R. salina* (cellular C was 68% in Fe-deficient *R. salina*), and 42% of that in Fe-replete *I. galbana* (cellular C was 55% in Fe-deficient *I. galbana*) (Tables 1, 2).

A notable difference in saturated fatty acids (SFAs) between Fe-replete and Fe-deficient cells was seen for 14:0, for *T. oceanica* and *I. galbana*, where 1.6-1.7 times higher contents were measured for Fe-replete cells. For monounsaturated fatty acids (MUFAs), the largest difference was seen for 16:1 in *T. oceanica*, where the contents of 16:1ω9 and 16:1ω7 were 2 times and 12 times as high, respectively, in Fe-replete cells (Table 2).

Among the PUFAs, 18:4ω3 was the only FA that was consistently higher (about double) in Fe-replete algae than in their Fe-deficient counterparts (Table 2). Fe-replete *T. oceanica* and *R. salina* had significantly higher concentrations of 20:5ω3 than Fe-deficient cells, whereas Fe-replete *I. galbana* had significantly higher 22:6ω3 than Fe-deficient cells ($p < 0.01$; Table 2). There was no consistent pattern among the three algal species of C_{20}:C_{22} ratios between Fe-replete and Fe- deficient cells (Table 2), but combined C-normalized 20:5ω3 + 22:6ω3 concentrations declined by 18%, 25%, and 35% from Fe-replete to Fe-deficient cells for *T. oceanica*, *R. salina*, and *I. galbana*, respectively (Table 2).

While the C normalized concentration of total unsaturated FAs of Fe-replete algae were indeed significantly higher than Fe-deficient algae for all three algal species (119 mg g^{-1} C in Fe-replete *T. oceanica vs* 90 mg g^{-1} C in Fe-deficient *T. oceanica*, 114 mg g^{-1} C in Fe-replete *R. salina vs* 73 mg g^{-1} C in Fe-deficient *R. salina*, 116 mg g^{-1} C in Fe-replete *I. galbana vs* 82 mg g^{-1} C in Fe-deficient *I. galbana*) ($p < 0.01$; Table 2), the proportion of total FAs that was unsaturated did not show much difference between Fe treatments (71% and 68%

in Fe-deficient and Fe-replete *T. oceanica*, 76% and 79% in Fe-deficient and Fe-replete *R. salina*, 63% and 66% in Fe-deficient and Fe-replete *I. galbana*) (Table 3). However, both Fe-replete *R. salina* and *I. galbana* had significantly higher UI values (270 for *R. salina* and 176 for *I. galbana*) than Fe-deficient cells (250 for *R. salina* and 134 for *I. galbana*) ($p < 0.001$), while the UI of Fe-deficient *T. oceanica* (233) was not significantly different from Fe-replete *T. oceanica* cells (207) ($p = 0.094$; Table 3). However, Fe-replete *T. oceanica* had a slightly higher ratio of monounsaturated to saturated FA ratio (0.55) than did the low-Fe *T. oceanica* cells (0.49). For *R. salina* and *I. galbana*, PUFA to MUFA ratios were 1.6-1.7 times as high in Fe-replete cells ($p < 0.001$).

Phytoplankton sterols

Total sterol contents (normalized to C mass of the algal cells) of Fe-replete algal cells were 1.6 times as high as in Fe-depleted cells of *T. oceanica*, 1.2 times as high as in Fe-depleted cells of *R. salina*, and 3.8 times as high as in Fe-depleted cells of *I. galbana* as in Fe-replete cells of the same species ($p <0 .05$; Table 4). Total sterol per cell (fg cell^{-1}) in Fe-depleted cells was 75% of that in Fe-replete cells of *T. oceanica* (compared with 46% for C cell^{-1}), 85% of that in Fe-replete cells of *R. salina* (compared with 68% for C cell^{-1}), and 2.12 times that in Fe-replete cells of *I. galbana* (compared with 55% for C cell^{-1}) (Table 4).

The sterol composition of the three phytoplankton species was simpler than the FA composition, with only one sterol dominating for each species. The dominant sterol of *T. oceanica* is 24-methylcholesta-5,24(28)-dien-3β-ol, accounting for 97-98% of its total sterol content (Table 5). For both *R. salina* and *I. galbana*, the dominant sterol is 24-methylcholesta-5,22-dien-3β-ol, which comprised 94-95% of their total sterol content in Fe-replete cells (Table 5). In Fe-deficient *I. galbana*, the proportion of 24-methylcholesta-5,22-dien-3β-ol declined to 55% due to an increase in other sterols (Table 5). Both *R. salina* and *I. galbana* contain a small amount of cholest-5-en-3β-ol (< 11%) whereas *T. oceanica* does not. Fe-replete algae of all species had 1.2 to 2.2 times lower C-normalized concentrations of their dominant sterol (Table 4).

Copepod fatty acids

Copepods had a 20% and 70% higher total FA concentration after feeding on Fe-rich *T. oceanica* and *R. salina* cells, respectively, than Fe-deficient cells of those species, but a lower FA concentration (70%) after feeding on Fe-rich *I. galbana* cells than Fe-deficient *I. galbana* (Table 6). The dominant FAs in the copepods were 14:0, 16:0, 18:0, 20:5ω3, and

22:6ω3 (Table 6). Copepods from all treatments had 2-11 times higher concentrations of 22:6ω3 than 20:5ω3 regardless of the C_{20}:C_{22} ratio of the food, and the copepods that fed on Fe-deficient algae had higher concentrations of 22:6ω3 than those that fed on Fe-replete cells for all dietary species.

Copepod sterols

Copepods feeding on Fe-deficient *T. oceanica* and *R. salina* had lower concentrations of total sterols than those feeding on Fe-rich cells (72% and 62%, respectively), but a diet of *I. galbana* produced the opposite effect (27% higher, Table 7). Cholest-5-en-3β-ol accounted for 85-95% of the total sterol content of the copepods, regardless of diet. No consistent difference in cholesterol was observed between Fe treatments among the algal species. There was a decline in 24-methylcholesta-5,22-dien-3β-ol in copepods fed Fe-deficient *T. oceanica* and *R. salina*, but not *I. galbana* (Table 7).

Discussion

Fatty acids

Generally, the FA concentration and composition of the Fe-replete algae were comparable to those in previous studies using Fe-enriched media. For *T. oceanica*, 14:0, 16:0, 16:1, 18:4ω3, and 20:5ω3 are the principal FAs (accounting for 81.4% of total FAs), in agreement with what Fisher and Schwarzenbach (1978) reported for the same clone (previously identified as *T. pseudonana* clone 13-1). Knuckey et al. (2002) reported similar principal FAs for *T. oceanica*, although the relative abundance of the FAs was different and varied with growth phase. The principal FAs of *R. salina*, 14:0, 16:0, 18:1, 18:3ω3, 18:4ω3, 20:5ω3 and 22:6ω3, accounted for 89.6% of total FAs and was similar to those reported by Veloza et al. (2006), although their total C normalized FA concentration was about 1.7 times of what we found. This difference may have been due to the physiological state of the cells, since FA concentrations increase strikingly as cells go from log-phase growth in nutrient-enriched media to stationary phase (Fisher and Schwarzenbach 1978, Knuckey et al. 2002). For *I. galbana*, the principal FAs, 14:0, 16:0, 18:1, 18:2ω6, 18:4ω3, and 22:6ω3, accounted for 79.6% of total FAs, and were similar to the FA profiles reported by Patil et al. (2007) and George et al. (2008).

The total C normalized concentration of FAs was consistently higher in Fe-replete phytoplankton cells than in Fe-deficient cells for all algal species, perhaps because FA synthesis is energy-intensive and Fe is used in electron transport chains for energy production.

The *de novo* synthesis of 1 mole of 16:0 from acetyl-CoA in cytosol requires 49 moles of adenosine-5'-triphosphate (ATP) (Gilbert 2000), indicating a high energy requirement for FA synthesis. We also observed higher C normalized concentration of total PUFAs as well as total 20:5ω3 (eicosapentaenoic acid, EPA) and 22:6ω3 (docosahexaenoic acid, DHA) combined in Fe-replete cells than in Fe-deficient cells for all algal species. This finding is consistent with reports that these FAs promote reproduction in marine copepods (Jónasdóttir 1994, Kleppel et al. 1998, Arendt et al. 2004) and are consistent with our findings of Fe promotion of egg production in *A. tonsa* (Chen et al. 2011a).

Comparison of FA composition between Fe-replete and Fe-deficient algae did not show consistent differences in any principal FAs among the three algal species examined except for 18:4ω3, which was consistently higher in all Fe-replete algae. Because 18:4ω3 is a precursor of long chain PUFAs such as EPA and DHA (George et al. 2008), synthesis of 18:4ω3 influences PUFA formation in these algae. The higher concentration of 18:4ω3 in Fe-replete algae suggests that 18:4ω3 synthesis involving 18:3ω3 desaturation may be sensitive to Fe availability in these algae.

Being an essential component of FA desaturases (Los and Murata 1998, Shankling and Cahoon 1998), Fe serves at the catalytic center of these enzymes by forming a reactive complex with oxygen (Fe-O-Fe) that reacts with C-C single bonds and converts them to double bonds. We therefore hypothesized that higher Fe levels in phytoplankton cells would result in more unsaturated FAs. Indeed, both Fe-replete *R. salina* and *I. galbana* had higher UI than their Fe-deficient counterparts. However, *T. oceanica* displayed the opposite pattern. Diatoms are more enriched in MUFAs than other phytoplankton (Brett et al. 2009), suggesting that the desaturation from SFAs to MUFAs in diatoms may be a more vulnerable step of desaturation to Fe limitation than the second step (from MUFAs to PUFAs). The lower UI of Fe-replete *T. oceanica* cells could be a result of the greater abundance of SFAs in these cells, a possible indication of increased energy for SFA synthesis. In the marine diatom *Asterionella japonica* desaturation of 16:0 to 16:1ω9 was found to be affected by copper but not zinc (Gillan et al. 1983); the ratio of 16:0 to 16:1 was also significantly affected by Fe in the diatom *T. oceanica* ($p = 0.029$; Table 3). Toxic concentrations of cadmium and mercury also reduced production of EPA in *Asterionella glacialis* (Jones et al. 1987) and toxic concentrations of Zn decreased production of 18:4 in the freshwater chlorophyte *Selenastrum capricornutum* (McLarnon-Riches et al. 1998). Unlike in the diatom, the desaturation from MUFAs to PUFAs in *R. salina* and *I. galbana* was significantly affected by Fe limitation ($p < 0.001$). Further study on the effect of the ambient Fe concentration on FA desaturation in

diverse algal species is needed. This may be particularly important in influencing the ability of Fe-deficient algae to grow in colder waters where a greater proportion of unsaturated FAs would be required to maintain membrane fluidity.

The most abundant FAs in the copepod *A. tonsa* were 16:0, 18:0, 20:5ω3, and 22:6ω3, consistent with earlier findings (Støttrup et al. 1999; Veloza et al. 2006). *A. tonsa* fed diatoms were richer in EPA than those fed *I. galbana* or the cryptophyte *Rhodomonas baltica* (Støttrup et al. 1999), similar to our findings for Fe-replete diets. As MUFAs are primarily involved in synthesis of storage wax esters and the highly unsaturated FAs EPA and DHA are primarily involved in structural phospholipids (Brett et al. 2009), the dominance of 20:5ω3 and 22:6ω3 and comparatively small amount of MUFAs suggests that this copepod stores little wax esters in the summer (when the copepods were collected). This is consistent with findings of Støttrup et al. (1999) who showed that wax esters in adult *A. tonsa* account for ≤ 3% of total lipids. Compared to their diet, *A. tonsa* accumulates DHA more than EPA, which can be considered as either an over-wintering strategy or a requirement for developing their nervous system (Brett et al. 2009).

Neither individual FA nor total FA concentration of copepods ingesting Fe-replete and Fe-deficient algae showed consistent differences for all dietary species. Total FA concentrations were higher in copepods that ate Fe-replete *T. oceanica* and *R. salina* than their Fe-deficient counterparts, but lower in copepods that ate Fe-replete *I. galbana*. Lee et al. (1971) reported that storage lipids of calanoid copepods are strongly affected by diet whereas the structural phospholipids are not. In adult *A. tonsa*, 21-44% of total lipids are triacylglycerols (Støttrup et al. 1999). Thus, it appears that FAs of copepods feeding on Fe-replete and Fe-deficient algae were not consistently affected by dietary FAs because most of the lipids of *A. tonsa* are structural.

Certain FA ratios such as C_{20}:C_{22} ratios and ω3:ω6 ratios have been widely used as an index of nutritional value of food and have been suggested to affect copepod reproduction. Diets with a lower C_{20}:C_{22} ratio and higher ω3:ω6 ratio promoted copepod egg hatching success (Jónasdóttir 1994, Broglio et al. 2003, Shin et al. 2003), and in a few studies, promoted egg production as well (Jónasdóttir 1994, Kleppel et al. 1998). However, neither of these two ratios showed a consistent correlation with the egg production rate of *A. tonsa* feeding on the algae in our study (Chen et al. 2011a). This could be either because C_{20}:C_{22} and ω3:ω6 ratios are stronger indicators of egg viability than egg production, which may be controlled by different mechanisms (Arendt et al. 2005), or because the direct effect of Fe on energy and total FA production outweighs the indirect effect on FA composition.

Sterols

The composition of sterols of phytoplankton and copepods are much simpler as only one sterol typically dominates each species. The dominant sterol of each species agrees with previous studies (Volkman and Hallegraeff 1988, Patterson et al. 1994, Tremblay et al. 2007) and the proportion of 24-methylcholesta-5,22-dien-3β-ol (98%) and cholest-5-en-3β-ol in Fe-replete *R. salina* matches what Chu et al. (2008) reported (99%). Cholest-5-en-3β-ol enrichment has been shown to enhance copepod egg production rate and this may be related to the cholest-5-en-3β-ol content of the algal diet (Hassett 2004). Both *R. salina* and *I. galbana* contain a small amount of cholest-5-en-3β-ol (< 11%) while *T. oceanica* does not. However in our study, the cholest-5-en-3β-ol content of both *R. salina* and *I. galbana* increased inversely with Fe content, and fewer eggs were produced by copepods that ingested Fe-deficient algae (Chen et al. 2011a), indicating that Fe availability had a greater effect than cholest-5-en-3β-ol on egg production. The concentration and composition of the dominant sterols of the algae changed with Fe, most clearly in *I. galbana*, but reasons for this effect are not yet apparent. We are unaware of any other study which assessed the effects of Fe on sterol composition.

Overall, in Fe-deficient algae compared to Fe-replete algae, the sterol content in cells was more conserved than carbon (75% for sterols *vs* 46% for C for *T. oceanica*, 85% *vs* 68% for *R. salina*, and 212% *vs* 55% for *I. galbana*), and much more conserved than the FAs (32% for *T. oceanica*, 45% for *R. salina*, and 42% for *I. galbana*). During Fe limitation, some cellular C, especially that stored in energy reserves, is presumably metabolized more rapidly than sterols, which have a more regulatory and structural function. It also appears that FA production and metabolism are more susceptible to Fe nutrition than cellular carbon and especially sterols in these algae. Explanations for differences in sterol and FA regulation in these cells in response to Fe are not obvious, but may be tied to the energy requirements for FA synthesis.

In copepods, the dominant sterol shifts to cholest-5-en-3β-ol, in agreement with previous studies that crustaceans, which do not biosynthesize sterols de novo, tend to accumulate cholest-5-en-3β-ol from algal diets and convert algal sterols to cholesterol via dealkylation and hydrogenation (Kanazawa 2001). There was no consistent pattern relating cholest-5-en-3β-ol concentration of copepods to the Fe content of the algal diet. The 93% decline in concentration of 24-methylcholesta-5,22-dien-3β-ol in the copepods fed Fe-deficient *T. oceanica* cells, compared to copepods fed Fe-replete diatoms, matches the 86%

decline in egg production in copepods fed Fe-limited diatoms (Chen et al. 2011a), suggesting a possible relationship between this sterol and egg production in *A. tonsa*.

Table 1. Comparison of cellular C concentration, Fe:C ratio, cell volume, and photosynthetic efficiency (F_v/F_m) between Fe-replete and Fe-deficient phytoplankton cells. Cellular C concentration and Fe:C ratios from Chen et al. (2011a).

		cellular C (fmol μm^{-3})	Fe:C ($\mu mol\ mol^{-1}$)	cell volume (μm^3)	F_v/F_m
T. oceanica	Fe-replete	13.5±1.6	31.5±3.6	145	0.58
	Fe-deficient	7.1±0.5	0.6±0.5	125	0.17
R. salina	Fe-replete	17.4±1.7	23.5±3.5	154	0.73
	Fe-deficient	19.8±0.9	0.5±0.2	92	0.34
I. galbana	Fe-replete	21.2±0.5	36.7±5.1	42	0.58
	Fe-deficient	18.2±0.4	0.7±0.1	27	0.30

Table 2. C normalized FA concentrations in phytoplankton (mg g^{-1} C) grown under Fe-limiting and Fe-replete conditions. Means of 4 replicate samples ± 1 SD. nd: not detected.

	T. oceanica		*R. salina*		*I. galbana*	
	low Fe	high Fe	low Fe	high Fe	low Fe	high Fe
14:0	22.2±0.8	34.6±2.3	10.4±0.4	11.3±0.5	20.0±0.7	33.6±0.8
iso-15	nd	nd	0.6±0.0	0.2±0.0	0.5±0.2	nd
anteiso-15	nd	nd	0.4±0.0	0.2±0.0	0.3±0.2	nd
15:0	1.5±0.0	1.7±0.1	0.6±0.0	0.3±0.0	0.5±0.3	0.4±0.0
16:0	8.9±0.4	11.0±4.6	7.6±0.3	15.5±0.5	18.1±0.5	22.8±0.4
16:1ω9	9.2±0.4	18.0±0.3	1.3±0.1	0.7±0.0	1.8±0.6	3.9±0.1
16:1ω7	0.3±0.0	3.7±0.1	nd	nd	nd	nd
16:2ω6	0.3±0.1	1.9±0.0	nd	nd	nd	0.3±0.0
16:2ω4	nd	nd	0.3±0.0	1.3±0.1	0.6±0.3	2.9±0.1
17:0	1.0±0.8	6.7±0.1	1.6±0.1	1.2±0.1	nd	nd
18:0	2.1±0.3	3.5±3.8	1.2±0.1	0.9±0.1	1.4±0.5	0.7±0.1
18:1ω9	1.8±1.6	3.5±4.5	0.6±0.0	0.4±0.0	27.3±0.8	26.3±0.6
18:1ω7	4.3±1.6	3.6±0.9	2.8±0.1	3.6±0.1	4.4±1.0	1.8±0.0
18:1ω5	1.7±3.0	2.1±1.2	8.4±0.3	9.5±0.3	nd	nd
18:2ω6	8.8±0.1	1.1±1.1	3.3±0.1	6.1±0.2	11.5±0.9	12.9±0.4
18:3ω6	0.5±0.0	0.7±0.0	1.2±0.1	1.8±0.1	1.5±0.7	1.5±0.0
18:3ω3	5.5±0.1	0.7±0.0	6.3±0.2	18.0±0.7	2.1±0.8	7.2±0.2
18:4ω3	13.2±0.8	29.1±1.2	21.3±1.0	37.0±1.6	7.0±1.6	13.9±0.6
18:5ω3	nd	nd	nd	nd	0.7±0.8	5.6±0.4
20:0	nd	nd	nd	nd	6.3±0.8	0.9±0.0
20:1	nd	nd	1.2±0.0	0.6±0.0	2.1±0.6	4.1±0.4
20:4ω3	0.2±0.2	0.2±0.2	0.3±0.0	0.9±0.0	nd	nd
20:5ω3	32.0±2.2	45.9±2.4	12.4±0.5	20.7±0.8	10.1±1.8	8.1±0.4
22:6ω3	11.8±1.0	7.5±0.6	13.1±0.4	13.3±0.6	12.6±1.7	27.0±1.1
SFA	35.7±1.2	57.5±10.1	22.5±0.8	29.6±1.0	47.2±1.8	58.4±0.9
MUFA	17.4±0.3	31.9±6.4	14.7±0.6	15.2±0.5	35.6±2.3	36.1±0.6
PUFA	72.4±4.3	87.1±4.7	58.1±2.4	99.2±4.0	46.0±8.3	79.4±2.9
total	126±3	176±17	95.3±3.7	144±5	129±12	174±4
C_{20}:C_{22}	2.70±0.05	6.11±0.3	0.95± 0.01	1.55± 0.01	0.80± 0.04	0.30±0.01
FA_{tot} $cell^{-1}$ (pg $cell^{-1}$)	1.35±0.03	4.17±0.40	2.10±0.08	4.63±0.17	0.77±0.07	1.85±0.04

Table 3. FA composition of phytoplankton (weight % of total FAs) grown under Fe-limiting and Fe-replete conditions. Means of 4 replicate samples ± 1 SD. nd: not detected.

	T. oceanica		*R. salina*		*I. galbana*	
	low Fe	high Fe	low Fe	high Fe	low Fe	high Fe
14:0	17.7±1.1	19.7±1.6	10.9±0.1	7.9±0.1	15.7±2.0	19.3±0.6
iso-15	nd	nd	0.6±0.0	0.2±0.0	0.3±0.2	nd
anteiso-15	nd	nd	0.5±0.0	0.1±0.0	0.3±0.2	nd
15:0	1.2±0.0	1.0±0.0	0.6±0.0	0.2±0.0	0.4±0.2	0.2±0.0
16:0	7.1±0.5	6.1±1.9	7.9±0.0	10.7±0.1	14.2±1.4	13.1±0.1
16:1ω9	7.4±0.4	10.3±0.8	1.4±0.0	0.5±0.0	1.4±0.3	2.2±0.0
16:1ω7	0.2±0.0	2.1±0.2	nd	nd	nd	nd
16:2ω6	0.3±0.1	1.1±0.1	nd	nd	nd	0.2±0.0
16:2ω4	nd	nd	0.3±0.0	0.9±0.0	0.4±0.2	1.6±0.0
17:0	0.8±0.7	3.8±0.4	1.7±0.1	0.8±0.1	nd	nd
18:0	1.7±0.3	1.8±1.8	1.2±0.0	0.6±0.0	1.1±0.3	0.4±0.0
18:1ω9	1.5±1.3	1.8±2.2	0.6±0.0	0.3±0.0	21.3±2.1	15.1±0.1
18:1ω7	3.4±1.3	2.0±0.3	2.9±0.0	2.5±0.0	3.4±0.5	1.1±0.0
18:1ω5	1.4±2.4	1.2±0.7	8.8±0.0	6.6±0.1	nd	nd
18:2ω6	7.0±0.2	0.6±0.7	3.5±0.0	4.2±0.0	8.9±0.2	7.4±0.1
18:3ω6	0.4±0.0	0.4±0.0	1.3±0.0	1.3±0.0	1.1±0.5	0.8±0.0
18:3ω3	4.4±0.1	0.4±0.0	6.6±0.0	12.5±0.0	1.6±0.6	4.2±0.1
18:4ω3	10.5±0.4	16.6±1.6	22.3±0.2	25.7±0.2	5.4±0.8	8.0±0.1
18:5ω3	nd	nd	nd	nd	0.5±0.6	3.2±0.2
20:0	nd	nd	nd	nd	4.9±0.3	0.5±0.0
20:1	nd	nd	1.3±0.0	0.4±0.0	1.6±0.3	2.4±0.2
20:4ω3	0.1±0.1	0.1±0.1	0.3±0.0	0.6±0.0	nd	nd
20:5ω3	25.5±1.1	26.2±2.3	13.0±0.0	14.4±0.1	7.8±0.7	4.7±0.2
22:6ω3	9.4±0.6	4.3±0.3	13.7±0.2	9.3±0.1	9.8±0.5	15.5±0.4
SFA	28.5±1.7	32.4±2.8	23.6±0.2	20.6±0.2	36.8±2.4	33.6±0.6
MUFA	13.9±0.3	18.0±1.9	15.4±0.0	10.6±0.1	27.7±1.1	20.8±0.2
PUFA	57.6±1.8	49.6±4.7	61.0±0.2	68.9±0.3	35.5±3.5	45.6±0.8
UI	233±10	207±19	250±1	270±1	134±14	176±4

Table 4. C normalized sterol concentrations in phytoplankton (mg g^{-1} C) grown under Fe-limiting and Fe-replete conditions. Means of 4 replicate samples ± 1 SD. nd: not detected.

	T. oceanica		*R. salina*		*I. galbana*	
	low Fe	high Fe	low Fe	high Fe	low Fe	high Fe
cholesta-5,22-dien-3β-ol	nd	nd	nd	nd	nd	nd
cholest-5-en-3β-ol	nd	nd	0.26±0.02	0.07±0.01	0.32±0.08	0.02±0.01
24-methylcholesta-5,22-dien-3β-ol	nd	nd	4.77±0.31	3.98±0.41	1.57±0.25	0.72±0.07
24-methylcholest-5,24(28)-dien-3β-ol	8.45±0.52	5.19±0.11	nd	nd	0.56±0.13	nd
24-methylcholest-5-en-3β-ol	nd	nd	nd	nd	0.35±0.07	0.01±0.01
24-methylcholesta-24(28)-dien-3β-ol	0.21±0.02	0.10±0.02	nd	nd	nd	nd
24-ethyl-cholest-5-en-3β-ol	2.41	nd	nd	nd	nd	nd
24-ethylcholesta-5,24(28)-dien-3β-ol	nd	nd	nd	nd	0.07±0.01	nd
Total	8.66±0.55	5.29±0.11	5.03±0.33	4.05±0.42	2.86±0.54	0.76±0.09
$Sterol_{tot}$ $cell^{-1}$ (fg $cell^{-1}$)	93.5±8.1	125±3	111±7	130±14	17.1±3.1	8.05±0.92

Table 5. Sterol composition of phytoplankton (weight % of total sterols) grown under Fe-limiting and Fe-replete conditions. Means of 4 replicate samples ± 1 SD. nd: not detected.

	T. oceanica		*R. salina*		*I. galbana*	
	low Fe	high Fe	low Fe	high Fe	low Fe	high Fe
cholesta-5,22-dien-3β-ol	nd	nd	nd	nd	nd	nd
cholest-5-en-3β-ol	nd	nd	5.1±0.2	1.7±0.1	10.9±0.9	2.9±1.3
24-methylcholesta-5,22-dien-3β-ol	nd	nd	94.9±0.2	98.3±0.1	55.1±2.3	95.9±2.1
24-methylcholesta-5,24(28)-dien-3β-ol	97.5±0.2	98.1±0.3	nd	nd	19.4±1.1	0.6±0.5
24-methylcholest-5-en-3β-ol	nd	nd	nd	nd	12.0±0.3	0.6±0.7
24-methylcholest-24(28)-dien-3β-ol	2.4±0.1	1.9±0.3	nd	nd	nd	nd
24-ethyl-cholest-5-en-3β-ol	nd	nd	nd	nd	nd	nd
24-ethylcholesta-5,24(28)-dien-3β-ol	nd	nd	nd	nd	2.5±0.1	nd

Table 6. FA concentrations in *A. tonsa* after feeding on 3 different algal species (mg g^{-1} C) that had been grown under Fe-limiting and Fe-replete conditions. Means of duplicate samples ± variance. nd: not detected.

	T. oceanica		*R. salina*		*I. galbana*	
	low Fe	high Fe	low Fe	high Fe	low Fe	high Fe
14:0	1.2±0.1	4.4±0.4	0.2±0.0	0.2±0.0	2.1±0.2	1.4±0.0
iso-15	nd	nd	0.3±0.1	0.4±0.1	0.1±0.0	nd
anteiso-15	nd	nd	nd	0.2±0.1	nd	nd
15:0	0.3±0.0	0.7±0.0	0.1±0.1	0.2±0.1	0.2±0.1	0.1±0.0
16:0	7.2±0.2	10.5±0.2	5.3±0.3	11.0±2.2	4.2±1.1	4.0±0.0
16:1ω9	0.2±0.1	0.4±0.1	0.3±0.2	0.3±0.1	nd	nd
16:1ω7	0.4±0.0	2.0±0.1	0.3±0.0	0.8±0.2	0.3±0.0	0.1±0.0
16:1ω5	0.1±0.1	0.2±0.0	nd	nd	nd	nd
16:2ω6	nd	0.3±0.0	nd	nd	nd	0.2±0.0
16:2ω4	nd	nd	0.1±0.1	1.0±1.1	0.1±0.0	0.1±0.0
17:0	0.4±0.5	0.2±0.0	1.0±0.1	1.3±1.5	nd	nd
18:0	5.9±0.0	6.7±0.2	4.3±0.6	6.2±1.2	2.1±0.9	2.0±0.1
18:1ω9	1.0±0.1	1.2±0.1	0.3±0.4	0.9±1.1	3.4±0.1	1.8±0.0
18:1ω7	1.0±0.1	2.0±0.0	1.1±0.5	2.9±0.7	0.8±0.1	0.4±0.0
18:1ω5	0.6±0.0	1.0±0.0	0.7±0.7	1.9±2.4	nd	nd
18:2ω6	nd	nd	0.2±0.0	1.9±0.3	1.3±0.0	0.8±0.0
18:3ω6	nd	nd	0.2±0.3	0.2±0.0	0.2±0.0	0.1±0.0
18:3ω3	nd	0.2±0.0	nd	2.7±0.2	0.3±0.0	0.3±0.0
18:4ω3	0.1±0.1	0.8±0.0	0.1±0.1	2.0±0.2	0.7±0.1	0.6±0.0
18:5ω3	nd	nd	nd	nd	0.1±0.0	0.2±0.0
20:0	nd	nd	nd	nd	0.7±0.1	0.1±0.0
20:1	nd	nd	0.1±0.1	0.2±0.0	0.2±0.0	0.1±0.0
20:4ω3	nd	nd	nd	0.1±0.1	nd	nd
20:5ω3	6.4±0.1	9.7±0.8	2.5±3.3	5.8±0.8	1.7±0.2	0.6±0.0
22:6ω3	23.3±0.2	16.8±2.2	15.2±3.2	13.7±2.9	9.3±3.6	6.8±0.3
total	47.4±0.2	57.2±3.7	32.0±7.8	53.9±9.3	27.9±5.5	19.4±0.5

Table 7. Sterol concentrations in *A. tonsa* after feeding on 3 different algal species (mg g^{-1} C) that had been grown under Fe-limiting and Fe-replete conditions. Means of duplicate samples ± variance. nd: not detected.

	T. oceanica		*R. salina*		*I. galbana*	
	low Fe	high Fe	low Fe	high Fe	low Fe	high Fe
cholesta-5,22-dien-3*β*-ol	0.02±0.00	0.04±0.03	nd	nd	0.04±0.01	0.02±0.00
cholest-5-en-3*β*-ol	2.63±0.05	3.42±0.55	1.68±0.27	2.70±0.36	2.93±0.01	2.52±0.04
5α(H)-cholestan-3*β*-ol	0.05±0.00	0.09±0.04	0.02±0.00	0.05±0.00	0.05±0.00	0.05±0.00
24-methylcholesta-5,22-dien-3*β*-ol	0.01±0.00	0.09±0.11	0.05±0.01	0.10±0.02	0.25±0.13	0.12±0.00
24-methylcholesta-5,24(28)-dien-3*β*-ol	0.02±0.00	0.09±0.04	nd	nd	0.07±0.04	0.01±0.02
24-methylcholest-5-en-3*β*-ol	0.02±0.00	0.06±0.01	nd	nd	0.09±0.03	0.01±0.01
24-methylcholesta-24(28)-dien-3*β*-ol	nd	nd	0.02±0.01	0.02±0.00	nd	nd
24-ethyl-cholest-5-en-3*β*-ol	0.01±0.01	nd	0.01±0.00	0.01±0.01	0.02±0.00	nd
24-ethylcholesta-5,24(28)-dien-3*β*-ol	0.01±0.01	0.05±0.06	nd	nd	nd	nd
Total	2.77±0.05	3.83±0.82	1.78±0.27	2.88±0.38	3.45±0.20	2.72±0.04

Chapter 4

Influence of algal iron content on the assimilation and fate of iron and carbon in a marine copepod

Abstract

We conducted pulse-chase experiments to study the assimilation and excretion of iron and carbon by the copepod *Acartia tonsa* fed three algal species cultured under Fe-replete or Fe-depleted conditions. Mean assimilation efficiencies (AEs) of Fe ranged from 47% to 53% for the diatom *Thalassiosira oceanica*, 43% to 45% for the cryptophyte *Rhodomonas salina*, and 50% to 69% for the prymnesiophyte *Isochrysis galbana*. Carbon AEs ranged from 82% to 90% for all species. No consistent pattern of Fe or C AE was found between Fe treatments among the three algal diets. The AEs of Fe were linearly correlated with the fraction of Fe contained in algal cytosol ($r^2 = 0.75$, $p = 0.02$). Contrary to the expectation based on the homeostasis theory, the efflux rate constants of Fe were generally higher when *A. tonsa* were fed Fe-depleted cells, especially for *R. salina* and *I. galbana*, contrary to expectations if the copepods were maintaining elemental homeostasis. Mean respiratory rates were 30-70% higher for the copepods fed Fe-depleted food among the three algal diets, suggesting those animals were either "dumping" excess carbon, experiencing greater physiological stress or foraging more actively. Biokinetic modeling based on parameters measured here suggests that *A. tonsa*, and possibly other mesozooplankton, cannot maintain stoichiometric homeostasis of their tissue Fe:C ratios. The lack of regulation of tissue Fe contents is in marked contrast to the well established regulation of the macronutrients nitrogen and phosphorus.

Introduction

Many metazoan consumers can maintain relatively constant elemental composition when confronted with variability in the elemental content of their food (Sterner and Elser 2002). This ability, defined as stoichiometric homeostasis, can be achieved by increasing the assimilation of the limiting nutrient element and preferentially releasing those elements that are in excess of requirements. For example, carbon assimilation efficiency of *Daphnia magna* was positively related to phosphorus content of food, while increasing P-deficiency in food led to higher C respiration and excretion rates and lower P excretion rate (DeMott et al. 1998). Similarly, freshwater zooplankton communities that were dominated by *Daphnia* excreted N and P in ratios that are similar to N:P ratios in their prey, indicating a tendency to maintain their body N:P balance (Urabe 1993). The idea that organisms regulate retention of elements from food to limit variability in elemental composition has been built into models of zooplankton production and nutrient cycling (e.g. Anderson et al. 2005), and dynamic energy budget models (e.g. Kuijper et al. 2004).

As an essential element, iron limits phytoplankton productivity in over one third of the ocean (Moore et al. 2004; Boyd et al. 2007). Phytoplankton experiencing different degrees of Fe limitation can vary in their Fe content by several orders of magnitude (Sunda and Huntsman 1997). Variation in Fe content of food also influences growth and reproduction of heterotrophic flagellates (Chase and Price 1997) and copepods (Chen et al. 2011a). Currently little is known about whether Fe content of marine herbivores is regulated as closely as are the contents of N and P. However, a review of the literature suggests that the Fe content of mesozooplankton varies little among regions and less than the Fe content of algae (Chen et al. 2011a). While the Fe content of marine zooplankton may indeed be regulated, there are several reasons to believe that, due to the specific biological roles and distinct chemistry of Fe, stoichiometric regulation of Fe may pose a different challenge to organisms than does the regulation of N and P contents.

First, the primary use of Fe in heterotrophs is as a cofactor for enzymes associated with energy yielding reactions of the electron transport system (Fraústo da Silva and Williams 2001). Low availability of Fe in food may therefore limit an organism's ability to perform energy yielding processes. Indeed, in marine heterotrophic bacteria Fe deficiency

decreases electron transport chain activity as well as glucose and amino acid respiration, resulting in lower bacterial growth rate and growth efficiency (Tortell et al. 1996, Kirchman et al. 2003). One way to deal with a C rich food is to convert excess organic C to CO_2 via respiration (DeMott et al. 1998). By limiting respiration, low dietary Fe may render such a strategy impossible, forcing the organism to rely on other less energetically demanding mechanisms for regulating Fe content, such as excretion of organic C or reduction of C assimilation. By contrast, P in the diet is unlikely to pose a short term energetic constraint on stoichiometric regulation, while the constraints imposed by N will not be limited to energy yielding processes.

Second, the chemical behavior of Fe influences its distribution within food and thus the assimilation and utilization of Fe by consumer organisms. Fe is a particle-reactive metal that adsorbs readily to the external algal cell wall (Hutchins et al. 1995). It is also contained within sulfur-iron complexes of the proteins for which it acts as a cofactor, some of which are membrane bound and others are free in the cytoplasm (Fraústo da Silva and Williams 2001). Only Fe associated with the cytoplasm of algal food is readily assimilated by copepods (Hutchins et al. 1995). By contrast, N and P distribution among these fractions varies relatively little among organisms or ambient conditions, with the exception of the ability of many phytoplankton to store large amounts of P as polyphosphate bodies (Raven and Knoll 2010). Therefore, both the subcellular distribution of Fe in the algal food and the regulation of uptake by copepods should be considered when studying Fe assimilation of copepods. Thus far, the correlation between Fe subcellular distribution and assimilation efficiency has only been investigated in a few studies (Hutchins et al. 1995, Wang and Dei 2001).

In this study, we measured the assimilation efficiency and loss of Fe and C by copepods feeding on different species of algae grown under Fe-replete and Fe-depleted conditions to determine if copepods engage in stoichiometric regulation of Fe. We also studied the subcellular distribution of Fe in the algal food to examine the cause of differences in Fe assimilation efficiency when copepods are fed algae experiencing varying degrees of Fe limitation. Finally, we measured copepod O_2 consumption to study the effect of dietary Fe on copepod respiration.

Materials and methods

Copepod and algae maintenance

The locally dominant calanoid copepod *Acartia tonsa* was used in experiments because it is readily available, easily cultured and manipulated. Individual *A. tonsa* copepodites and adults collected from Stony Brook Harbor landing during the incoming tide were carefully picked out of the zooplankton mixture under a dissecting microscope, and then maintained in 0.22-μm filtered and aerated Stony Brook Harbor water (salinity ~ 28) at 21 °C on a 14:10 hour light:dark cycle. *A. tonsa* were fed a mixed diet of the cryptophyte *Rhodomonas salina* (clone CCMP 1319) and the prymnesiophyte *Isochrysis galbana* (CCMP 1323) which are commonly used as food for crustaceans in aquaculture and are considered high quality food. The algal food species were cultured in sterile filtered seawater (salinity ~ 34, collected 10 km off Southampton, NY) enriched with f/2 levels of nutrients (Guillard and Ryther 1962), at 17 °C under a 14:10 hour light:dark cycle.

For all assimilation and respiration experiments, we compared Fe-replete and Fe-depleted cells of the diatom *Thalassiosira oceanica* (CCMP 1005), *R. salinas* and *I. galbana* as food species. *T. oceanica* is similar in size to the other food species and, as an oceanic species, exhibits moderate growth rates under Fe-depleted conditions (Sunda et al. 1991). All algae used in experiments were grown in chelexed (Price et al. 1988/1989) sterile-filtered Southhampton seawater to which f/2 levels of the macronutrients nitrate, phosphate and silicate were added (Guillard and Rhyther 1962). A trace metal solution that included ethylenediaminetetraacetic acid (EDTA) was added to achieve final concentrations in the media of 0.1 mmol L^{-1} EDTA, 50 nmol L^{-1} $MnCl_2$, 40 nmol L^{-1} $CuCl_2$, 100 nmol L^{-1} $ZnCl_2$, 40 nmol L^{-1} $CoCl_2$ and 100 nmol L^{-1} $NiCl_2$ (Sunda and Huntsman 1995). Cultures were maintained at 17°C on a 14:10 hour light:dark cycle with 120 μmol quanta m^{-2} s^{-1} illumination provided by cool-white fluorescent lamps.

In the Fe-replete cultures, $FeCl_3$ was added along with the trace metal-EDTA solution to achieve a final total Fe concentration of 100 nmol L^{-1}. No Fe was added to Fe deficient media but up to 0.1 nmol L^{1} of dissolved Fe was transferred to the chelexed media with the initial inoculum of cells. The phytoplankton cell densities and sizes were determined daily by a Multisizer™ III Coulter Counter to verify that growth rates were slower in the Fe-

deficient cultures (Table 1). To assess the degree of physiological Fe stress experienced by the cells, the quantum yield of fluorescence, F_v/F_m, was also measured (Geider 1993, Table 1). Cellular Fe:C ratios cultured under each Fe conditions were analyzed as described in Chen et al. (2001). The phytoplankton cultures were used in experiments once limited growth and photosynthetic efficiency of Fe-depleted cells remained stable for 3 days.

Assimilation and efflux rate

Assimilation and efflux of Fe were measured using a pulse-chase radiotracer approach. Both Fe-replete and Fe-depleted media were radiolabeled by adding 75.7 kBq of $^{59}FeCl_3$ to each 200 ml culture flask, which corresponded to an addition of 1 nmol L^{-1} of $FeCl_3$. Algal cells were then allowed to grow for a week to allow uniform radiolabeling. To prepare the experimental feeding suspension, algal cells were centrifuged at 465 g for 20 min and the sedimented cell pellets were resuspended into filtered seawater as food. Thus, the ^{59}Fe in the feeding solutions was, initially, entirely associated with the algal cells. Final cell densities in feeding chambers were adjusted so that the particulate organic carbon concentration equaled 500 μg C L^{-1} based on regressions relating cell volume and carbon (Menden-Deuer and Lessard 2000). Actual densities ranged from 2.5 to 7.6×10^4 cells mL^{-1}, depending on the algal species and sizes. Three replicate batches of adult copepods, each containing 50 individuals were added to each 50 ml aliquot of the feeding suspension and allowed to feed for 30 min. The feeding time was chosen to be shorter than the gut passage time of calanoid copepods at 21°C on a variety of foods (Dam and Peterson 1988; Irigoien 1998) so as to avoid overestimating elemental assimilation. They were then removed from the suspensions using 250 μm nitex netting and rinsed with an EDTA-seawater solution and filtered chelexed seawater. After rinsing they were transferred to unlabeled food suspensions with the same C concentration as labeled food to purge their guts of any remaining radiolabeled food for 25 h.

The gamma emissions from ^{59}Fe retained by copepods were assayed immediately after feeding and at each time prior to being resuspended in unlabeled feeding suspensions during depuration. Emissions near the principle emission peak at 1292 keV were assayed using a NaI(Tl) well detector (Canberra Instr.). Each sample was counted for 5 min, with the analytical precision of < 5%. The percent of radioactivity retained in the copepods at any one

time was divided by the initial radioactivity observed immediately after feeding. The calculation of assimilation efficiency (AE) and the efflux rate constant (k_e, h^{-1}) is described below (*statistics* session).

The assimilation and retention of carbon were determined in separate experiments. Fe-replete and Fe-depleted phytoplankton cultures were labeled with 616 kBq of $NaH^{14}CO3$ (an addition of 8.33 nmol L^{-1} $NaHCO_3$), and allowed to grow for a week. The β-emissions associated with ^{14}C in copepods were measured with a Packard Tri-Carb™ liquid scintillation analyzer. The same number of copepods and replicates were used in the C experiment as in the Fe analysis described above. Because this method is destructive, it proved unfeasible to reproduce the degree of temporal resolution we accomplished for the ^{59}Fe depuration curves. To address this problem, we used a mass balance method to study the fraction of C allocated to each possible compartment by copepods after being ingested. Past studies have indicated that five hours should have been long enough to allow for defecation of all unassimilated radioactivity in the gut (Reinfelder and Fisher 1991). Consequently, we estimated the C AE by dividing the radioactivity in copepods after 5 h of depuration by the radioactivity measured immediately after the initial 30 min pulse feeding period. To prepare samples for liquid scintillation, copepods were first separated from the food suspension by gravity filtration through a 250-µm mesh and rinsed with seawater. They were then transferred to 20-ml scintillation vials that each contained 1 ml Perkin Elmer™ Solvable. The vials were placed in a 60°C oven for 1 h to help digest the copepod tissues. After cooling, each vial received 200 µl H_2O_2 and was heated at 60°C for an additional 30 min for further tissue digestion. After cooling, 10 ml Ultima Gold liquid scintillation cocktail was added to each vial. Radioactivity of each sample was measured with a Tri-Carb™ Liquid Scintillation Analyzer.

The food suspension that passed through the 250-µm netting contained fecal pellets and dissolved C produced by copepods over the 5 h depuration period. Fecal pellets were collected by filtering through a 20-µm mesh. The fecal pellets collected were treated as described above for copepods and analyzed for radioactivity of ^{14}C. The remaining water and algal cells were distributed among 5 20-ml scintillation vials and 10 ml of scintillant was added to each vial prior to analysis of radioactivity. The radioactivity associated with this

fraction was considered to be ^{14}C that was lost through respiration and excretion during the 5 h depuration.

Subcellular fractionation of Fe

Measurement of phytoplankton subcellular fraction of Fe was conducted following the method of Fisher et al. (1983). Briefly, the algal cells were uniformly labeled with ^{59}Fe as described above, centrifuged at 465 g for 20 min, and the supernatant with culture medium was pipetted out, after which the cells were resuspended into Milli Q water to promote cell lysis. The cell suspensions were then centrifuged with a Sorvall® RC-5C Plus centrifuge at 4°C at 10,000 g for 15 min to separate cytosol from cellular debris. Radioactivity of the pellets and the final supernatant (cytosol) were determined with a NaI(Tl) gamma spectrometer as described above. Each analysis was conducted for 4 replicates. The proportions of Fe contained in algal cytosol were plotted against the assimilation efficiencies of Fe in copepods fed respective food.

The separation technique described above does not reveal details of the intracellular Fe distribution that may affect assimilation of this metal. To measure the subcellular distribution of Fe within phytoplankton cells, radiolabeled algal cells were rinsed with an oxalate reagent (Tovar-Sanchez et al. 2003) to remove the loosely adsorbed Fe on the cell walls. After being lysed in Milli Q water, the cell suspensions were first centrifuged at 750 g for 5 min to separate cell walls, plasmalemma, and nuclei. The remaining supernatant was then centrifuged at 2,000 g for 15 min to separate mitochondria, lysosomes, and peroxisomes, and then again at 10,000 g for 15 min to separate endoplasmic reticulum, golgi bodies, ribosomes and polysomes. The final supernatant contained cytosol, which includes lipids, soluble enzymes and small molecules (Sheeler 1981). The Fe in cytosol and in contents of pellets following 2,000 g and 10,000 g constituted the Fe in cytoplasm. Radioactivity in pellets from each sequential centrifugation was measured as described above. Each analysis was conducted for 4 replicates.

O_2 consumption rate

To determine if Fe content in food affects copepod respiration, O_2 consumption rates

were measured for copepods fed on food grown under different Fe concentrations. Before the experiment, copepods were acclimated to Fe-replete or Fe-depleted algal food for ~2 d. Oxygen consumption of *A. tonsa* was measured by a Hansatech® Oxygengraph System connected with a Clark-type polarographic microcathode electrode. The respirometer was calibrated by first measuring the voltage signal after removing O_2 from the aliquot of seawater within the incubation chamber by adding $Na_2S_2O_4$. The other end point was determined on an aliquot of filtered seawater that was saturated with O_2 at 21°C. The change in voltage readings were converted to the volume of O_2 dissolved in the seawater based on the solubility of O_2 in Southampton seawater at 21°C (Weiss 1970).

At the start of the incubation, the magnetic stirrer was turned off to prevent damage to the copepods. Three replicate batches of copepods each containing 30 individuals for each food condition were analyzed for O_2 consumption rate. Each batch of 30 copepods were transferred to 2 ml of food suspension within the sealed incubation chamber of the respirometer. After incubating copepods for 1 h, stirring was resumed and the O_2 was measured. This procedure was repeated for three separate replicate batches of copepods at each Fe level for all algal food species. The change in O_2 concentrations in 2 ml of food suspension without copepods was also measured after 1 h as a control. The difference between the declines in O_2 with and without copepods was used to estimate the O_2 consumed by copepods, expressed as nmol O_2 ind.$^{-1}$ h^{-1}.

Stoichiometric modeling analysis

To quantify the overall ability of *A. tonsa* to regulate Fe homeostatically, we used a biokinetic model to evaluate the combined effects of C and Fe metabolism on stoichiometric homeostasis (Wang and Fisher 1999). Traditionally, such models are used to predict steady state metal content within animals using the concentration of the substance in the food and aqueous phase and experimentally determined rates of assimilation, loss and growth. Here we use the approach to predict how the ratio between the Fe content of copepod tissues and the Fe content of their food will differ given Fe-replete and Fe-depleted food. If the copepods are maintaining stoichiometric homeostasis, we expect that this copepod to food ratio will be greater when copepods are fed Fe-depleted algae.

To calculate the ratio at steady state of Fe:C in tissues of copepods to Fe:C in their algal food (θ*), we simplified the model of Wang and Fisher (1999) to exclude the negligible contribution of sorption of aqueous Fe.

$$\frac{(\mathrm{Fe:C})_z{}^*}{(\mathrm{Fe:C})_\mathrm{f}} = \frac{IR \times AE_{Fe}}{k_{Fe} + g} = \theta^* \quad (1)$$

where $(Fe:C)_z^*$ is the predicted molar Fe:C ratio (mol mol^{-1}) in copepods at steady state, $(Fe:C)_f$ is the Fe:C ratio (mol mol^{-1}) in the food, *IR* is the specific carbon ingestion rate (d^{-1}), AE_{Fe} is the assimilation efficiency of Fe (unitless), k_{Fe} is the excretion rate constant for Fe (d^{-1}), and *g* is the specific growth rate of copepods in terms of C (d^{-1}). We estimate g by calculating the scope for growth using our measurements of carbon metabolism.

$$g = IR \times AE_C \times (1\text{-}E_C) - R \quad (2)$$

where AE_C is assimilation efficiency of C (unitless), E_C is fraction of assimilated carbon lost to the dissolved phase during the depuration period (unitless), and *R* is the specific respiration rate in carbon terms (d^{-1}). It is important to realize that, calculated in this way, *g* is a growth potential based solely on carbon balance while realized growth may be constrained by other factors, including dietary Fe. Average values for each parameter under each experimental treatment were used to calculate θ*. Ingestion rates were from Chen et al. (2011) and are shown in Table 2. Oxygen consumption was converted to carbon units assuming a respiration quotient of 0.86 at 18°C (Kiørboe et al. 1985). Ingestion and respiration rates were converted to specific rates assuming a copepod carbon content of 3.3 μg (Kiørboe et al. 1985).

Statistics

The Fe assimilation efficiency (AE) and the efflux (k_e, h^{-1}) and defecation (*f*, h^{-1}) rate constants for ^{59}Fe were estimated by fitting a double exponential decay model of the form $Y = Ue^{-ft} + AEe^{-ket}$ to the time series of fraction ^{59}Fe retained for each replicate group of copepods (Fig. 1), where Y is the percentage of initial radiolabel remaining in the copepods. The parameter U in this model refers to unassimilated and ultimately egested fraction of Fe. This model was constructed to assume that the total loss of Fe from copepods comprises two parts of depuration in exponential modes: a rapid loss from defecation (Ue^{-ft}) and a slower loss through excretion (AEe^{-ket}). The parameters were estimated using an iterative

Marquardt-Levenberg algorithm implemented in SigmaPlot v11.0 (SPSS).

For all variables in the experiments, analysis of variance (ANOVA) was used to test for significant differences among the levels for the two main factors as well as any interaction between them. We used Tukey's honestly different significant test (Tukey's HSD) for post-hoc tests of differences among group means. Fractional values (i.e., AE, U) were arcsin square root transformed prior to analysis. Any p-level < 0.05 was presumed to denote a statistically significant result, but we report all p-values as well.

Paired two-tailed *t*-test was used for comparing θ* under Fe-replete and Fe-depleted conditions across algal species, because measurement of parameters were from different experiments.

Results

Some of the ^{59}Fe ingested by copepods was rapidly lost within the first 4 h of depuration, followed by a slower loss thereafter (Fig. 1). The initial loss rate of ingested ^{59}Fe during the first 4 h ranged from 0.28 to 1.61% h^{-1}. Between 18 and 45% of ^{59}Fe ingested was retained in the animals at the end of 25 h depuration. AEs of Fe in copepods feeding on Fe-replete and Fe-deficient algae were on average higher for copepods fed on Fe-depleted food ($p = 0.048$), with the largest difference for copepods fed on *I. galbana* (Fig. 2). The AE of Fe did not differ significantly between Fe-replete and Fe-depleted diets for copepods fed on *R. salina*.

I. galbana had a higher fraction of cellular Fe in cytosol than the other two species (Fig. 3). There was no consistent effect of Fe level on the proportion of Fe in cytosol among the algal species; a lower fraction of Fe was contained in cytosol for *T. oceanica* and *I. galbana* grown under Fe-replete conditions, while similar fraction of Fe was contained in cytosol under Fe-replete conditions for *R. salina*. The proportion of Fe in cytosol was positively correlated with the AE of Fe in the copepods ($r^2 = 0.75$, $p = 0.02$, Fig. 4). Subcellular partitioning of Fe after rinsing with oxalate revealed that 58-87% of the intracellular Fe was contained in the cytosol fraction (Fig. 5). Moreover, a higher proportion of the Fe was found within organelles (pellets 2 and 3) in Fe-depleted cells than in Fe-replete cells ($p < 0.05$, Fig. 5).

There was no evidence that copepods fed on Fe-depleted cells retained Fe more efficiently than those fed on Fe-replete cells. In fact, copepods ingesting Fe-depleted diets showed on average higher efflux rates than those ingesting Fe-replete food ($p = 0.018$). Efflux rate constants of copepods fed on Fe-depleted *R. salina* and *I. galbana* were on average 2 fold greater than those fed on Fe-replete diets. However, efflux rates for copepods fed *T. oceanica* did not differ significantly between Fe-replete and Fe-depleted treatments. Given the same ingestion rate, copepods ingesting Fe-replete *R. salina* and *I. galbana* should concentrate Fe more effectively from food than those ingesting Fe-depleted cells, indicated by higher AE/k_e ratios (Fig. 2C). Copepods fed on *T. oceanica* showed the opposite pattern, due to higher Fe AE when fed Fe limited food.

Carbon AEs ranged from 76% to 93% among all treatments. Neither C AE nor the fraction of assimilated C lost to the dissolved pool showed any difference between copepods fed on Fe-replete and Fe-depleted diets ($p = 0.54$ for AE, $p = 0.18$ for C loss, Fig. 6). The fraction of ingested C that was released by the copepods into solution over the 5 h depuration period ranged from 0.4 to 0.8. Copepods fed *T. oceanica* released a greater fraction of assimilated C than those fed the other two species of algae.

Copepod O_2 consumption rates ranged from 0.33 to 1.34 nmol O_2 ind.$^{-1}$ h^{-1}. Copepods fed on Fe-depleted food had higher O_2 consumption rates than those fed on Fe-replete diet in general ($p = 0.045$). O_2 consumption rates with Fe-depleted diets were 30-60% higher than with correspondent Fe-replete diets. Copepods fed *I. galbana* had the highest O_2 consumption rates, which is 30% higher than the other two species in Fe-depleted treatments and 70% higher in Fe-replete treatments (Fig. 7).

The mean values of the experimentally determined parameters presented above and results of ingestion rate from Chen et al. (2011) used in Equations (1) and (2) were presented in Table 3. The ratio at steady state of Fe:C in tissues of copepods to Fe:C in their algal food (θ^*) were lower under Fe-depleted conditions (($p = 0.028$, paired two-tailed *t*-test).

Discussion

Previous studies have shown that crustacean zooplankton exposed to varying N:P and P:C ratios in their food alter assimilation, excretion and respiration rates in ways that tend to maintain stoichiometric homeostasis with respect to tissue N, P and C (Urabe 1993, DeMott et al. 1998, He and Wang 2008). We expected that *Acartia tonsa* would do likewise when faced with extreme variations in the Fe:C ratio of their food. While in fact some of the responses observed are consistent with stoichiometric regulation, other responses are not. In the following discussion, we examine each set of responses, placing them in context of previous measurements. We then try to evaluate the effect of these responses in sum on the ability of *A. tonsa* to maintain stoichiometric homeostasis with respect to Fe and C. Finally, we discuss some of the ways in which stoichiometric regulation of Fe and other trace metals may differ from regulation of P and N.

Copepods did not consistently assimilate Fe more efficiently in response to reduced Fe content of their food. We did observe significantly higher AEs of Fe when copepods were fed on Fe-depleted *I. galbana* diets than when Fe-replete *I. galbana* was used ($p = 0.008$). This pattern is consistent with regulation of nutrient intake by copepods when they are faced with low nutrient food. However, this response was not observed for the other food species; specifically, AEs for Fe did not differ when copepods were fed Fe-replete and Fe-deplete *R. salina*. A comparison across all experiments suggests that it is actually the fraction of Fe in the cytosol of algal food that determines the Fe AE. Previous work has shown that AEs for a wide range of elements show a 1:1 relationship with the fraction of each element that is found in cytoplasm (Reinfelder and Fisher 1991). In our data, a linear regression between the AE for Fe and the fraction of Fe in cytosol was highly significant ($r^2 = 0.75$, $p = 0.02$). Moreover, the points all cluster about the 1:1 relationship between Fe AE and the fraction of metal in algal cytosol (Fig. 3), which comprised the majority of cytoplasmic Fe in all cases (Fig. 5). For the two species that exhibited differences between Fe treatments, Fe AE changed in proportion to the change in the percentage of Fe associated with the cytosol. Consequently, the variability of Fe AE by copepods is less a function of stoichiometric regulation but more a function of the different Fe subcellular distributions in algal cells.

An increase in the percentage of Fe in cytoplasm in oxalate rinsed cells may be a general response of algae to Fe limitation, although other factors clearly play a role. The

amount of Fe bound to cell wall ligands should decline as free Fe ion declines in the media (Sunda and Huntsman 1995). At the same time, concentrations of Fe in certain intracellular pools may be relatively conserved so as to maintain critical respiratory and photosynthetic capacity under Fe-depleted conditions. Consistent with this reasoning, we found that Fe-depleted cells after oxalate rinse for all algal species allocated a higher proportion of intracellular Fe to organelles (pellet 2 and pellet 3) and lower proportion of Fe to cytosol (supernatant) than rinsed Fe-replete cells ($p < 0.05$, Fig. 5). One factor mediating against lower ratios of adsorbed Fe under Fe-depleted conditions are the smaller cell sizes and greater cellular surface area:volume ratios observed for all species under Fe limiting conditions. All else being equal, a greater surface area:volume ratio pattern should lead to a greater fraction of surface-adsorbed Fe. Certain species under Fe limitation may also produce more surface bound ligands for Fe acquisition, which could alter the adsorption for those cells.

Our AEs for Fe are generally higher than previously reported values for *Acartia* (e.g. Hutchins and Bruland 1994, Hutchins et al. 1995, Schmidt et al. 1999). AEs for Fe may be overestimated if the copepods reabsorbed Fe released from fecal material or via excretion during depuration. However, Fe is released very slowly from copepod fecal pellets (Hutchins et al. 1995, Xu and Wang 2001) and is therefore unlikely to contribute significantly to Fe content of copepods during the depuration period. It is possible that some Fe excreted by copepods was taken up by algae and reassimilated. However, a similar process would have also occurred in previous studies employing the same methodology so this cannot explain why AEs in this study should differ from the previous ones. In fact, the total fraction of Fe retained in copepods after depuration and Fe excreted from the assimilated reservoir during the depuration could be as high as 80% of ingested Fe (Hutchins et al. 1995, 1999). A more plausible explanation for differences in Fe AEs may be differences in the algal cytoplasmic fraction of Fe among the various studies (accompanied by corresponding differences in AEs). Studies that have previously reported lower AEs for Fe have also reported lower fractions of Fe in cytoplasm or cytosol, and the 1:1 relationship between cytoplasmic distributions of Fe in algal food and Fe AEs in copepods is consistent among these studies (Hutchins et al. 1995). Differences in cellular distribution among studies may be due to variations among algal species, variations in physiological state of the cells at the time of analysis, and differences in

water chemistry between media formulations. Consequently, our Fe AEs are not very likely to be overestimated and are reasonable values.

Contrary to expectations, excretion rates of Fe were on average faster for copepods ingesting Fe-depleted *R. salina* and *I. galbana* than Fe-replete cells. Schmidt et al. (1999) observed a similar pattern when a mixed group of calanoid copepods was fed Fe-replete and Fe-limited *T. weissflogii* cells. Under Fe-replete conditions, Fe excretion rates averaged ~ 0.2 d^{-1} and did not vary among algal species ($p = 0.09$), consistent with findings for other metals (Wang et al. 1996). Grazers may not be able to regulate trace metals to the same degree as macronutrients such as N and P. Further evidence of lack of regulation of metals in copepods was provided by Xu et al. (2001) who found no effect of elemental concentrations of algal food on copepod efflux rates of Cd, Se and Zn. The reason for higher excretion rates under Fe-depleted conditions is currently unclear.

One way to assess the influence of the Fe treatments on bioaccumulation of Fe is to compare the trophic transfer potential (TTP) of this metal. This value is calculated as the ratio of AE to k_e (Reinfelder et al. 1998). Values of TTP can be directly compared as long as ingestion rates are similar, as is true for our experiments (Table 2). Our results show that under Fe-replete conditions there is a similar potential of trophic transfer of Fe regardless of the dietary algal type and species. However, under Fe-depleted conditions, copepods retain less Fe but regenerate Fe faster when fed *R. salina* and *I. galbana*, especially for the Fe-depleted *R. salina* treatment where TTP was < 1. A high TTP was obtained when copepods were fed Fe-depleted *T. oceanica*. This high TTP can be attributed to the Fe-depleted efflux rates measured relative to Fe-depleted *R. salina* and *I. galbana* treatments (Fig. 2B). On average, our results were opposite what we expected if the organisms were maintaining stoichiometric homeostasis, which predicts that Fe assimilated from Fe-depleted food is more likely to be retained whereas Fe assimilated from Fe-replete food is more likely to be released back to the media.

The absence of a difference between Fe-depleted and Fe-replete treatments was observed in C AEs or in the fractions lost to the dissolved phase. Our C AEs are within the range reported for *A. tonsa* in other studies (51-95%, Kiørboe et al. 1985, Wang et al. 1996, Besiktepe and Dam 2002). We also observed little difference in C AEs between treatments

among algal species ($p = 0.11$), unlike Wang et al. (1996) and Besiktepe and Dam (2002) who reported that C AEs differed with algal diet. The loss of assimilated C noted in our study was comparable to that measured in other studies (e.g. Schmidt et al. 1999). The contribution of sloppy feeding to the dissolved C measured can be ignored, as sloppy feeding is only important to DOC production when copepod-to-prey size ratios are < 55 (Molles 2005), and our copepod-to-prey sizes ranged from 195 to 893 (assuming body volume of *A. tonsa* is 25 mm^3; Tang 2005). Therefore, we conclude that the observed loss of C to the dissolved phase during the depuration period was largely the result of respiration and excretion, rather than sloppy feeding.

Copepods fed on Fe-depleted food uniformly displayed higher O_2 consumption rates, consistent with expectations if the organisms were releasing excess C assimilated from Fe-depleted food. However, this response is opposite to what we expected based on hypothesized effects of Fe-depleted supply on respiratory electron transport system activity. It is common for organisms to maintain electron transport potential well in excess of that needed to sustain basal metabolism, possibly to support bursts in activity related to foraging or predatory avoidance. The influence of lower dietary Fe may therefore only be observed as a reduced ability to sustain such bursts. Increased respiration observed under Fe-depleted conditions may also not be related to stoichiometric homeostasis. An alternative explanation is that the higher O_2 consumption rate in response to Fe-depleted food reflects more active swimming as copepods attempt to locate better quality food, although this was not quantified in our study. Such behavior would be predicted by optimal foraging theory. Food particle size may also affect copepod respiration. Copepods feeding on algae of smaller size may need to spend more energy in capturing more cells for the same amount of C. For example, *I. galbana* is the smallest cell among the three species of algae we tested, and produced the highest O_2 consumption rates of copepods among all treatments. Similarly, Fe-depleted algae had smaller cell size than Fe-replete algae, so copepods feeding on Fe-depleted algae were presumably more active in filtration, and thus had higher respiration rates.

The stoichiometric modeling analysis verified that overall, *A. tonsa* had little ability to adjust Fe retention, even though the observed differences in respiration and the excretion of Fe under Fe-replete and Fe-depleted conditions can support opposite conclusions regarding

the ability of copepods to regulate their tissue Fe:C ratios. For example, on one hand, the copepods do not retain Fe more efficiently when they are fed Fe-depleted food, suggesting that they are incapable of stoichiometric homeostasis. On the other, the greater respiration rates observed when copepods are fed Fe-depleted food suggests that copepods may be able to maintain stoichiometric homeostasis by "dumping" excess carbon. The modeling analysis assists resolving this ambiguity by comparing the steady state Fe:C ratios in copepod tissues with that in their diets.

In every case, the ratio at steady state of Fe:C in tissues of copepods to Fe:C in their algal food (θ*) was greater for copepods fed Fe-replete food than for those fed Fe-depleted food, indicating that copepods actually were somewhat less efficient at accumulating Fe from their diet when it was Fe-limited than if it was Fe-replete ($p = 0.028$, paired two-tailed *t*-test). The reason for this non-intuitive pattern varied depending on the algal food species. Copepods accumulated dietary Fe less efficiently when fed Fe-depleted *I. galbana* and *R. salina* because they excreted Fe more rapidly than when fed Fe-replete algae. In contrast, copepods fed Fe-depleted *T. oceanica* lost substantially less assimilated C to solution, resulting in lower Fe:C ratios than predicted when fed Fe-replete algae. Despite the large relative differences in respiration under Fe-replete and Fe-depleted diets, the effect of respiration on θ* was negligible because the absolute differences in respiration were small compared to differences in other parameters related to C assimilation. Kiørboe et al. (1985) reported daily C-specific respiration rates that were significantly higher than those reported here (~ 0.15 d^{-1}). If we have underestimated respiration rates, we could be underestimating the absolute differences in C loss among treatments. When we assume that respiration rates were 3-fold higher than we measured, θ* is still lower under Fe-depleted conditions than Fe-replete conditions, indicating that our results are robust to such errors.

In contrast to previous work suggesting strong stoichiometric homeostasis of tissue N and P contents in crustacean zooplankton, our measurements and model results indicate that *A. tonsa* has little if any ability to maintain stable tissue Fe:C ratios in the face of variations in the Fe of its food. The ability of *A. tonsa* to concentrate Fe from food varied by less than 50% between Fe-replete and Fe-depleted conditions (Table 2). By comparison, ratios of Fe:C in the algae varied by at least 30-fold among the Fe-replete and Fe-depleted treatments (Chen et

al. 2011a). Similar levels of variability in food Fe:C are likely to exist in the field depending on Fe availability (Moore et al. 2004, Boyd et al. 2007, Twining et al. 2011). As proper metabolic function depends on maintaining tissue Fe:C levels above a critical minimum value, the inability to maintain stoichiometric homeostasis makes *A. tonsa* particularly prone to mineral limitation by dietary Fe (Chen et al. 2011a). As *A. tonsa* predominately reside in coastal and estuarine environment, where Fe limitation is rare, uture work should determine the extent to which results from *A. tonsa* can be extrapolated to other copepod species. Such research would help determine if variations in Fe content of algae may be an important constraint on secondary production in the ocean.

Table 1. Cellular characteristics of food algae under Fe-depleted and Fe-replete conditions from Chen et al. (2011). Each value is the mean of three replicates. F_v/F_m = the ratio of variable fluorescence to maximum fluorescence, which is a measure of Fe stress (see text).

Algal species	Fe treatment	Cell volume (μm^3)	Growth rate (d^{-1})	F_v/F_m	Fe:C (μmol mol^{-1})
T. oceanica	Fe-replete	128±7	1.46±0.10	0.55±0.03	31.5±3.6
	Fe-depleted	78±4	0.74±0.06	0.20±0.004	0.6±0.5
R. salina	Fe-replete	128±10	1.27±0.09	0.72±0.04	23.5±3.5
	Fe-depleted	85±4	0.43±0.01	0.39±0.01	0.5±0.2
I. galbana	Fe-replete	46±3	1.21±0.06	0.65±0.03	36.7±5.1
	Fe-depleted	28±2	0.49±0.01	0.39±0.01	0.7±0.1

Table 2. Parameters used to calculate the efficiency of dietary Fe accumulation by *A. tonsa* fed three algal species grown under Fe-depleted and Fe-replete conditions. *IR* is the specific carbon ingestion rate (d^{-1}), AE_{Fe} is the assimilation efficiency of Fe (unitless), k_{Fe} is the excretion rate constant for Fe (d^{-1}), AE_C is assimilation efficiency of C (unitless), E_C is fraction of assimilated carbon lost to the dissolved phase during the depuration period (unitless), *R* is the specific respiration rate in terms of C (d^{-1}), *g* is the specific growth rate of copepods in terms of C (d^{-1}), θ* is the ratio at steady state of Fe:C in tissues of copepods to Fe:C in their algal food.

		IR	AE_{Fe}	k_{Fe}	AE_C	E_C	*R*	*g*	θ*
T. oceanica	Fe-depleted	0.71	0.53	0.23	0.89	0.55	0.06	0.22	0.82
	Fe-replete	0.75	0.47	0.26	0.90	0.75	0.03	0.13	0.90
R. salina	Fe-depleted	0.56	0.43	0.57	0.82	0.57	0.06	0.14	0.34
	Fe-replete	0.49	0.45	0.27	0.87	0.48	0.04	0.19	0.49
I. galbana	Fe-depleted	0.55	0.69	0.58	0.87	0.55	0.08	0.14	0.53
	Fe-replete	0.57	0.50	0.28	0.84	0.51	0.06	0.18	0.63

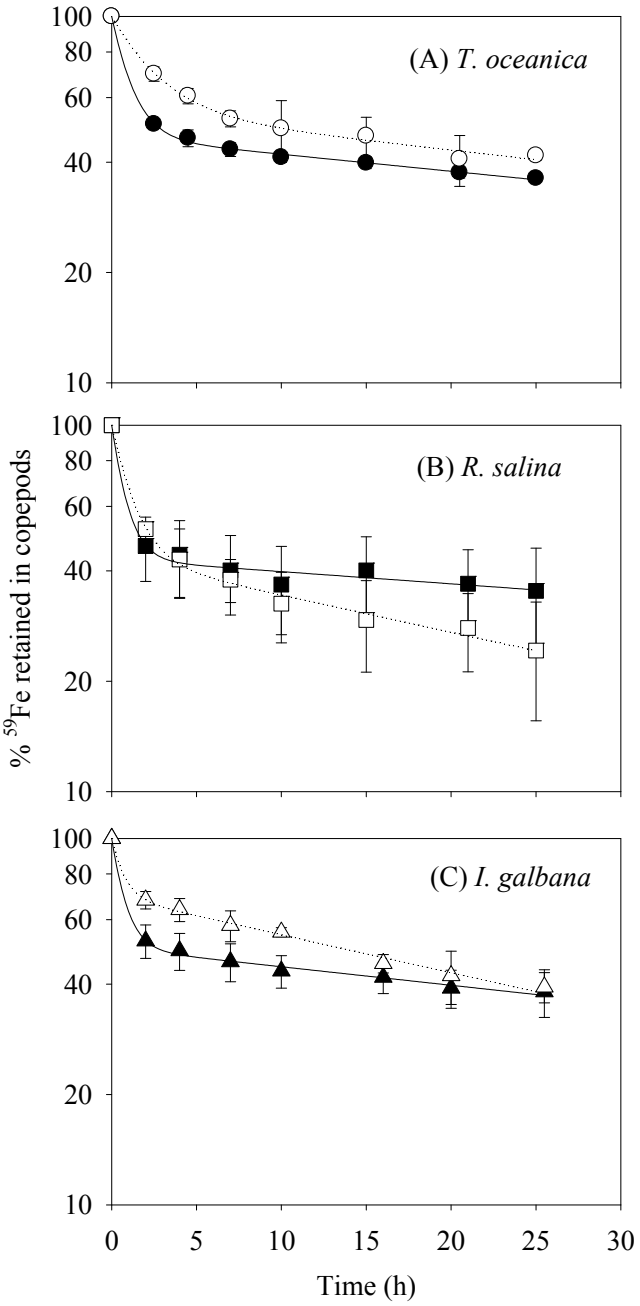


Fig. 1 Change in percent of ingested ^{59}Fe retained by *A. tonsa* fed Fe-depleted (open symbols) and Fe-replete (filled symbols) cells of (A) *T. oceanica*, (B) *R. salina*, and (C) *I. galbana* over 25 h depuration. Data points are means of 3 experiments of 50 copepods each ± 1 standard error (SE).

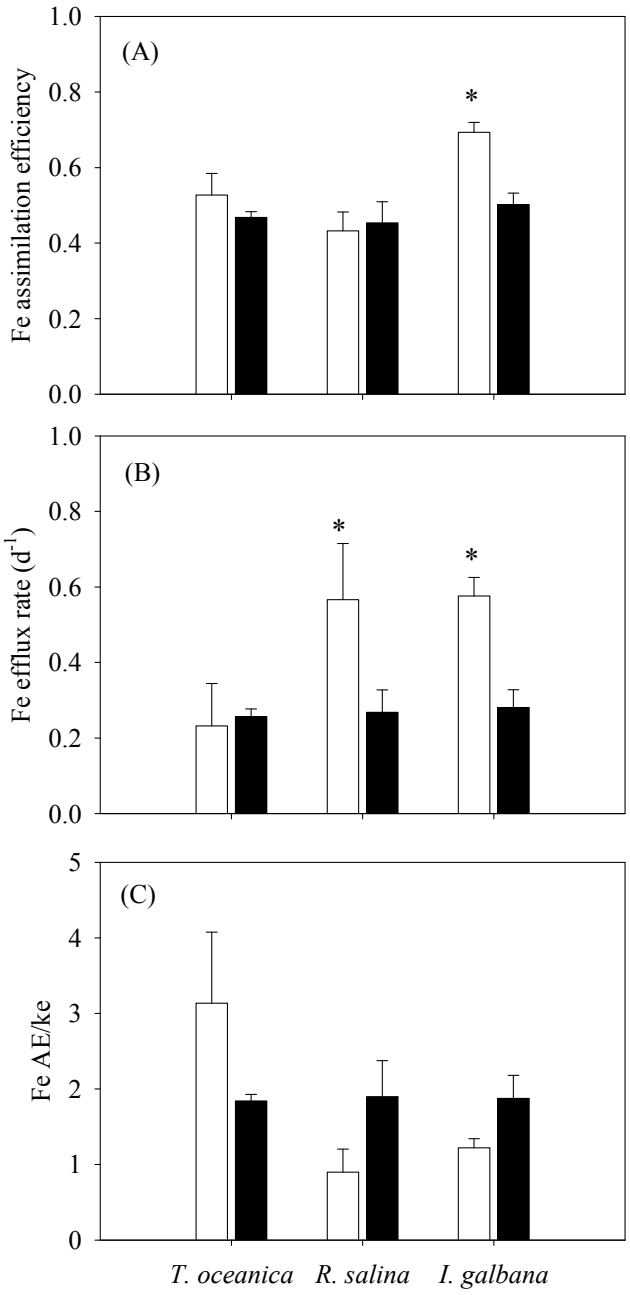


Fig. 2 The (A) assimilation efficiency (AE), (B) efflux rate (k_e), and (C) trophic transfer potential of Fe (AE/k_e) for *A. tonsa* fed Fe-depleted (open bars) and Fe-replete (filled bars) algae. Data are means of 3 experiments of 50 copepods each ±1 SE.

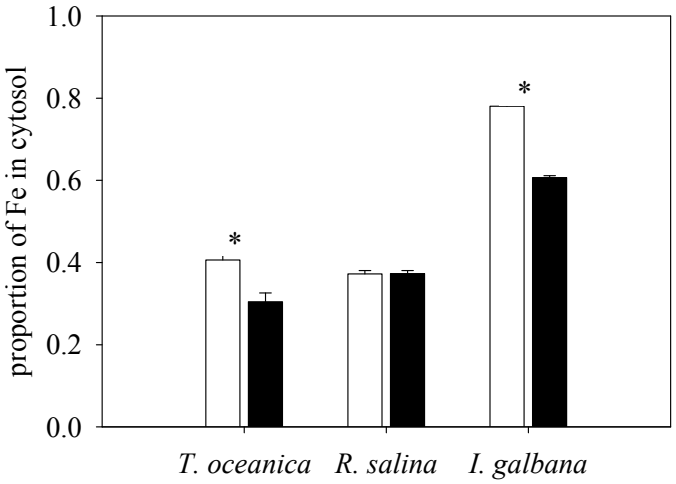


Fig. 3 The proportion of Fe located in the cytosol of each algal food species under Fe-depleted (open bars) and Fe-replete (filled bars) conditions. Data are means of 4 replicate batches of algae ± 1 SE.

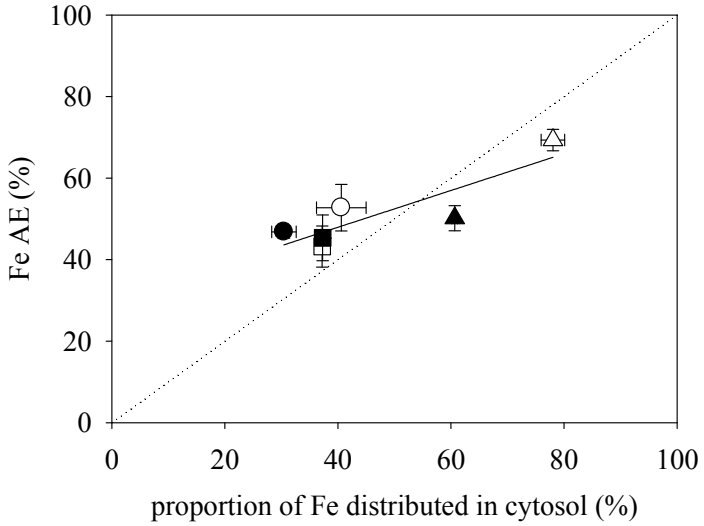


Fig. 4 The relationship of Fe AE (%) with the distribution of Fe in the cytosol (%) of algae *T. oceanic* (circle), *R. salina* (square), and: *I. galbana* (triangle) cultured under Fe-depleted (open symbols) and Fe-replete (filled symbols) conditions. The solid line represents the linear regression of the data with the equation y = 29.90 + 0.45 x (r^2 = 0.75. p = 0.02). The dotted line represents the 1:1 line.

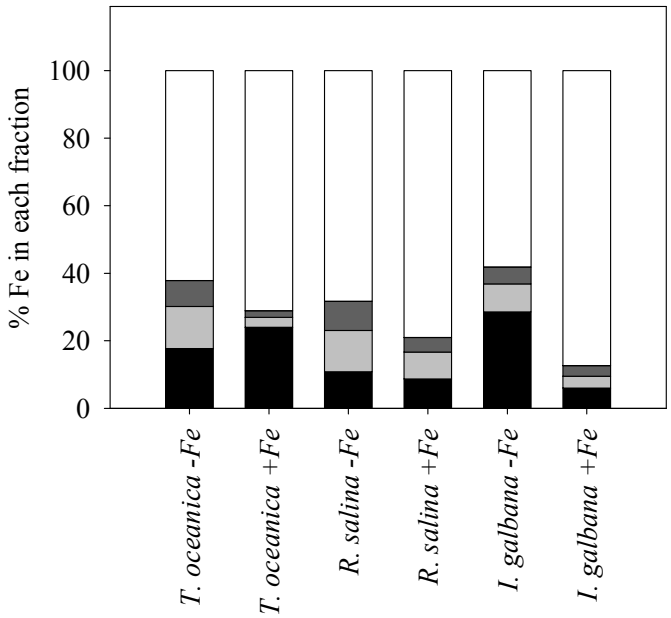


Fig. 5 Subcellular distribution of intracellular Fe within food algae under Fe-replete and Fe-depleted conditions. The four fractions in each bar from the bottom to top represent: (1) pellet 1 after the first centrifugation at 750 g for 5 min, (2) pellet 2 after the second centrifugation at 2,000 g for 15 min, (3) pellet 3 after the third centrifugation at 10,000 g for 15 min, and (4) the final supernatant containing cytosol. See text for content of various fractions. Data shown are means of 4 replicate batches of algae.

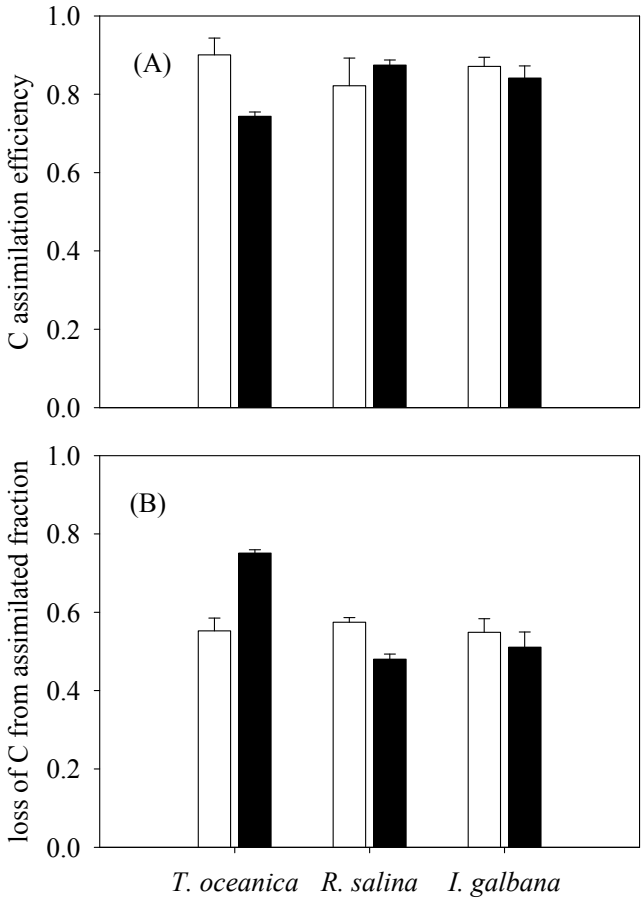


Fig. 6 The (A) AE of C, and (B) the proportion of C loss from the assimilated fraction after 5 h by *A. tonsa* fed Fe-depleted (open bars) and Fe-replete (filled bars) algae. Data are means of 4 replicate experiments of 50 copepods each ± 1 SE.

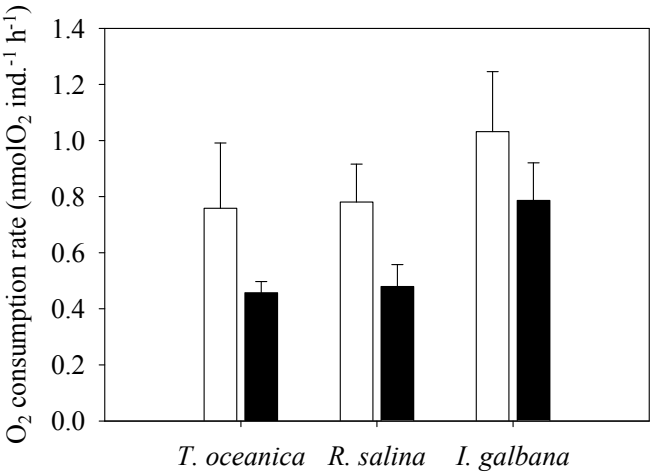


Fig. 7 The O_2 consumption rates of *A. tonsa* fed Fe-depleted (open bars) and Fe-replete (filled bars) algae. Data are means of 4 replicate experiments of 30 copepods each ± 1 SE.

Chapter 5

Iron limitation of a copepod common to HNLC regions

Abstract

Egg production of *Acartia tonsa*, an estuarine copepod species that almost never experiences Fe limited conditions in the field, has been shown to be influenced by the Fe content of its food. In this study, we examined whether egg production of the copepod *Calanus pacificus*, a species which experiences Fe limitation in its natural environment, also varies with the Fe content of its algal diet. In laboratory experiments, *C. pacificus* was fed a fixed ration of each of three algal species grown under Fe replete and Fe deplete conditions for up to 9 days. When fed on the diatom *Thallasiosira weissflogii, C. pacificus* produced up to 8 eggs ind.$^{-1}$ d^{-1} on an Fe-replete diet, however no eggs were produced when *C. pacificus* was fed Fe-depleted *T. weissflogii*. This result was reversible within 1 day after the Fe content of the diet was changed from high to low Fe, or low to high Fe. Differences in clearance and ingestion rates among the three algal food species could be attributed to differences in cell size of the food algae ($r^2 = 0.89$). The low ingestion rate of *C. pacificus* on the smaller algal species *Rhodomonas salina* and *Thalassiosira oceanica* resulted in low or zero egg production. Despite experiencing low Fe conditions within its natural ecological range, it appears that *C. pacificus* is also prone to limitation by dietary Fe, much like *A. tonsa*. The results of this study suggest that Fe limitation of marine zooplankton may be more common than previously considered, and highlight the need for field studies of zooplankton dietary constraints in Fe-deficient systems.

Introduction

Iron is a limiting factor for primary production in over 30% of the world's ocean (Martin et al. 1991, Hutchins et al. 1998, de Baar et al. 2005). Phytoplankton in these Fe-limited regions exhibit Fe contents low enough that dietary Fe content, rather than assimilated energy, may control growth and reproduction of copepods and other zooplankton. Indeed, reproduction and naupliar survivorship of the coastal marine copepod *Acartia tons*a was reduced when fed Fe-limited phytoplankton (Chen et al. 2011a). However, *A. tonsa* is an Atlantic coastal and estuarine species and, as such, rarely if ever experiences Fe limitation in its natural range. Natural selection on species that do experience Fe-limitation could result in lower Fe requirements or more efficient assimilation and retention of dietary Fe, making such species less prone to Fe limitation. Thus, the extent to which Fe limitation of copepods can be generalized from the experiments with *A. tonsa* remains uncertain.

By comparison, the copepod *Calanus pacificus* is an excellent model organism for use in experiments on Fe limitation of copepods. *C. pacificus* is widely distributed in the north Pacific, including the Gulf of Alaska and the northern California upwelling system (Bucklin and LaJeunesse 1994), where Fe limitation has been observed (Martin et al. 1991, Hutchins and Bruland 1998). It is also a dominant copepod species off the coast of southern California, where it exhibits active egg production all year (Mullin 1991, Ohman 1998). As a consequence, reproductively viable individuals are widely and consistently available for use in experiments. In addition, *C. pacificus* is an active suspension-feeder whose reproductive rate is tightly correlated with food conditions (Runge 1985) and responsive to dietary changes within 24 hours (Uye 1996), making it a good candidate for laboratory studies.

We used *C. pacificus* females collected from the southern California Current System (sCCS) in the present study. The sCCS is a region of complex hydrography south of Point Conception, California, where low concentrations of dissolved Fe concentrations (< 0.1 nmol L^{-1}) lead to areas of low chlorophyll (< 1 μg L^{-1}) and significant surface concentration of nitrate (< 4 μmol L^{-1}) (King and Barbeau 2007). On the southern border of the sCCS, the coastal waters off La Jolla had higher concentration of dissolved Fe (0.5-4.5 nmol L^{-1}) at the time of our study. However, the copepods existing there are mixed widely with those from Fe-limited areas to the north and offshore. Subpopulations of the subspecies *C. p.*

californicus from Oregon, California and Japan cannot be distinguished using mitochondrial DNA haplotypes, suggesting a large degree of genetic exchange between these regions (Nuwer et al. 2008). Thus, we expect that responses to low Fe food by individual *C. pacificus* collected near La Jolla will resemble those of copepods more commonly exposed to Fe limitation further north in the California Current (e.g. Hutchins et al. 1998).

In this study, we replicated previously reported experiments with *A. tonsa* (Chen et al. 2011a) using *C. pacificus* collected from the wild. The main goal of the study was to determine if variations in Fe content of food influence the ability of *C. pacificus* to produce eggs. In addition, we address the effect of algal food species and food size on our results. Finally, we discuss the implications of our results for patterns of secondary production in the ocean.

Materials and methods

In our experiments, the copepod diet consisted of one of three species of algae, *Thalassiosira weissflogii, T. oceanica* and *Rhodomonas salina. T. oceanica* and *R. salina* were successfully used in a previous study where lowered egg production in *A. tonsa* was related to low Fe content of food. The diatom *T. weissflogii* was selected due to its larger biovolume, as previous studies indicated a preferential feeding of *C. pacificus* on larger algal cells (Frost 1972). Culture media were made in chelexed (Price et al. 1988/1989) 0.1-µm filtered seawater collected from the pier of the Scripps Institution of Oceanography enriched with f/2 levels of the macronutrients nitrate, phosphate and silicate (Guillard and Ryther 1962). A trace metal solution containing ethylenediaminetetraacetic acid (EDTA) was added to achieve final concentrations in the media of 0.1 mmol L^{-1} EDTA, 50 nmol L^{-1} $MnCl_2$, 40 nmol L^{-1} $CuCl_2$, 100 nmol L^{-1} $ZnCl_2$, 40 nmol L^{-1} $CoCl_2$ and 100 nmol L^{-1} $NiCl_2$, as described by Sunda and Huntsman (1995). Media were either amended with $FeCl_3$ at a final total dissolved Fe concentration of 100 nmol L^{-1} (High Fe treatment) or not (Low Fe treatment).

The Fe-replete or Fe-depleted media were both inoculated with a similar density of algal cells from stock cultures. The total concentration of dissolved Fe in the Fe-depleted media after inoculation was <1 nmol L^{-1}, calculated from the carryover Fe from the stock

culture. Cultures were maintained at 17 °C on a 14:10 hour light:dark cycle with 105 μmol quanta m^{-2} s^{-1} illumination provided by cool-white fluorescent lamps. The phytoplankton cell densities and sizes were determined daily by an Elzone® II 5390 Particle Size Analyzer (Micromeritics, GA). The Fe-replete and Fe-depleted cells were harvested after 6-8 days and 10-12 days of growth, respectively, for copepods feeding before they entered stationary growth phase. To assess the degree of physiological Fe stress experienced by the cells, the quantum yield of fluorescence, F_v/F_m, was also measured at the beginning of feeding (Geider 1993).

Zooplankton samples were collected at 150-m depth 1.5 km off La Jolla using 200-µm Nitex net. Female adults of *C. pacificus* were sorted under a dissecting microscope. Three replicate 200-ml batches of algal suspension (containing 500 µg C L^{-1}) held in acid-washed flasks each received 5 individual female copepods for feeding. The algal carbon contents of their diets were calculated from their biovolume (Menden-Deuer and Lessard 2003). Food suspensions were replaced every 24 hours, and algal cell densities and biovolumes before and after feeding were counted with the particle analyzer. Copepods and eggs and feces were separated from the old food suspension using 500-µm and 60-µm Nitex screens. The number of living copepods and eggs produced were counted under a dissecting microscope. After 7 days of feeding, copepods that had been fed Fe-replete and Fe-depleted *T. weissflogii* and *R. salinia* were switched from high Fe food to low Fe food, and vice versa, and allowed to feed for an additional 2 days.

The significance of differences in egg production rate between Fe treatments was analyzed by the Mann-Whitney Rank Sum Test because the data were not normally distributed. The significance of differences in ingestion rate was analyzed by a *t*-test or Mann-Whitney Rank Sum Test, depending on data normality.

Results

Growth of *R. salina* and *T. weissflogii* started to show signs of limitation after 3 days of culture in Fe-depleted media, *T. oceanica* after 5 days of growth (Fig. 1). Cells were harvested for food before entering stationary growth phase, which was about 6-8 days after the initiation of growth for Fe-replete conditions, and 10-12 days for Fe-depleted conditions.

Because *T. oceanica* and *T. weissflogii* had higher growth rates than *R. salina* and entered stationary phase earlier, the diatoms were harvested for food prior to *R. salina*. Quantum yield efficiencies (F_v/F_m) of Fe-depleted cells (0.28-0.61, Table 1) before copepod feeding were about half of the Fe-replete cells (0.70-0.89). Feces produced from copepods fed Fe-depleted algae were lighter in color, indicating lower pigment content (Fig. 2).

Algal biovolume did not show a consistent difference between Fe-replete and Fe-depleted cells. Fe-replete *T. oceanica* and *R. salina* cells had higher biovolumes (range 130-170 μm^3 for *T. oceanica*, and 148-171 μm^3 for *R. salina*) than Fe-depleted cells (range 75-94 μm^3 for *T. oceanica*, and 113-157 μm^3 for *R. salina*), but this was not the case for *T. weissflogii* (range 1179-1353 μm^3 for Fe-replete *T. weissflogii* cells, and 1034-1603 μm^3 for Fe-depleted cells).

After 3 days of feeding, copepods given an Fe-replete *T. weissflogii* diet produced significantly more eggs (Mann-Whitney Rank Sum Test, $p < 0.001$) than those fed Fe-depleted *T. weissflogii* or the two other algal species. Egg production per individual reached 8 eggs day^{-1} after 7 days of feeding (Fig. 3). The daily egg production rate of copepods ingesting Fe-depleted *T. weissflogii* dropped from 2 to 0 after the fourth day of the experiment (Fig. 3). After copepods were switched from an Fe-depleted to an Fe-replete *T. weissflogii* diet, egg production increased within 1 day. Conversely, egg production of copepods that had fed on Fe-replete cells dropped to close to 0 within 1 day when their diet was changed to Fe-depleted *T. weissflogii* (Fig. 3).

For copepods feeding on *R. salina*, the average egg production in Fe-depleted treatments in the first 7 days was a little higher than for an Fe-replete diet, opposite to what was shown in the *T. weissflogii* experiment (Mann-Whitney Rank Sum Test, $p = 0.017$, Fig. 4), but the egg production rates for both Fe culture treatments dropped to 0 on the 7th day. Copepods that were later switched between Fe-depleted and Fe-replete *R. salina* diet resumed production a few eggs $ind.^{-1}$ d^{-1}, but the increases were small. Egg production rates of copepods feeding on a *T. oceanica* diet are not shown in the figure because no eggs were produced with this diet throughout the experiment, regardless of Fe enrichment.

Copepod ingestion rates differed significantly among algal diets ($p < 0.001$) with highest rates for *T. weissflogii* (7.5-12.1 μg C $ind.^{-1}$ d^{-1}), lowest rates for *T. oceanica* (2.0-5.7

μg C ind.$^{-1}$ d^{-1}), and intermediate rates for *R. salina* (2.9-6.7 μg C ind.$^{-1}$ d^{-1}, Fig. 4). Ingestion rates differed modestly but significantly during the first 7 feeding days between Fe-replete and Fe-depleted diets for *T. weissflogii* (*t*-test, $p = 0.017$) and *T. oceanica* (*t*-test, $p = 0.011$), but no difference was observed between Fe-replete and Fe-limited *R. salina* diets (Mann-Whitney Rank Sum Test, $p = 0.352$).

Discussion

Our data indicate that egg production of the copepod *C. pacificus* differed when fed *T. weissflogii* that was grown under Fe-replete and Fe-depleted conditions. Previous studies have indicated that phytoplankton grown under the different culture conditions varied in their Fe:C ratios by 53-fold (Chen et al. 2011a). In the present study, the Fe status of food algae was seen as differences in growth rates and F_v/F_m values of cells grown under the different Fe conditions. The different colors of copepod fecal pellets produced from feeding on algae grown under the different Fe conditions (Fig. 2) reflected the lower chlorophyll concentrations in Fe-depleted algal cells, a further indication of algal Fe limitation. Given that Fe:C ratios in Fe-deficient phytoplankton are low enough to limit copepod growth (Sunda and Huntsman 1995, Chen et al. 2011a), it seems likely that the differences in egg production response resulted from differences in the Fe contents of the *T. weissflogii* cells.

Algal cell size played a large role in the responses of *C. pacificus* to different algal food species. Clearance rates of *C. pacificus* in this study were positively related to algal cell biovolume (Fig. 5, $r^2 = 0.89$). Similarly, Frost (1972) found a positive linear correlation between log diatom cell biovolume and the clearance rate of *C. pacificus* when ingestion rates were below maximal values. The slopes of the relationship between clearance rate and algal cell biovolume in the two studies were essentially identical, although the rates for our study were higher, a difference which likely reflects our higher experimental temperatures (~19°C compared to 12.5°C). While cell size differed between Fe-depleted and Fe-replete *R. salina* and *T. oceanica*, these differences did not result in significant effects on clearance rate. Interestingly, copepods fed somewhat less efficiently on Fe-limited *T. weissflogii* even though they did not differ significantly in size from Fe-replete cells. It is possible that this reflects the reduced physiological condition of copepods feeding on Fe-poor food, or more

subtle differences in morphologies or chemical cues of diatoms grown under Fe-replete and Fe-depleted conditions. At present, we are not able to discriminate among these hypotheses.

Size related differences in ingestion rate probably explain most of the variation in egg production among algal food species used here. Egg production was undetectable when *C. pacificus* was fed the smallest species, *T. oceanica*, and low when fed the slightly larger *R. salina*. In a pilot study, female *C. pacificus* fed an even smaller alga, the prymnesiophyte *Isochrysis galbana*, also stopped producing eggs (data not shown). While Fe-treatment differences in egg production rates were rather inconsequential for the smaller food species used in these experiments, the results clearly establish that *C. pacificus* cannot benefit directly from the smaller class of cells that should be competitively superior in taking up Fe under Fe-limiting conditions. Only when the larger diatom *T. weissflogii* was used as food were egg production rates substantial. This pattern is consistent with results from related copepod species of similar size. Becker et al. (2005) showed that the RNA:DNA ratio, an indicator of animal growth and fecundity, of *C. finmarchicus* was positively correlated with the concentration of food particles with biovolumes larger than 1000 μm^3, but negatively correlated with those smaller than 1000 μm^3, indicating a preference of *C. finmarchicus* for food particles > 1000 μm^3. Among the three algal species used in our study, *T. weissflogii* is the only one > 1000 μm^3, while the other two are much smaller (75-171 μm^3). While egg production rates of *C. pacificus* were substantial when fed on Fe-replete *T. weissflogii*, they approached zero on a diet of Fe-depleted *T. weissflogii*. This occurred despite the fact that the copepods ingested Fe-limited *T. weissflogii* cells at only a marginally lower rate than Fe-replete cells.

Egg production rates observed here for *C. pacificus* are lower than have been reported elsewhere because ingestion rates of *T. weissflogii* were constrained by the amount of algae initially added to the flasks. Egg production rates in this study reached about 13 eggs $ind.^{-1}$ d^{-1}. By comparison, Ohman (1998) found maximum egg production rates of 40 eggs $ind.^{-1}$ d^{-1} for *C. pacificus* females feeding on a mixed natural assemblage of microplankton sampled near San Diego. The values were even higher (80 eggs $ind.^{-1}$ d^{-1}) when the natural plankton was supplemented with culture-grown *T. weissflogii*. In our experiments, the initial carbon concentration after daily replenishment of the algal food (500 $\mu g\ C\ L^{-1}$) exceeded the

threshold (300 μg C L^{-1}) for maximum ingestion rates for *C. pacificus* feeding on a diatom of similar size to *T. weissflogii*, *T. fluviatilis* (Frost 1972). However, such saturation of ingestion rates would only have been transient. Given an average clearance rate of 4.5 ml $ind.^{-1}$ h^{-1}, five individuals would have reduced the concentration of organic carbon below the 300 μg C L^{-1} after only 4.5 h, while final concentrations were <10% of initial values. Were *T. weissflogii* concentrations held constant above this value for the entirety of the 24-h feeding period, ingestion would probably have been at least 3-fold greater than the maximum rate observed during these experiments.

Food quality factors other than Fe content played a minor role in determining egg production in our experiments. *R. salina* contains slightly higher composition of polyunsaturated fatty acids (61.0-68.9%) than does *T. oceanica* (49.6-57.6%, Chen et al. 2011b), especially 18:4ω3 (10.5-16.6% in *T. oceanica* vs. 22.3-25.7% in *R. salina*) and 22:6 ω3 (4.3-9.4% in *T. oceanica* vs. 9.3-13.7% in *R. salina*). In addition, the siliceous cell wall of *T. oceanica* is also likely to cost copepods more energy for digestion. Differences in nutritional quality and toxin content may explain why some other studies have found that copepods exhibit lower egg production and egg viability when fed certain diatoms species, including *T. weissflogii* (e.g. Uye 1996, Pierson 2005). Nutritional factors could explain why copepods fed *R. salina* exhibited higher egg production and ingestion rates than did copepods fed the similarly sized *T. oceanica*, although this difference is also consistent with a higher capture rate for the more elongate cryptophytes. In any case, the possible effect of food quality on egg production was negligible compared to the effect of algal cell size as *C. pacificus* fed *T. weissflogii* produced significantly more eggs than those fed *R. salina* ((Mann-Whitney Rank Sum Test, $p = 0.021$), which is not toxic and contains essential nutrients, and typically supports robust reproduction of *Acartia tonsa*.

The exact mechanism by which dietary Fe influences *C. pacificus* reproduction is unknown. The specific roles that Fe plays in zooplankton biochemistry, and the physiological effects of Fe limitation are not well studied in this and other zooplankton. The most important role of Fe in heterotrophs generally is the respiratory electron transport chain (Frausta da Silva and Williams 1991, Chase and Price 1997). The energy cost associated with biosynthesis of new material, including growth and reproduction, accounts for most of

the increase in respiration related to feeding (the specific dynamic action, SDA, Kiørboe et al. 1985). Thus, reduced production of electron transport system (ETS) proteins under Fe limitation could result in less energy available to support biosynthesis of ovaries and eggs, especially as these are disproportionately composed of energy-dense lipids (Chen et al. 2011a). Studies of other arthropods suggest that production of eggs may entail an additional Fe requirement specific to egg production. For the insect *Sarcophaga peregrine*, oocytes take up the Fe transport protein transferritin from hemolymph during oogenesis (Kurama et al. 1995). If production of this vitellogenic protein is constrained by supply of dietary Fe, it could have immediate consequences for development of eggs. For *Drosophila melanogaster*, the Fe storage protein ferritin is actively synthesized in female adult ovaries and largely stored in the eggs for use during embryo development, especially when the diet is supplemented with Fe (Georgieva et al. 2002). Although no similar studies have been done for copepods, these other studies hint that arthropods may have specific and significant Fe requirements for egg development.

Our results are reminiscent of previous studies using *A. tonsa* in that Fe status of algal food significantly affected reproduction of copepods fed diatoms as food. However, *C. pacificus* differs from *A. tonsa* in that it is common in both coastal and open ocean regions of the temperate and subarctic north Pacific where Fe limitation of phytoplankton growth is known to occur. Indeed, *C. pacificus* may encounter physiologically Fe-limited phytoplankton within the southern California Current ecosystem that we sampled (King and Barbeau 2007) as well as further north off California and Oregon (Hutchins et al. 1998). *C. pacificus* collected from these regions are genetically indistinguishable from one another, and indeed from coastal populations on the western Pacific (Nuwer et al. 2008), suggesting that the coastal forms at least comprise a mixed population. Consequently, we would expect that *C. pacificus* is likely to respond to Fe-limited food in these other regions similar to the responses observed here for *T. weissflogii*. As *C. pacificus* is commonly a dominant member of these zooplankton communities, such limitation could have large implications for the spatial and temporal variability in secondary production within the California Current system and its associated upwelling centers.

How broadly such effects can be extrapolated to copepods existing in more

chronically Fe-limited habitats remains an interesting question for future research. Copepods can hypothetically sidestep problems associated with low Fe content of food by having lower requirements, by adjusting assimilation and retention of dietary Fe and C, by behaviors such as selective feeding on high Fe particles, or by altering vertical migratory behaviors in ways that optimize their zonation along the coast (e.g. Peterson et al. 1979) with respect to Fe-deficient areas. However, some of these adaptations to low Fe food may be associated with reductions in performance evident under non-Fe limiting conditions. Coastal populations of *C. pacificus* encounter Fe-limited algae only sporadically, and over spatial scales that are small compared to potential scales of genetic exchange. Consequently, the apparent lack of any adaptation to low Fe food in *C. pacificus* may reflect the predominance of selection for traits that are favorable under non-Fe limiting conditions. It would be interesting to compare responses of coastal *C. pacificus* to those from the open subarctic Pacific, a region of extensive and predictable seasonal Fe limitation that is home to a genetically distinct population of *C. pacificus*. Such comparisons would be more fruitful if more were known about the mechanisms by which Fe-limitation affects copepod biology. Future molecular analysis on copepods analogous to that on insects could provide helpful information on the direct roles that Fe may play in egg production, in addition to respiration and the accompanying energy generation. Such studies would provide a more focused assessment of the tradeoffs and challenges associated with adapting to Fe-limited conditions.

Table 1. Cell biovolumes and F_v/F_m values for the phytoplankton species -- *Thalassiosira weissflogii*, *T. oceanica* and *Rhodomonas salina* – fed to *Calanus pacificus* in this study. Data for cell biovolumes are shown as ranges; data for F_v/F_m are shown as means of 3 replicates ± standard deviations (SD).

		cell volume (μm^3)	F_v/F_m
T. weissflogii	Fe-replete	1179 - 1353	0.74 ± 0.04
	Fe-depleted	1034 - 1603	0.29 ± 0.01
T. oceanica	Fe-replete	130 - 170	0.72 ± 0.02
	Fe-depleted	75 - 94	0.31 ± 0.02
R. salina	Fe-replete	148 - 171	0.83 ± 0.06
	Fe-depleted	113 - 157	0.62 ± 0.01

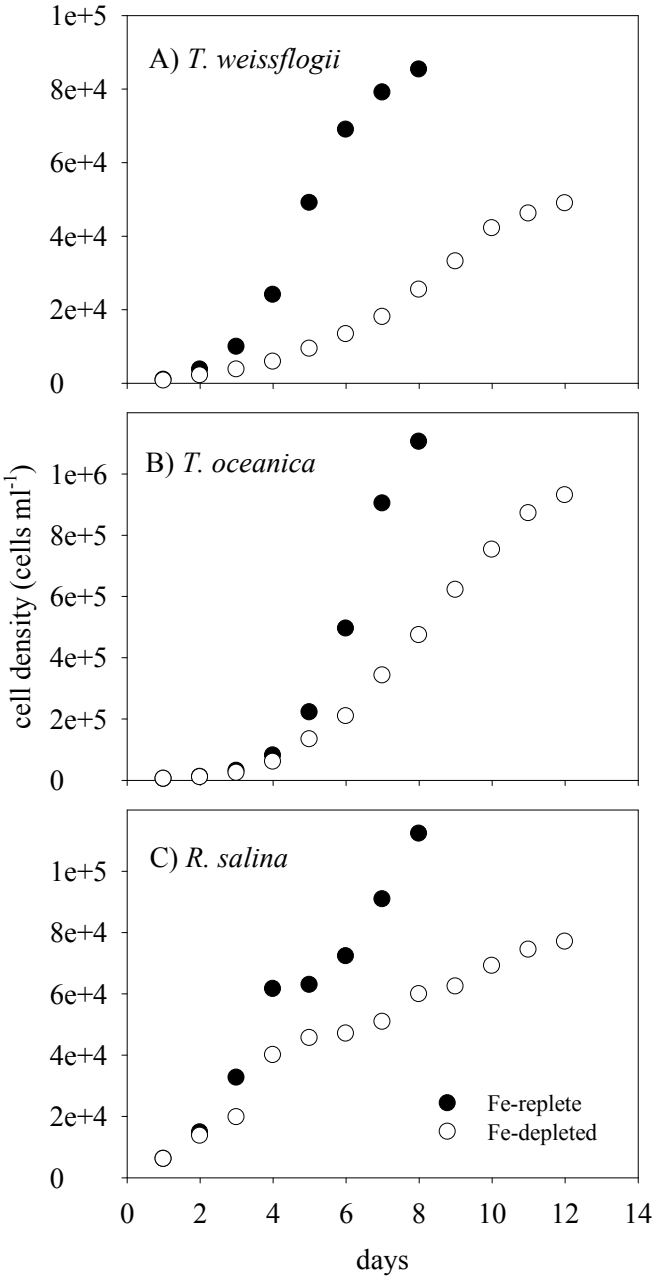


Fig. 1. Growth rates of algae cultured under Fe-replete (filled symbols) and Fe-depleted (open symbols) conditions. Cells were cultured into stationary phase for pinpointing the late log phase/stationary phase transition, and the cells used as diets were cultured in separate batches and were harvested in log phase.

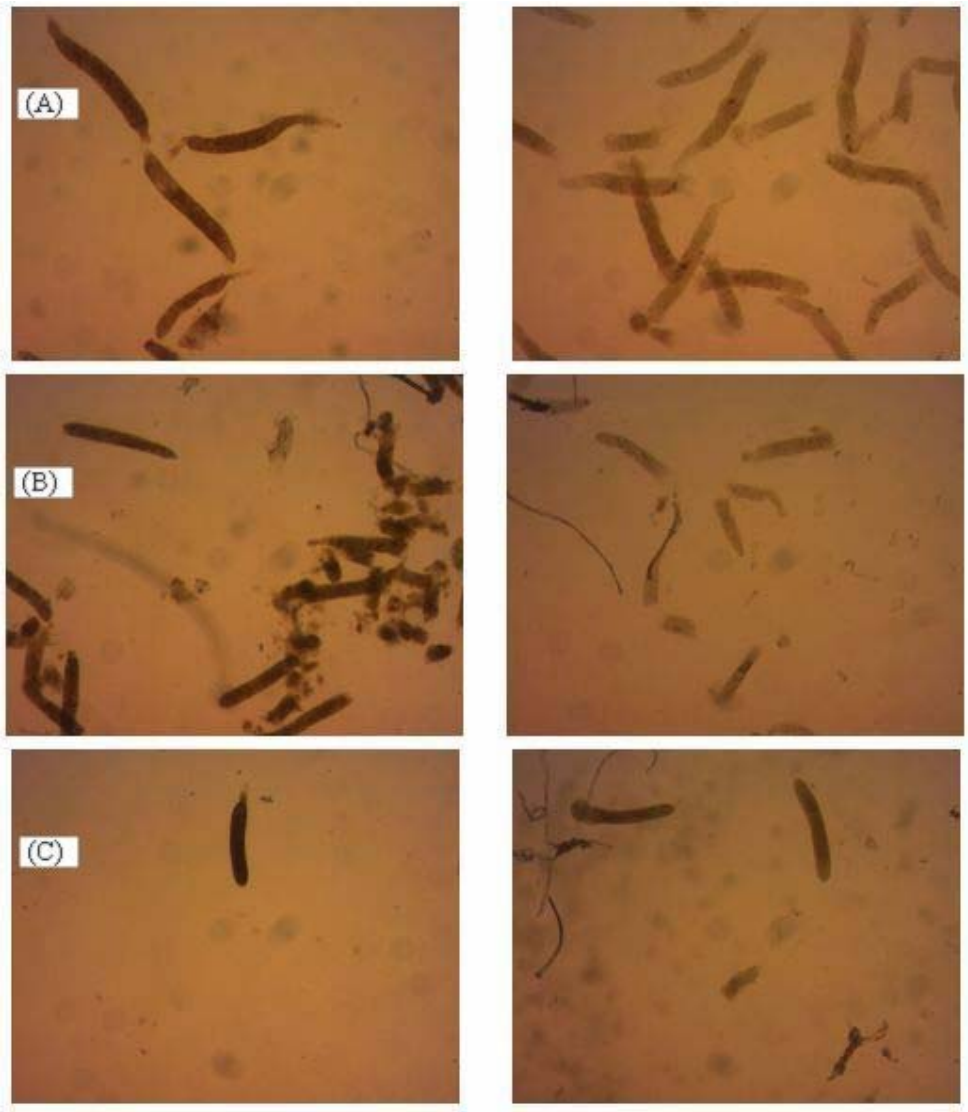


Fig. 2. Images of fecal pellets of *Calanus pacificus* feeding on (A) *Thalassiosira weissflogii*, (B) *T. oceanica*, and (C) *Rhodomonas salina* cells. The left side images show Fe-replete treatments; the right side images represent Fe-depleted treatments.

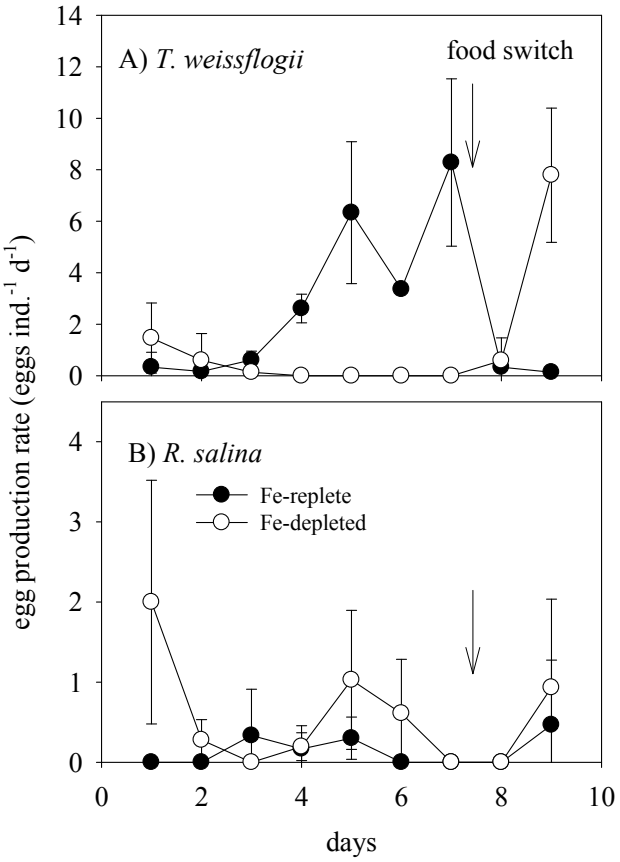


Fig. 3. Egg production rates of *Calanus pacificus* feeding on Fe-replete (filled symbols) and Fe-depleted (open symbols) cells of (A) *Thalassiosira weissflogii* and (B) *Rhodomonas salina* over a 7-d period. Diet switch between Fe treatments occurred after 7 days of the initial feeding (shown as the downward arrows). Data points are means of 3 replicates ± 1 SD.

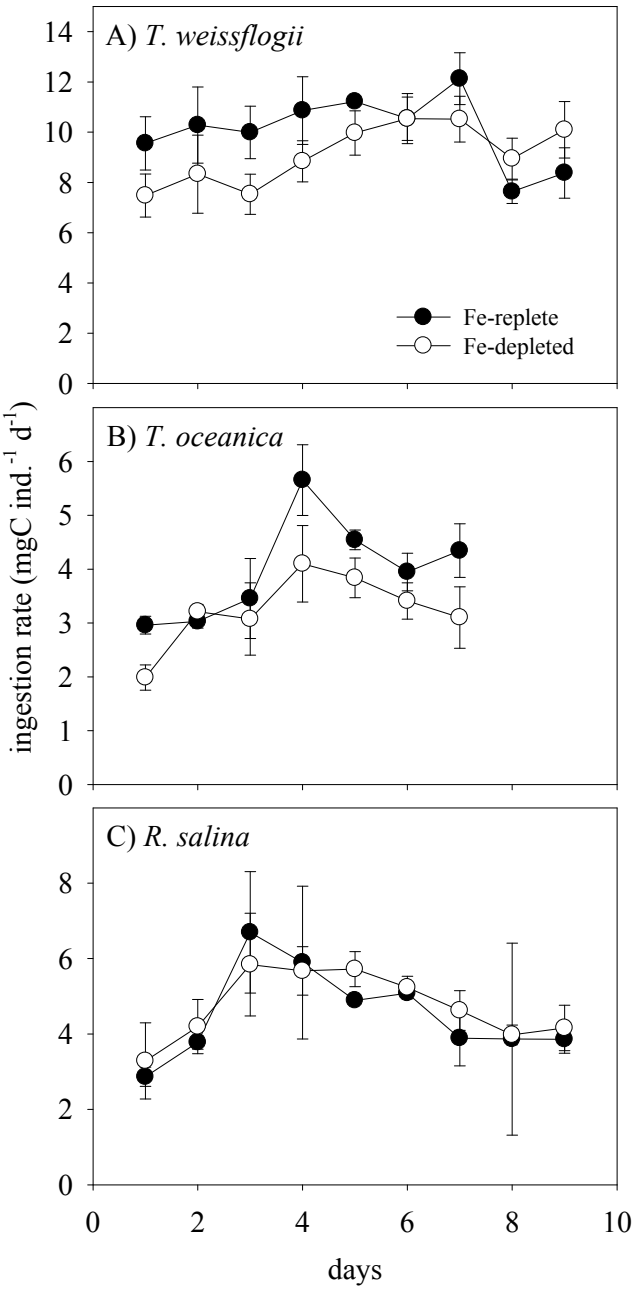


Fig. 4. Ingestion rates of *Calanus pacificus* feeding on Fe-replete (filled symbols) and Fe-depleted (open symbols) cells of (A) *Thalassiosira weissflogii*, (B) *T. oceanica*, and (C) *Rhodomonas salina* over a 7-d period. Data points are means of 3 replicates ± 1 SD.

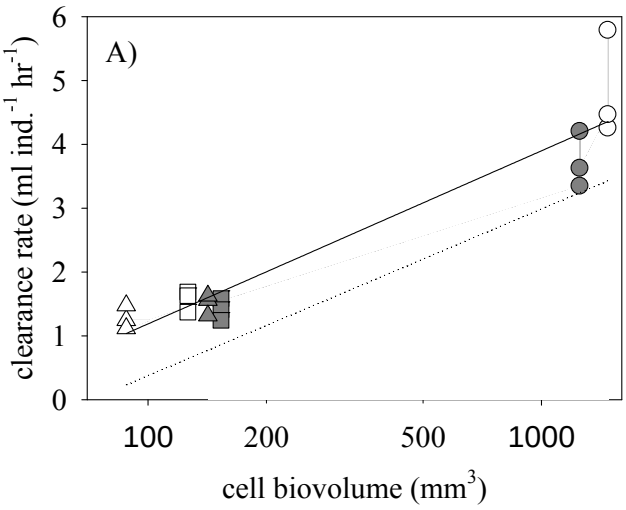


Fig. 5. Relationship between clearance rates of *Calanus pacificus* and algal cell biovolume. Each data point represents the average algal cell size and copepod clearance rate from day 1 to day 7 in one replicate feeding container. The regression (solid line) has the best linear fit with the equation: y = -4.234+ 1.177 ln x (r^2 = 0.89, p < 0.0001). The dotted line represents the relationship between clearance rate and cell biovolume from Frost (1977).

Chapter 6

Elemental stoichiometry and RNA:DNA ratios of zooplankton in the Costa Rica Upwelling Dome

Abstract

We measured the tissue concentrations of the nutrient elements carbon, nitrogen, phosphorus, iron, zinc and the ratios between these elements in zooplankton samples collected from Costa Rica Upwelling Dome (CRD). The sampling location ranged between 87.4°-92.9° W and 6.6°-9.6° N, and has been identified as a High-Nutrient Low-Chlorophyll (HNLC) region, with low dissolved Fe and Zn concentrations in the water. Zooplankton samples were collected at different hours and fractionated into 4 groups by size: 0.2-0.5, 0.5-1, 1-2 and 2-5 mm. The average Fe and Zn concentrations in zooplankton were 1579 and 566 nmol g^{-1} dry weight, respectively. The average C:N:P was 123:26:1. Carbon, N and Fe concentrations showed a pattern of decrease with zooplankton size, while P and Zn concentrations increased with zooplankton size. Concentrations of all elements showed a strong diel pattern in the large size group (2-5 mm), with C, N and P concentrations having the lowest values at mid-day, while Fe and Zn concentrations showed the opposite. The average RNA:DNA ratio was 2.14 for all samples and showed no variation with size and sampling time of a day. Concentrations of all elements, especially Fe and Zn, as well as RNA:DNA ratios were generally low compared to zooplankton in other regions. Results of this study suggest that secondary production in the CRD may be limited by Fe and Zn.

Introduction

Secondary production by marine zooplankton is generally considered to be limited by food availability in High Nutrient Low Chlorophyll (HNLC) areas. An *in situ* Fe enrichment experiment showed that adding Fe increased algal abundance and enhanced zooplankton feeding (Rollwagen Bollens and Landry 2000), suggesting an energy limitation of zooplankton in HNLC areas. However, it is also possible that zooplankton production is directly affected by low Fe intake from food. Our laboratory experiments on two model copepods *Acartia tonsa* and *Calanus pacificus* both showed that zooplankton production can be reduced directly by feeding on Fe-deficient food (Chen et al. 2011a, Chapter 5). Whether similar limitation of zooplankton production takes place in HNLC regions remains unstudied.

Mesozooplankton are believed to maintain constant tissue elemental concentrations of N and P through post-absorptive homeostatic regulation (Sterner and Elser 2002). Whether this idea applies to trace metals as well is an open question. For example, in pulse chase radioisotope experiments, the copepod *A. tonsa* did not retain Fe more efficiently when fed Fe-limited algal food (Chapter 4). Similarly, freshwater zooplankton failed to maintain a constant Zn concentration when the Zn content in seston was below 68 μg gdw^{-1}, a possible limiting condition and above 272 μg gdw^{-1}, a possible toxic condition (Zauke et al. 1998, Sterner and Elser 2002). To better understand the true ability of zooplankton to maintain homeostasis with respect to trace elements, studies on the trace metal contents in zooplankton from waters of different trace metal concentrations are necessary. Few studies have measured the concentration of Fe, the most widely limiting trace metal for marine production, in zooplankton from natural waters in addition to Martin and Knauer (1973), Fowler (1977), and Masuzawa et al. (1988). The content of another possible limiting trace metal, zinc, in zooplankton has been somewhat more studied, particularly in polar and subpolar regions (Zauke et al. 1996, Ritterhorf and Zauke 1997, Kahle and Zauke 2003), but little attention was given to the extent to which Zn limits zooplankton in these waters. Trace element concentrations and requirements could vary with body size of zooplankton. Most previous work has focused on larger mesozooplankton species, as they are easy to sort. However, small zooplankton may have elemental contents that differ from larger ones. For example, they may contain some juvenile zooplankton which have higher nutrient requirements

compared to adults (Boersma and Kreutzer 2002). Also, zooplankton of smaller size may feed more exclusively on organisms at the base of the food web, which can be strongly depleted in Fe and Zn. Larger zooplankton often migrate to deep water during the day to avoid predation and return to the surface water at night for feeding (Zaret and Suffern 1976, Baars and Oosterhuis 1984). This behavior may allow larger zooplankton to exploit resources at depth where trace element availability and trace element content of food is likely to be higher than in surface waters, where trace elements like Zn and Fe are often depleted (Boyd and Ellwood 2010). Depending on the swimming ability and the strategy applied by different animals balancing the tradeoffs between feeding and predator avoidance, samples collected at different depths and times of day may have very different zooplankton composition, which may further result in different elemental content. Such behavior should result in variations in trace element stoichiometry of the larger zooplankton size fraction over diel cycles.

In addition to elemental stoichiometry, information on zooplankton physiological activity is needed to determine whether zooplankton production can be limited by trace metals. Animal production in the laboratory is often measured as growth rates for juvenile or reproductive rates for adults. However, because of the diversity of natural zooplankton communities, it is difficult to measure the community level zooplankton growth and reproductive rates in situ. The variation of zooplankton biomass usually also takes a long time (3-10 d) to manifest. As nucleic acids, especially ribosomal RNA, is required for protein synthesis and tissue production, RNA concentration or RNA:DNA ratios have been widely used as a reliable index of instantaneous somatic growth or reproduction of aquatic organisms ((Dortch et al. 1983, Kerkhof and Ward 1993, Buckley et al. 1999), including Copepods (Saiz et al. 1998, Wagner et al. 1998) and Daphnids (Vrede et al. 2002). Correlations between RNA:DNA ratios and Fe and Zn contents may indicate limitation of zooplankton growth and reproduction by these elements.

The Costa Rica Upwelling Dome (CRD) is an upwelling region in the eastern tropical Pacific, centered at 8ºN, 90ºW, with the diameter of 100-900 km, depending on the strength of the initiating seasonal wind stress curl (Hofmann et al. 1981, Saito et al. 2005). During the upwelling prevailing seasons which are summer and early fall, this area has relatively high

concentrations of macronutrients (NO_3^-: 4.4 μmol L^{-1}, PO_4^{3-}: 0.9 μmol L^{-1}, Franck et al. 2003), low dissolved Fe (0.07-0.13 nmol L^{-1}) and presumably low dissolved Zn (data not available, Franck et al. 2003). The biomass in surface waters is dominated by prokaryotic picoplankton *Synechococcus* (Saito et al. 2005), which builds the foundation of local food-webs. A deeper community of larger eukaryotes often exists below the persistently shallow thermocline (<15m), and this community may not experience the same level of metal limitation as in surface waters. This HNLC feature of CRD makes it an ideal environment to examine the in situ trace metal limitation of secondary production.

Here I present the data on the concentration of C, N, P, Fe and Zn in mesozooplankton collected during a cruise in CRD in June and July, 2010. I fractionated the zooplankton samples into four groups by animal size to investigate the variation of zooplankton elemental stoichiometry with size. I also compared samples collected at different times of day to better understand the diel variation of zooplankton stoichiometry. In addition, I assessed RNA:DNA ratios of zooplankton samples in each size fraction and compared my results with other studies to discuss the fitness of zooplankton from this HNLC region.

Materials and methods

Sampling

Mesozooplankton samples were collected at the CRD on a cruise between 22 June and 26 July, 2010. Sampling was conducted within 6.6-10.0°N and 87.5-92.9°W (Fig. 1). The entire cruise included 1 large transect through the region and 5 lagrangian drift cycles that followed the evolution of the plankton community within a body of water sampled over four days using drogues. During the transect through the CRD region, mesozooplankton samples were collected every 5-8 h, regardless of time of day. During the lagrangian cycles, samples were collected at the onset and end of each cycle at each station (day and night).

Zooplankton samples were conducted with a standard 1-m^2 ring net with 202-μm Nitex mesh. Samples were collected from surface to 150-m depth and were then fractionated into 4 size ranges: 0.2-0.5, 0.5-1, 1-2, and 2-5 mm by sieves. Each fraction of the mesozooplankton samples was treated in two ways depending on the purpose of analysis. Subsamples used for RNA:DNA analysis were transferred quickly into microcentrifuge tubes

and stored at -80°C on board. Because RNA:DNA may vary significantly among taxa, 15 cyclopoid and 15 calanoid copepod individuals were also picked out for separate RNA:DNA analysis. Subsamples for elemental analysis were rinsed with 10 ml of oxalate reagent for 10 min (Tovar-Sanchez et al. 2003) and then 30 ml of trace-metal free seawater for 10 min before being transferred into trace-metal clean centrifuge tubes and stored in a -80°C freezer. Trace-metal free seawater was obtained by being collected with trace-metal clean rosette and then chelexed with acid-washed chelex-100 resin. Before elemental analysis, samples were freeze-dried in trace-metal clean containers in the lab before further processing.

Elemental analysis

Subsamples of freeze-dried zooplankton were homogenized and weighed before being analyzed for C and N masses. Carbon and N masses were analyzed using a Flash EA1112 CN Analyzer (CE Elantech). Atropine was used as a standard; analytical precision was <1%. Another portion of each freeze-dried zooplankton sample was analyzed for P. The sample was crushed and weighed before being transferred into glass scintillation vials. The remaining approach followed Solórzano and Sharp (1980). Specifically, vials with samples were then combusted at 450°C for 5 h to release orthophosphate from zooplankton tissues. After cooling, 10 mL of 0.5 mol L^{-1} HCl was added to each vial to allow P extraction at 90°C for 2 h. After cooling, 2.5 mL of the extracted solution was pipetted from each vial and 0.5 mL of a mixed reagent of ammonium molybdate, sulfuric acid, ascorbic acid, and potassium antimonyl tartrate was added. After 2 h in the dark, absorbance of each sample was read at 885 nm wavelength with a Varian Cary 50 Bio UV-Vis spectrophotometer (Varian Inc., NC). Absorbance of samples was compared to a standard curve to obtain P mass.

A third portion of freeze-dried zooplankton samples was digested and analyzed for Fe, Zn and P concentrations by inductively coupled plasma mass spectrometry (ICP-MS) using a Thermo-Finnigan Element with an ESI ApexHF desolvating nebulizer and a CETAC ASX-100 autosampler (Twining et al. 2011). Instrument parameters were optimized to maximize sensitivity to a 0.5 mg L^{-1}. Carbon, N and P concentrations were calculated by dividing elemental mass by sample dry weight and further correlated with the Fe and Zn concentration obtained from ICP-MS for the calculation of elemental ratios.

RNA:DNA ratio analysis

Nucleic acids of zooplankton samples were analyzed following Speekmann et al. (2007), with some modifications due to the large biomass in each sample. RNA and DNA of samples were extracted and stained with fluorochrome RiboGreen for fluorescence measurement. Before measurement, RNase and DNase free water was made by adding diethyl pyrocarbonate (DEPC) (0.1% final concentration) to distilled, deionized water. The obtained water was then incubated at room temperature for 24 h, before being autoclaved at 120°C at 15lbs/sq. inch for 30 min. TE assay buffer was made by diluting the 20×TE buffer (200 mM Tris–HCl, 20 mM EDTA in RNase/DNase-free water, pH 7.5, Molecular Probes) with nuclease-free water. Extraction buffer was made with 1×TE buffer containing 0.1% Triton X-100 and 0.1 mg mL^{-1} protease (type VIII, 7-15 units mg^{-1}, Sigma) on the day of analysis. Quant-iT™ RiboGreen RNA quantitation reagent (Molecular Probes®) was diluted by 200-fold with 1×TE buffer in the dark within an hour of analysis. RNA standard (16S and 23S rRNA from Escherichia coli, Molecular Probes®) and DNA standard (lambda DNA, Invitrogen™) stocks were diluted with 1×TE buffer into 4 µg mL^{-1} and 2.5 µg mL^{-1}, respectively, as working standards. RNase (5.0 mg mL^{-1}) was made by dissolving RNase A (type III-A, 85-140 Kunitz unit, Sigma) in 0.01M sodium acetate (pH 5.0) and heating to 100ºC for 15 min. After cooling down, bovine albumin serum (BSA) was added at 1.0 mg mL^{-1} and adequately mixed. pH was adjusted to 7.4 with 1M Tris-HCl before use.

On the day of analysis, RNA and DNA standards were diluted with 1×TE buffer to 0-2000 ng mL^{-1} and 0-1250 ng mL^{-1}, respectively. Each Eppendorf tube of zooplankton samples received 0.5 mL of extraction buffer and was crushed with an RNase/DNase/pyrogen free plastic pellet pestle. Less than 20 µL of subsamples was transferred into a new Eppendorf tube with addition of another 0.5 mL extraction buffer and then crushed again. Subsamples were incubated at room temperature on a TSZ scientific™ orbital shaker for 1 h. After incubation, 100 µL of extracted sample or standards was added into a black Nunc™ U96 MicroWell™ Plate with 100 µL diluted RiboGreen reagent, and then incubated in the dark for 5 min. Fluorescence was measured by a spectrofluorometer integrated with an Infinite® F500 filter-based multimode microplate reader for RNA and DNA combined (FL_C).

Each well was then added with 25 μL RNase and incubated in the dark for 30 min before measuring fluorescence for DNA (FL_D). Fluorescence for RNA was then calculated as $FL_R = FL_C - FL_D$. RNA and DNA concentrations in samples were calculated from standard curves.

Statistical analysis

All continuous variables including zooplankton elemental contents and RNA:DNA ratios were log transformed to obtain normal distributions. The significance of results among size fractions and between samples collected during daytime and nighttime was analyzed by two-way analysis of variance (ANOVA). The diel patterns of variables were analyzed by fitting data points with polynomial regression. All analyses were performed by JMP 7.01 (SAS).

Results

The elemental contents and RNA:DNA in zooplankton samples of different sizes are listed in Table 1 and statistical results are given in Table 2. The average contents of C, N and P were 27, 5.7 and 0.22 mmol gdw^{-1}, respectively. Carbon and N content were tightly correlated (($r^2 = 0.87$, $p < 0.0001$, Table 3). The average contents of Fe and Zn were 1623 and 565 nmol gdw^{-1}, respectively.

Concentrations of all measured elements in zooplankton were affected by body size, although patterns differed among elements. The contents of Fe and Zn showed opposite patterns, with Fe content tending to decline with size in the 0.2-2 mm size range ($p = 0.003$, Table 2), while the Zn content increased with size ($p < 0.0001$). Both C and N concentrations declined as size increased ($p < 0.0001$ for C, $p = 0.009$ for N, Table 2). The P content, however, increased subtly with size ($p = 0.031$). Concentrations of all these three macronutrient elements varied greatest in the largest size fraction (2-5 mm). As expected based on the variations in elemental content among size fractions, the Zn:C and Zn:P ratios of zooplankton increased significantly with size ($p < 0.0001$) while C:P decreased with size ($p = 0.0002$). The Fe:C and Fe:P ratios increased with size fractions ($p < 0.005$), while the C:N ratios decreased with size ($p = 0.01$).

Zooplankton elemental concentrations varied with time of day, especially for the two

larger size groups. Both the Fe and Zn contents of the 2-5 mm size group were highest at midday and lowest at midnight, displaying a parabolic distribution ($p = 0.054$ for Fe, $p = 0.015$ for Zn, Fig. 3, Table 2). In contrast, macro-elements C, N and P showed opposite trends, with the lowest concentrations at mid-day and higher concentrations at night ($p < 0.0001$ for all 3 elements in the 2-5 mm group, Fig. 3, Table 2). Because trace metals and macro-elements displayed different patterns of variation in the elemental contents with sampling time of a day, zooplankton Fe:C and Fe:P as well as Zn:C and Zn:P ratios showed even more significant parabolic distributions in both 1-2 and 2-5 mm size groups (Table 2).

The patterns in Fe and Zn among size classes differed between daytime and nighttime samples. The decreasing Fe:C and Fe:P with size were mostly driven by lower Fe concentrations in 1-2 mm and 2-5 mm zooplankton captured at night (Fig. 4). In contrast, the trend of increasing Zn:C and Zn:P with size was more noticeable in the daytime samples, although it was also evident at night (Fig. 4). Zinc:C and Zn:P ratios in the largest size fraction were ~2-fold greater than in the smallest size fractions during the day, but 5-fold larger during the night. The decline of C:N with size was also more apparent in nighttime samples (Fig. 4).

RNA:DNA ratios ranged between 0 and 23. However, the majority of the ratios were below 1, indicating low growth rates of the zooplankton. RNA:DNA ratios did not show any diel pattern nor variation with size group (Table 2). Instead, variation in RNA:DNA ratios was more a function of location ($p = 0.0005$). The RNA:DNA ratios of copepods were on average >1. The cyclopoid copepods had 76% higher RNA:DNA than calanoid copepods (Table 6). No significant correlation between RNA:DNA and trace metal contents were found.

Discussion

Since it has been hypothesized that Zn is limiting to phytoplankton in the CRD, we might expect that zooplankton in this region should exhibit relatively low Zn contents. Indeed, the zooplankton Zn content we report here is on the lower end of values for other regions of the ocean. Average Zn concentration of zooplankton in the Greenland Sea ranged over 10-fold, from 566 nmol gdw^{-1} in the decapod *Hymenodora glacialis* to 5950 nmol gdw^{-1}

in the copepod *Metridia longa* (Ritterhoff and Zauke 1997, Table 4), with 10 out of 13 species exhibiting Zn concentrations above 1000 nmol gdw^{-1}. In samples collected from the Fram Strait, the zooplankton Zn concentration ranged from 933 nmol gdw^{-1} in the amphipod *Themisto libellula* to 5476 nmol gdw^{-1} in the ostracod *Conchoecia borealis*, with a median of 1369 nmol gdw^{-1} (Table 4, Ritterhoff and Zauke 1997). Zooplankton collected from the North Sea and Weddell Sea ranged from 688 to 8014 nmol gdw^{-1} and 2799 to 7922 nmol gdw^{-1}, respectively (Table 4, Zauke et al. 1996, Kahle and Zauke 2003). By comparison, Zn concentrations averaged over the entire community in the CRD were 565 nmol gdw^{-1}. The smallest size fraction exhibited the lowest Zn contents yet observed for zooplankton. Even the largest fraction exhibited values at the low end of the range reported from other studies, although within the range of observations.

One possible reason for the lower zooplankton Zn concentration in our study is that surface adsorbed Zn in our samples were rinsed off by the oxalate reagent, which was not done in other studies. However, Kahle and Zauke (2003) have argued that Zn adsorption onto zooplankton exoskeleton was generally negligible, as the process of metal adsorption and desorption is at least 3 orders of magnitude faster than metal uptake from food. Therefore, it is more likely that it was the low Zn concentration in the environment that resulted in low zooplankton Zn content. Previous studies have shown that freshwater zooplankton were unable to regulate the tissue Zn concentration when the Zn content in seston was below 68 μg gdw^{-1}, although Zn concentrations in zooplankton remained fairly constant (1774) at higher environmental Zn levels (Zauke 1998). It is possible that at an environmental Zn concentration as low as in CRD, zooplankton have lost the ability to maintain the same level of Zn in tissue as those in other regions or have evolved to require low Zn contents.

The CRD region also exhibits relatively low Fe concentrations, which possibly reflect low Fe contents of organisms at the base of the food chain. The Fe content of zooplankton has been less studied than Zn. Fe contents of copepods and euphausiids in Monterey Bay measured by Martin and Knauer (1973) ranged from 967-5050 mmol gdw^{-1}, with an average of 2690 mmol gdw^{-1} (Table 5). The concentrations of Fe were even higher (1612-30799 mmol gdw^{-1}) in bulk zooplankton samples collected from transects in Hawaii. Fowler (1977)

and Masuzawa et al. (1988) measured Fe content of one single zooplankton species of each order. The per dry weight mass of Fe of the euphausiid *Meganyctiphanes norvegica* in Mediterranean Sea was measured as 1146 mmol gdw^{-1}, and values of 591, 1970, and 4298 mmol gdw^{-1}, were obtained respectively for the copepod *Calanus plumchrus*, the euphausiid *Thysanoessa longipes*, and the amphipod *Parathemisto japonica* in the Japan Sea, respectively. Our results are within the range of these studies, but again are on the low side. Generally the Fe contents measured by Martin and Knauer (1973) were somewhat lower for euphausiids than copepods, consistent with our finding that Fe contents decreased with size.

The reason for the different patterns of Zn and Fe concentrations among size classes of zooplankton is puzzling. The Zn pattern hints at possible Zn limitation of zooplankton feeding at the base of the food chain. Phytoplankton exhibit substantial stoichiometric flexibility in response to gradients in Zn availability (Sunda and Huntsman 1992). Moreover, phytoplankton biomass in CRD surface waters is dominated by *Synechococcus* (Saito et al. 2005), which has a lower cellular Zn concentration than eukaryotes, possibly making them better adapted to low Zn environments (Twining et al. 2011). While copepods and other crustacean zooplankton do not feed on these organisms directly, they do feed on consumers that do, including protozoa and mixotrophic algae. Organisms that feed further up the food chain in low Zn regions are not as subject to a limited dietary supply of Zn. Zooplankton tend to vary less in their Zn contents than do the particles that comprise their food (Zauke et al 1998). Copepods also assimilate metals, including Zn, much more efficiently when fed organisms which have obtained those metals primarily from food (Twining and Fisher 2004). Both of these factors might affect Zn accumulation through the food chain, which helps release Zn limitation for larger organisms feeding at higher trophic levels. It is also conceivable that larger, more complex animals have higher Zn demand, since animals use Zn primarily in finger proteins, which act as structuring agents for DNA and RNA as well as regulatory factors in DNA expression and DNA repair.

The different patterns of zooplankton Fe and Zn concentrations with size imply that the Fe and Zn of CRD zooplankton are regulated by different factors, even though both trace metals are essential and possible limiting nutrients and thereby have often been studied together (Franck et al. 2003). Interestingly, additional measurements of the same

zooplankton samples have revealed that the zooplankton Fe concentrations were in fact strongly coupled with aluminum ($r^2 = 0.601$) and titanium ($r^2 = 0.518$), both of which are often considered as tracers for terrestrial or sediment sources. Therefore, the coupling between Fe and Al/Ti in zooplankton samples may imply that the Fe content measured in zooplankton is in fact contained in the zooplankton guts, which might be enriched in clay and could not be rinsed off by oxalate reagent.

The concentrations of macronutrient elements C, N and P were not as different from those previously reported as were Fe and Zn. The average C content in zooplankton (32% of dry weight calculated from 27.15 mmol gdw^{-1}) is a little lower than what has previously been measured for crustacean zooplankton (e.g. 33-60% dry weight, Beers 1966, Kiørboe et al. 1985, Anderson and Hessen 1991, Walve and Larsson 1999). The average N (7.95% of dry weight calculated from 5.68 mmol gdw^{-1}) and P (0.67% of dry weight calculated from 0.22 mmol gdw^{-1}) contents were also on the lower end of the range that has been reported in those studies (5.3-12.7% of dry weight for N, 0.43-2.02% of dry weight for P). The decreasing C and N content in zooplankton with size may be related to the composition of zooplankton in each size group. For example, the most common organisms in the largest size group (2-5 mm) were euphausiids and copepods of the genus *Eucalanus*. The latter have particularly low C, P, protein and lipid contents, and high body-water contents compared to other copepods (Flint et al. 1991, Ohman 1997). This so-called "gelatinous" body type is considered a strategy to adapt to low food conditions (Flint et al. 1991). In contrast, the smaller size groups (0.2-0.5mm, 0.5-1 mm) usually comprise fast growing zooplankton juveniles and fast feeding adults, which require high investment in organic tissues.

The diel patterns of C and N contents indicate a clear vertical migration signal of zooplankton in the lagest size fraction (2-5 mm). Hays et al. (2001) have classified their zooplankton samples by size based on the same standard as in our study, and reported a much stronger diel vertical migration in the larger size groups (0.5-1 and 1-2 mm) than in the smallest size group (0.2-0.5 mm). The strongest diel change of C and N content in our largest size group (2-5 mm) confirmed that vertical migration is a more common phenomenon in large zooplankton. Hays et al. (2001) also observed higher individual C content in samples collected at dawn compared to those collected at dusk for all three species of the migrating

copepod *Pleuromamma*, which is somewhat similar to our result that C contents in nighttime zooplankton samples were higher. One possible contributing factor to this pattern may be that these large migratory zooplankton are actively feeding in surface water at night, and therefore are actively replenishing themselves with organic materials, whereas during the day, they are catabolizing C and N contents in deep water. A number of studies have shown that zooplankton have higher feeding rates and gut fluorescence at night (Baars and Oosterhuis 1984), and contribute a large amount of DOC and DON to deep water at daytime by excretion (Steinberg et al. 2002). The small zooplankton, instead, may lack the ability to travel deep enough during the day to display a significant variation in physiological activities. The cause for the increase in the Fe and Zn content of large zooplankton during the day is unclear. This pattern may be driven by the different compositions of zooplankton samples collected during daytime and nighttime. Unfortunately, information on community composition of different samples is not available at present.

The RNA:DNA ratios measured in this study are within the range but generally on the low side of what have been measured for samples collected from other open-ocean areas, e.g. 0.31-4.72 for mixed copepods in the North Pacific (Ikeda et al. 2007), and 1.72-18.36 for euphausiids in the Weddell Sea (Donnelly et al. 2004). The low RNA:DNA ratios match the low P content and low P:C in these samples, and are indicative of slow growth and reproductive rates of animals residing in the CRD, suggesting a resource limitation of these animals. The similar RNA:DNA ratios in all size groups implies that the degree to which the animals were limited is independent of animal size, or that the allometric scaling of zooplankton growth rate is obscured under Zn limitation. Even so, the fact that the average RNA:DNA in cyclopoid copepods, some of which have a parasitic lifestyle, were higher than that in calanoid copepods suggests that the limitation may be somewhat relieved by feeding on diets of diverse sources.

In conclusion, concentrations of the elements C, N, P, Fe and Zn in zooplankton as well as the ratios among the elements all varied with size and the sampling time of day, although some variables showed stronger patterns than others. The body contents of C, N, Fe and the ratios of C:N, C:P, Fe:C and Fe:P tended to decrease with increasing animal size. The concentrations of Zn and P and the ratios of Zn:C and Zn:P showed the opposite trend. Diel

patterns of elemental concentrations and ratios were stronger in the larger size groups (1-2, 2-5 mm). Specifically, the concentrations of C, N, P and the C:P ratio showed the lowest values at mid-day and declined with decreasing irradiance, whereas the concentrations of Fe, Zn and the ratios of C:N, Fe:C, Fe:P, Zn:C and Zn:P were the opposite. No size and diel pattern was shown for the animal RNA:DNA ratios. Generally, the zooplankton Fe, Zn and P content measured in this study were lower than values reported elsewhere, hinting that the production of zooplankton in the CRD may be limited by low environmental trace metal concentrations. Nevertheless, it is worth noting that time period of this cruise was presumably only at the onset of upwelling. Even stronger Fe and Zn limitation signals might be evident later in the season.

Table 1 Mean elemental concentrations and RNA:DNA ratios (± standard error) in different size fractions of zooplankton samples

	0.2-0.5 mm	0.5-1 mm	1-2 mm	2-5 mm	all size fractions
macro-elements (mmol gdw^{-1}) and ratios (mol mol^{-1})					
C	29.21 ± 0.40	28.33 ± 0.45	26.35 ± 0.66	24.20 ± 0.98	27.15 ± 0.36
N	5.97 ± 0.11	5.91 ± 0.13	5.48 ± 0.16	5.36 ± 0.26	5.68 ± 0.09
P	0.20 ± 0.01	0.21 ± 0.01	0.22 ± 0.01	0.25 ± 0.02	0.22 ± 0.01
C:N	4.91 ± 0.04	4.82 ± 0.05	4.85 ± 0.07	4.68 ± 0.07	4.81 ± 0.03
C:P	163.71 ± 6.07	155.78 ± 4.50	141.45 ± 6.34	131.96 ± 5.26	148.46 ± 3.01
N:P	33.39 ± 1.20	32.47 ± 1.02	29.24 ± 1.35	28.67 ± 1.30	30.97 ± 0.63
trace metals (nmol gdw^{-1}) and ratios (µmol mol^{-1})					
Fe	2227.22 ± 351.80	1546.35 ± 146.74	1256.98 ± 186.35	1262.06 ± 354.26	1579.07 ± 141.49
Zn	403.74 ± 59.26	424.43 ± 28.30	613.40 ± 38.89	833.75 ± 64.99	565.62 ± 29.71
Fe:C	76.97 ± 12.11	56.10 ± 6.03	57.54 ± 11.09	58.71 ± 17.33	62.37 ± 6.07
Fe:P	12469.2 ± 1473.8	7869.2 ± 1501.9	7099.3 ± 1473.8	4787.7 ± 1563.2	8152.42 ± 789.18
Zn:C	13.72 ± 1.96	15.17 ± 1.06	23.49 ± 1.79	37.42 ± 4.22	22.39 ± 1.53
Zn:P	2162.37 ± 333.26	2125.43 ± 339.61	2855.67 ± 333.26	3986.06 ± 353.48	2753.98 ± 182.39
RNA:DNA	2.00 ± 0.84	2.55 ± 1.30	2.01 ± 1.17	2.04 ± 1.05	2.14 ± 0.54

Table 2 Statistic analysis among variables. The significance of log transformed means of elemental concentrations and ratios as well as RNA:DNA between size fractions was determined by ANOVA analysis. Analytical results in the section of diel pattern regression indicate the fitness of quadratic polynomial models to data.

	comparison among size fractions (n=107)		diel pattern regression							
			0.2-0.5 mm (n=27)		0.5-1 mm (n=26)		1-2 mm (n=27)		2-5 mm (n=24)	
	r^2	*p*-value	r^2	*p*-value	r^2	*p*-value	r^2	*p*-value	r^2	*p*-value
log C	0.224	**<.0001**	0.022	0.768	0.007	0.925	0.238	**0.038**	0.650	**<.0001**
log N	0.105	**0.009**	0.031	0.685	0.007	0.921	0.386	**0.003**	0.642	**<.0001**
log P	0.082	**0.031**	0.069	0.423	0.077	0.399	0.219	0.052	0.559	**0.0001**
log C:N	0.104	**0.010**	0.028	0.708	0.037	0.647	0.232	**0.042**	0.275	**0.025**
log C:P	0.175	**0.0002**	0.013	0.854	0.106	0.276	0.096	0.299	0.347	**0.004**
log N:P	0.113	**0.006**	0.018	0.800	0.079	0.388	0.028	0.708	0.500	**0.0005**
log Fe	0.129	**0.003**	0.154	0.135	0.001	0.986	0.117	0.223	0.243	0.054
log Zn	0.289	**<.0001**	0.075	0.392	0.040	0.625	0.130	0.187	0.328	**0.015**
log Fe:C	0.074	**0.046**	0.159	0.125	0.000	0.996	0.133	0.179	0.334	**0.012**
log Fe:P	0.208	**<.0001**	0.163	0.119	0.013	0.858	0.148	0.147	0.422	**0.002**
log Zn:C	0.352	**<.0001**	0.096	0.300	0.045	0.591	0.254	**0.030**	0.558	**0.0001**
log Zn:P	0.208	**<.0001**	0.123	0.208	0.099	0.302	0.375	**0.004**	0.671	**<.0001**
log RNA:DNA	0.026	0.590	0.187	0.172	0.020	0.861	0.046	0.684	0.007	0.948

Table 3 Correlations between log transformed elemental concentrations

	log C	log N	log P	log Fe
log N	0.866			
log P	0.087	0.177		
log Fe	0.016	0.055	0.012	
log Zn	0.164	0.098	0.104	0.002

Table 4 Zn concentration (nmol gdw^{-1}) of zooplankton from other regions.

Taxon	Species	Region	Conc.	Reference
Amphipods	*Eusirus propeperdentatus*	Antarctic Peninsula	749	14
	Hyperia sp.	Northern North Sea	1101	16
	Paraceradocus gibber	Antarctic Peninsula	964	14
	Parathemisto japonica	Sea of Japan	1239	9
	Phrosina semilunata	Mediterranean	2294	3
	Phronima sedentaria	NW Mediterranean	1652-3013	13
	Themisto abyssorum	Fram Strait and Greenland Sea	1422-2401	17
	Themisto compressa	NE Atlantic	1162	10
	Themisto gaudichaudii	Antarctic	902-1009	10
	Themisto libellula	Fram Strait and Greenland Sea	1055-1315	17
Copepods	*Acartia clausi*	Mediterranean	2570	18
	Calanoides acutus	Weddell Sea	2799	19
	Calanus cristatus	Bering Sea	1851	6
	Calanus finmarchi./helgol	North Sea	1071-1973	16
	Calanus finmarchicus	Fram Strait and Greenland Sea	1346-2692	12, 17
	Calanus glacialis	Fram Strait and Greenland Sea	795	17
	Calanus hyperboreus	Fram Strait and Greenland Sea	902-5369	12, 17
	Calanus plumchrus	N Pacific, Sea of Japan	719-2019	6, 9
	Calanus propinquus	Weddell Sea	2921	19
	Euchaeta barbata	Fram Strait and Greenland Sea	5154	17
	Euchaeta glacialis	Fram Strait and Greenland Sea	2631	17
	Euchaeta norvegica	Fram Strait and Greenland Sea	3441	17
	Metridia curicauda	Weddell Sea	4252	19
	Metridia gerlachei	Weddell Sea	7923	19
	Metridia longa	Fram Strait and Greenland Sea	642-933	17
	Rhincalanus gigas	Weddell Sea	6608	19
	mixed species	Monterey Bay, California	1773	2
Chaetognaths	*Eukrohnia hamata*	Fram Strait and Greenland Sea	1193	17
	Sagitta elegans	Sea of Japan	2875	9
Decapods	*Chorismus antarcticus*	Weddel Sea	673	14
	Notocrangon antarcticus	Weddel Sea	704	14
	Systellaspis debilis	Atlantic (African coast)	642-1422	7
Euphausiids	*Euphausia pacifica*	N Pacific	195-2983	6
	Euphausia superba	Antarctic	505-1040	5, 8, 10, 14, 15
	Hymenodora glacialis	Fram Strait and Greenland Sea	566-1208	17
	Meganyctiphanes norvegica	Firth of Clyde, NE Atlantic, Greenland Sea, Mediterranean	658-1591	1, 4, 10, 11, 13, 17
	Thysanoessa inermis	Fram Strait and Greenland Sea	1315	17
	Thysanoessa longipes	Bering Sea, Sea of Japan	902-2677	6, 9
	mixed species	Monterey Bay, California	1081	2
Ostracod	*Conchoecia borealis*	Fram Strait and Greenland Sea	5476-5950	17

Mixed	Monterey Bay, California	1354	2
zooplankton	Sea of Japan	2554	9

Table 5 Fe concentration (nmol gdw^{-1}) of zooplankton from other regions.

Taxon	Species	Region	Conc.	Reference
Amphipods	*Parathemisto japonica*	Sea of Japan	4298	9
Chaetognaths	*Sagitta elegans*	Sea of Japan	3402	9
Copepods	*Calanus plumchrus*	Sea of Japan	591	9
	mixed species	Monterey Bay, California	3370	2
Euphausiids	*Meganyctiphanes norvegica*	Mediterranean	1146	4
	Thysanoessa longipes	Sea of Japan	1970	9
	mixed species	Monterey Bay, California	1934	2
Mixed		Monterey Bay, California	6169	2
zooplankton		Sea of Japan	8058	9

Notes:

1 Leatherland et al. 1973
2 Martin and Knauer 1973
3 Fowler and Benayoun 1974
4 Fowler 1977
5 Stoeppler and Brandt 1979
6 Hamanaka and Tsujita, 1981
7 Ridout et al. 1985
8 Yamamoto et al. 1987
9 Masuzawa et al. 1988
10 Rainbow 1989
11 Ridout et al. 1989
12 Pohl 1992
13 Romeo et al. 1992
14 Petri and Zauke 1993
15 Locarnini and Presley 1995
16 Zauke et al. 1996
17 Ritterhoff and Zauke 1997
18 Fisher et al. 2000
19 Kahle and Zauke 2003

Table 6 RNA:DNA ratios of zooplankton from this and other studies.

Taxon	Species	Region	RNA:DNA	Reference
Copepods	cyclopoid	CRD	2.51±0.41	This study
	calanoid	CRD	1.43±0.29	This study
Copepods	*Acartia bifilosa*	eastern Gotland basin	1.9-5	Gorokhova 2003
	Calanus finmarchicus	Raunefjord, Bergen	4-9	Hansen et al. 2003
	mixed Calanoid	N Pacific and Bering Sea	1.48-4.72	Ikeda et al. 2007
Euphausiids	*Cyllopus lucasii*	Weddell Sea	4.31±1.62	Donnelly et al. 2004
	Vibilia stebbingi	same as above	18.36±9.22	same as above
	Galiteuthis glacialis	same as above	2.53±0.33	same as above
	Euphausia triacantha	same as above	1.44±0.30	same as above
	Euphausia superba	same as above	1.87-2.93	same as above
	Thysanoessa macrura	same as above	2.18-4.59	same as above
	Cyphocaris faueri	same as above	2.54	same as above
	Cyphocaris richardi	same as above	2.11-4.69	same as above
	Cleonardo longipes	same as above	1.63	same as above
Amphipods	*Parandania boecki*	same as above	6.41	same as above
	Cyllopus lucasii	same as above	3.44-3.87	same as above
	Hyperiella antarctica	same as above	11.53	same as above
	Hyperoche medusarum	same as above	10.98	same as above
	Megalanceola remipes	same as above	9.5	same as above
	Megalanceola stephenseni	same as above	8.55	same as above
	Primno macropa	same as above	11.43-25.47	same as above
	Themisto gaudichaudi	same as above	5.19	same as above
	Vibilia stebbingi	same as above	15.06-18.45	same as above
Decapods	*Gennadas kempi*	same as above	1.09±0.09	same as above
	Nematocarcinus lanceopes	same as above	2.13±0.38	same as above
	Pasiphaea scotiae	same as above	2.79-4.95	same as above
	Petalidium foliaceum	same as above	0.66	same as above
Isopods	*Anuropus australis*	same as above	0.59	same as above
Mysids	*Boreomysis rostrata*	same as above	1.06	same as above
	Gnathophausia gigas	same as above	0.36	same as above
Ostracods	*Gigantocypris mulleri*	same as above	1.33-1.87	same as above

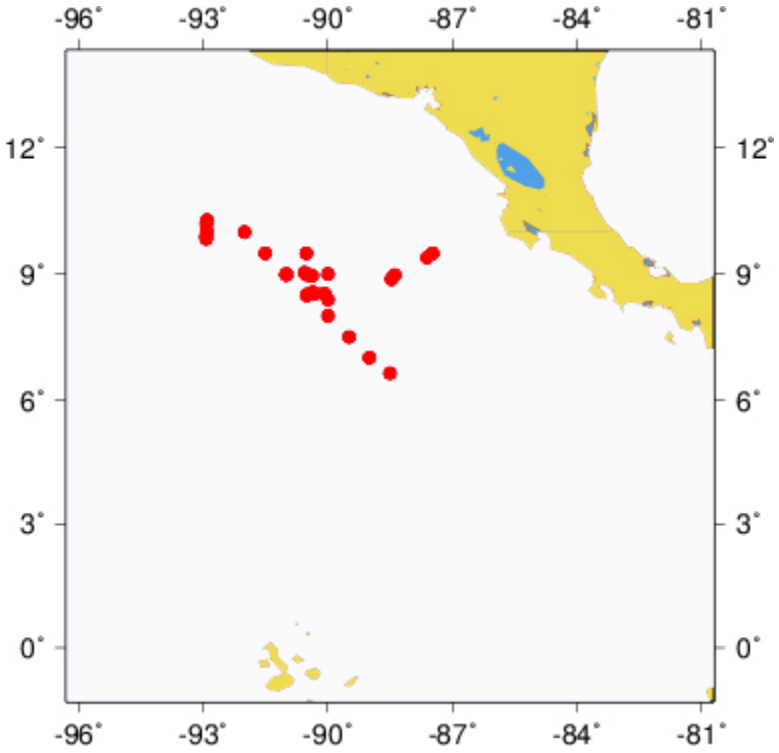


Fig. 1 Station map of CRD cruise. Dots showed the location of sampling stations.

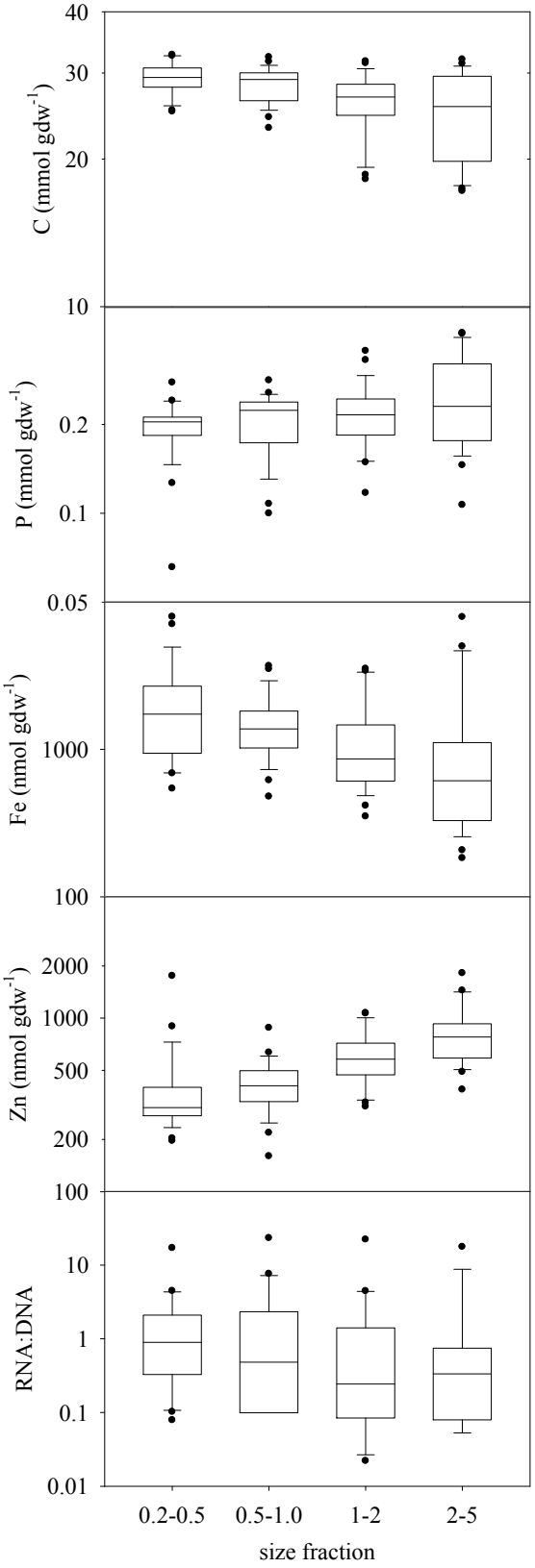

C (mmol gdw-1)
40
30
20
10
P (mmol gdw-1)
0.2
0.1
0.05
Fe (nmol gdw-1)
1000
100
Zn (nmol gdw-1)
2000
1000
500
200
100
RNA:DNA
10
1
0.1
0.01
0.2-0.5
0.5-1.0
1-2
2-5
size fraction

Fig. 2. Box plots of the concentrations of the elements C, N and P (mmol gdw^{-1}), Fe and Zn (nmol gdw^{-1}) and RNA:DNA ratios in zooplankton samples. The solid line represents the median, the dotted line represents the arithmetic mean, the box delineates the 25th and 75th percentile confidence intervals, and the error bars encompass the 10th and 90th percentile confidence intervals. Data falling outside of these ranges are plotted individually.

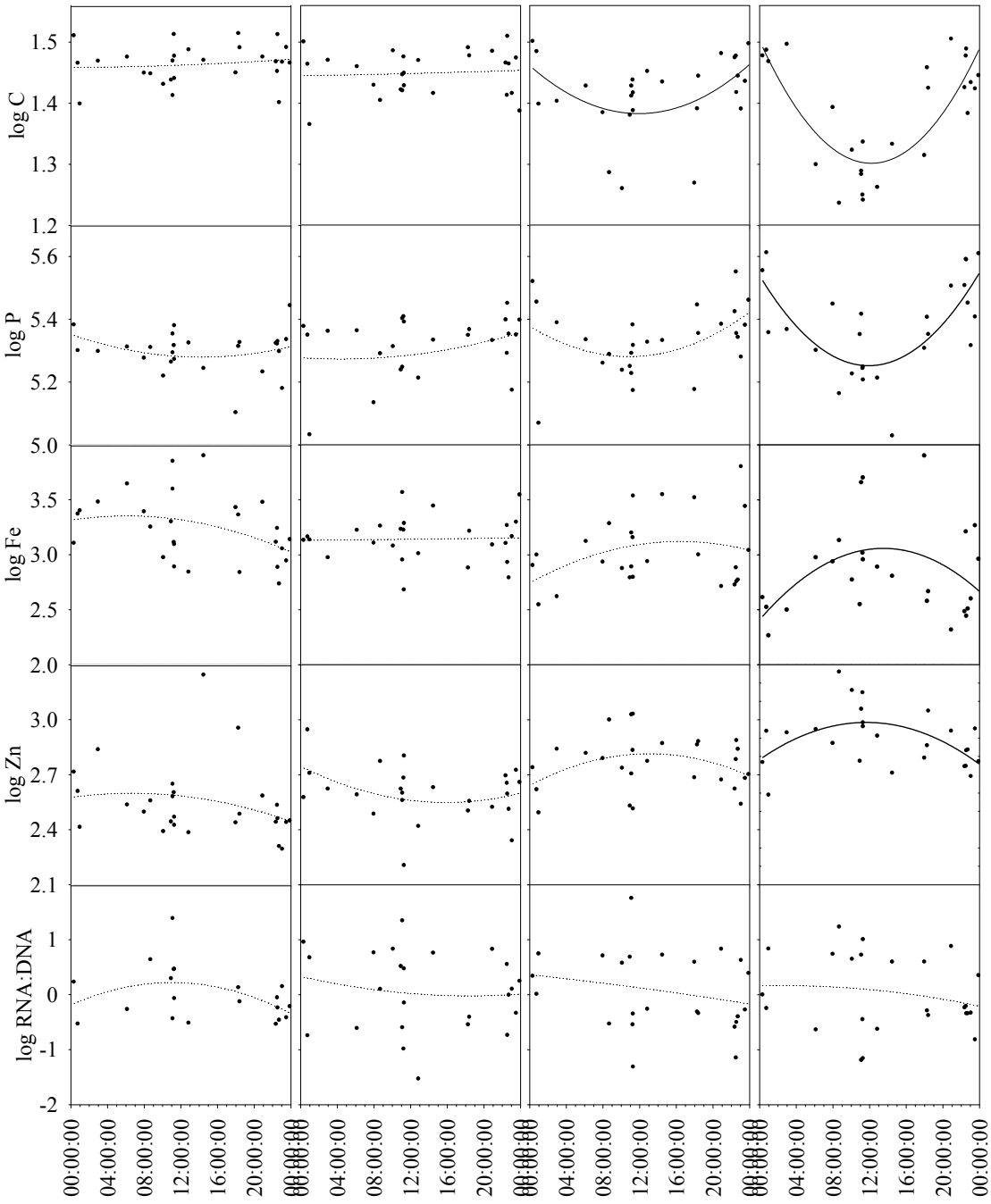


Fig. 3. The diel pattern of elemental concentrations and RNA:DNA ratios in zooplankton samples. The figures in each panel from left to right indicate the values from 0.2-0.5, 0.5-1, 1-2 and 2-5 mm size fractions, respectively. Data in each subpanel were fitted by a quadratic polynomial model. Solid regression line means regression is significant ($p < 0.05$). Dotted regression line means the regression is not significant ($p > 0.05$).

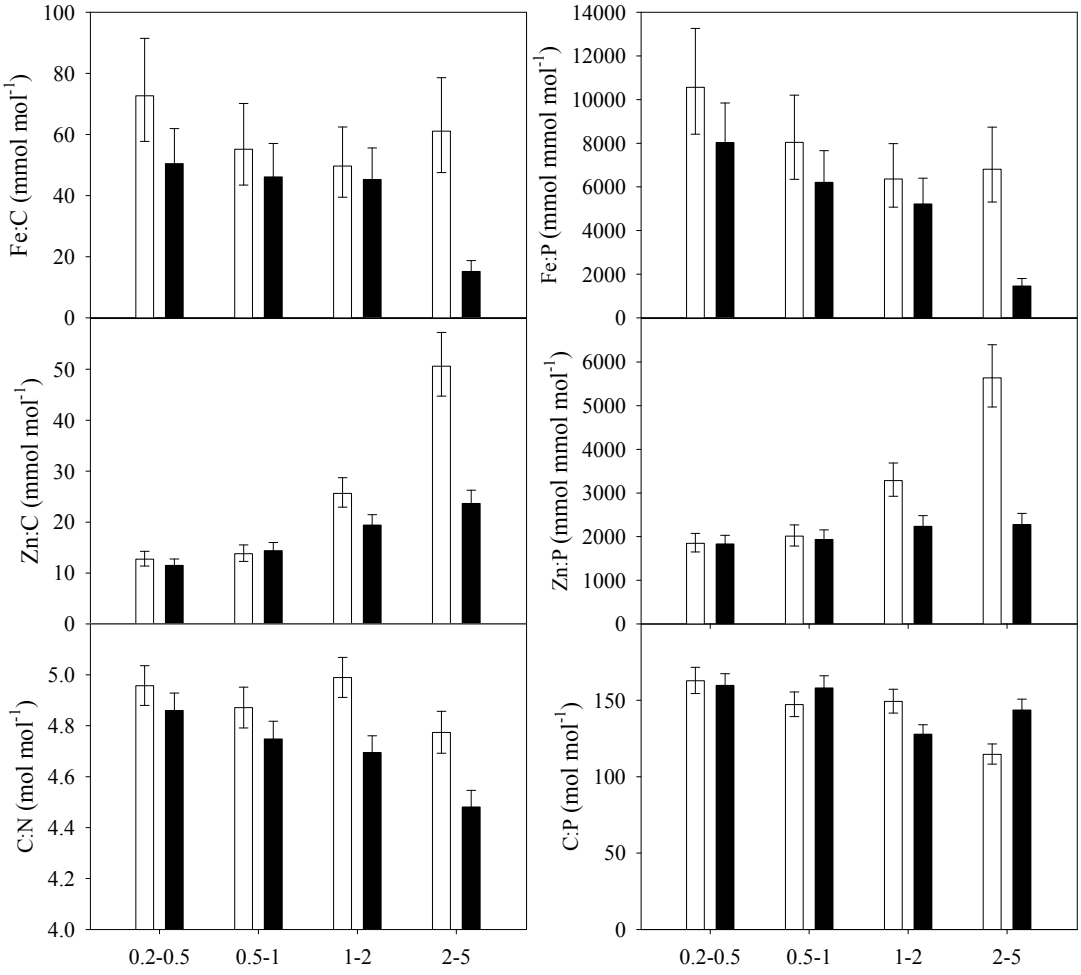


Fig. 4. Elemental ratios of Fe:C, Fe:P, Zn:C, Zn:P, C:N and C:P of different size groups in zooplankton samples collected in the CRD. Empty bars represent samples collected during the daytime; filled bars represent samples collected at night. Error bars represent the standard errors of the corresponding groups.

Chapter 7

Summary and conclusion

The general objective of this dissertation was to test whether the production of marine mesozooplankton can be limited by trace metals such as Fe. I also explored the mechanisms underlying the observed effects and assessed the applicability of laboratory experiments to field conditions. Many studies have examined Fe limitation of marine phytoplankton cells, and have explored the effect of dietary macronutrient deficiency (N, P) on mesozooplankton in aquatic ecosystems, but Fe limitation of mesozooplankton has not been well addressed in previous studies.

The Fe limitation of mesozooplankton is of interest because Fe is needed in zooplankton for respiration and other biological functions, yet it is limiting primary production in over 30% of the world's oceans due to its low solubility and the low bioavailability of its dominant chemical form, Fe (III). To better understand how Fe can affect zooplankton, I conducted a series of laboratory experiments by feeding two model marine copepods *Acartia tonsa* and *Calanus pacificus* with a variety of cultured phytoplankton including *Thalassiosira oceanica, T. weissflogii, Rhodomonas salina* and *Isochrysis galbana* containing either high or low Fe content, and then measuring copepod egg production rates, naupliar survivorship, ingestion rates, respiratory rates, elemental assimilation and excretion, and lipid content. The selected algal species included bacillariophytes, a cryptophyte, and a prymnesiophyte, which presumably have different nutritional values to their zooplankton grazers. To further examine the prevalence of Fe limitation to mesozooplankton, I also participated in a field study in an *in situ* low Fe region to assess the elemental stoichiometry and physiological status of the mesozooplankton residing there.

One of the key findings of this dissertation research is that marine mesozooplankton can indeed be limited by trace elements such as Fe. Specifically, in Chapter 2 and 5, both *A. tonsa* and *C. pacificus* showed reduced egg production rates when fed Fe-depleted phytoplankton cells, and the reduction of egg production was not related to ingestion rate, indicating a more direct effect of Fe. The direct positive correlation between the amount of Fe assimilated from algal food and copepod egg production rates was later confirmed by a radiotracer experiment in which the assimilation and retention of Fe and C were determined. The reduced concentration of total and polyunsaturated fatty acids in algae cultured under the Fe-depleted condition

may be partly responsible for the decline in copepod egg production, as shown in Chapter 3, because fatty acids are required by zooplankton for energy supply and cell membrane formation.

Another key finding is that *A. tonsa* did not seem to be able to regulate Fe by adjusting Fe assimilation and excretion when faced with Fe deficiency, as seen in Chapter 4. The result from this study is very different from previous studies on the macronutrients N and P, where animals were shown to minimize N or P excretion when these elements were scarce in food (DeMott et al. 1998, He and Wang 2008). Compensation for low Fe by increasing Fe assimilation efficiency was only seen for the *I. galbana* diet. Analysis of Fe subcellular distribution revealed that copepod Fe assimilation efficiency was tightly related to the cytosolic Fe distribution in the algal cells, the latter being uncontrolled by copepods. The reason for the observed pattern of Fe distribution in algal cells is not clear, but it may be related to the proportion of Fe adsorbed to the cell surface. Because cells grown in Fe-replete and Fe-depleted media often have different biovolumes and thereby different surface to volume ratios, the proportion of Fe adsorption onto the cell surface could be different, resulting in variant cytosolic Fe allocation. Contrary to expectation, the Fe excretion rate from copepods was even higher when animals were fed Fe-depleted diets *R. salina* and *I. galbana*. Explanations for this pattern were not evident from my study.

Combined, these findings may transform our views on factors controlling marine zooplankton productivity and on the ability of zooplankton to maintain homeostasis. Many models of marine foodwebs have been built on the assumption that secondary productivity is limited by food availability (e.g. White and Roman 1992, Kiørboe and Nielsen 1994). Results in this study suggest that nutrient content including trace metal concentration in diets can have a direct impact on zooplankton production. Future marine foodweb modeling should take the impact of trace metals contents in diets, in addition to other measurements of food quantity, into consideration. Another assumption on which many stoichiometric models have been built is that zooplankton are able to maintain homeostasis of elemental composition in tissues. This homeostatic regulation is primarily achieved in zooplankton by minimizing the excretion of limiting nutrients while releasing the nutrients that are in excess. However, the results from this study

contradict this idea. It is very likely that homeostasis does not apply to trace metals because trace metals play very specific biological roles in animals and cannot be stored in zooplankton tissues when their supply is in excess. Future studies should determine if other trace metals cannot be regulated post-absorptively by zooplankton.

Without a doubt, the laboratory experiments still have drawbacks. One potential drawback is the use of EDTA as an artificial ligand in algal culture media to control the concentration of free Fe ions. The downsides of using EDTA have been discussed in previous experimental studies and reviews. For example, EDTA is not a strong ligand for [Fe^{3+}], therefore, producing free [Fe^{3+}] concentration comparable to that in naturally Fe-deficient seawater requires addition of EDTA to at least 10^{-4} M, which is the concentration used in our culture media, and is not a negligible introduction of organic C (Gerringa et al. 2000). Furthermore, EDTA also influences the redox speciation of Fe (Gerringa et al. 2000). The effect of this introduction of EDTA may have been most problematic in the experimental treatments with *R. salina* and *I. galbana*. Unlike diatoms which bear silicified frustules, these two species only have a layer of soft periplast or scale, and are easy to deform or stress during filtration. To avoid this problem, the stock cultures of these two algal species were added directly into copepod food suspensions as a way of dilution. This methodology undoubtedly carried over a certain amount of EDTA into the feeding solutions. Options for solving this potential problem may include using naturally Fe-deficient seawater and natural organic ligands such as siderophores for making algal culture media. Because siderophores are stronger ligands for Fe than EDTA, they can be added to the media at a much lower concentration.

The laboratory study could certainly be expanded significantly by including more Fe gradients into the experiments. Currently, only two Fe concentrations were used, including one Fe-replete (100 nM of total dissolved Fe) and one Fe-depleted (around 1 nM of total dissolved Fe considering the carryover from the stock cultures) concentrations. However, a few more Fe concentrations between these two extremes could be used to determine the threshold Fe concentration that leads to copepod Fe limitation. Pulse-chase experiments coupled with the application of radiotracers on more Fe levels could also be conducted to elucidate whether copepods are able to regulate Fe under moderate Fe-limited conditions where they might be healthier and better equipped

physiologically to accommodate low Fe food. This information could further contribute to modeling studies that illustrate zooplankton Fe limitation from a broader perspective.

Future studies on the roles that Fe plays in the synthesis of biomass such as eggs are necessary to better understand the Fe impact on zooplankton. Having two valences, Fe(II) and Fe(III), Fe actively functions in many enzymes in animals participating in electron transports, including ferrodoxin reductase, fatty acid desaturases, catalases, peroxidases, and in protists, superoxide dismutase. The last three enzymes are related to antioxidation and important for animal immune systems in higher animals. However, no study has addressed whether Fe is involved in these enzymes in mesozooplankton. In addition to the enzymes above, there are maybe other biological functions of Fe in zooplankton that have not been recognized.

For the field part of this dissertation research, the elemental stoichiometry and RNA:DNA ratios of zooplankton samples from the Costa Rica Dome (CRD) upwelling region demonstrate that Fe and presumably Zn limitation in zooplankton not only takes place in laboratory experiments but may also be a widespread phenomenon in the field (Chapter 6). The concentrations of almost all elements and the RNA:DNA ratios in zooplankton were generally low compared to those previously reported for zooplankton elsewhere. Particularly, Fe, Zn and P were all on the very low end of the range of those in previous studies. Elemental concentrations and ratios had various patterns with animal size. Specifically, C:N and Fe:C decreased with animal size, whereas Zn:C and P:C increased with size. The zooplankton in the larger size groups (1-2, 2-5 mm) showed significant diel patterns of elemental concentrations and ratios, corresponding to the vertical migration of these animals. The zooplankton composition of different size groups is currently pending analysis, and could provide valuable information to help explain the patterns that I have seen.

Of course the field study can also be largely expanded by including more sampling depths and more High-Nutrient Low-Chlorophyll (HNLC) regions. In this CRD study, only one depth range was used (0-150 m) for zooplankton sampling. In fact, zooplankton composition can be very different at varient depth of water column. Particularly, CRD has a relatively shallow yet thick oxygen minimum zone (OMZ) with the upper boundary of only 40 m in depth and the thickness of up to 1000 m (Fiedler and

Talley 2006). The concentration of O_2 in the water column greatly affects the vertical distribution of zooplankton (Saltzman and Wishner 1997). In addition, strong diel vertical migration signal of large zooplankton (2-5 mm) was detected in this CRD study. Zooplankton inhabiting at different depths of the water column may use different strategies to deal with low Fe. The sampling of zooplankton at different depths of water column can provide us with more detailed information regarding these strategy utilized by different zooplankton.

As noted, the CRD is not only low in Fe, but is also known to be low in Zn. Therefore, the limiting effects observed in our study might be a combined effect of co-limitation by these metals. More studies are needed in areas where Fe is the dominant limiting factor for primary production. In addition, the CRD is a tropical region where the meszooplankton may possess some special traits, including less or no lipid accumulation, more pronounced vertical migration, and perhaps deeper habitation, in contrast to zooplankton inhabiting polar areas. As Fe limitation of primary productivity also takes place in polar regions such as the Southern Ocean, and subpolar regions such as the subarctic Pacific Ocean, studies in those regions are also needed, as polar and tropical zooplankton may well exhibit different responses and strategies in handling Fe limitation. Furthermore, some field experiments can be carried on in more detail. For instance, the growth and reproductive activities of zooplankton collected from CRD were measured as the RNA:DNA ratios of bulk samples. However, these bulk samples contain mixed species, and studying the bulk RNA:DNA ratios may not be ideal. The field studies would make more sense by including some deck-board growth and reproduction experiments on the dominant species in certain areas, especially in polar and subpolar areas where the mesozooplankton are often dominated by a few copepod and euphausiid species.

Overall, this dissertation study is a starting point addressing the roles that trace metals could play in marine secondary production. Important findings were obtained for Fe, the most commonly limiting trace metal for primary production. Future studies involving different metals over a range of concentrations, different animals, and different locations should be conducted to better understand the spatial and temporal patterns in marine zooplankton productivity and community structure.

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