

## The regeneration of highly bioavailable iron by meso- and microzooplankton

Jochen Nuester,<sup>1,\*</sup> Steve Shema,<sup>1,2</sup> Alexander Vermont,<sup>1</sup> David M. Fields,<sup>1</sup> and Benjamin S. Twining<sup>1</sup>

<sup>1</sup>Bigelow Laboratory for Ocean Sciences, East Boothbay, Maine

<sup>2</sup>University of Iceland, Reykjavik, Iceland

### Abstract

The micronutrient iron (Fe) is rapidly cycled in surface waters, and regenerated Fe supports much of the phytoplankton growth in open ocean waters. Meso- and microzooplankton grazing are both important mechanisms to regenerate Fe, but the chemical conditions in the respective digestive systems are different and might affect the bioavailability of Fe. We conducted radiotracer grazing experiments with the copepod *Acartia tonsa* or the heterotrophic dinoflagellate *Oxyrrhis marina* feeding on the diatom *Thalassiosira pseudonana* or the coccolithophore *Emiliania huxleyi*. Uptake of regenerated <sup>55</sup>Fe by a separate *T. pseudonana* culture was compared to the uptake of inorganic Fe. Iron regenerated by *A. tonsa* was taken up 4- to 7-fold faster than inorganic iron. In contrast, no difference was detected between the uptake rate of inorganic Fe and Fe regenerated by *O. marina*. Ingestion of different prey by *A. tonsa* revealed that Fe released during diatom digestion was taken up 1.8-fold faster than Fe released from coccolithophore digestion. Digestive systems and the chemical makeup of the ingested prey are crucial in determining the bioavailability of regenerated Fe. Differences in pH and oxygen saturation between digestive vacuoles and guts may affect the speciation of regenerated Fe, and the release of ferrous Fe might contribute to the bioavailability of regenerated Fe. The ecological structure determines the importance of regenerated Fe for a particular ecosystem.

The importance of iron (Fe) as a limiting nutrient for phytoplankton in vast areas of the ocean, such as high-nutrient, low-chlorophyll regions, has been established in the past two decades (De Baar et al. 2005). As marine phytoplankton contribute up to 40% of global biological carbon fixation, the supply of dissolved Fe in iron-limiting regions may influence atmospheric carbon dioxide concentrations over geological timescales (Martin 1990). The supply of dissolved Fe is regulated by a complex network of factors including biological recycling, Fe speciation, photochemistry, and biological Fe uptake. Because of the interconnection of these factors, questions remain about the importance of the different modes of supply of bioavailable iron (Boyd et al. 2005). Independent of the supply mode, chemical and biological processes control the speciation of iron and ultimately its bioavailability to cells for important processes such as photosynthesis, electron transport, nitrogen fixation, and nitrogen reduction.

There is general consensus that grazing is an important mechanism to regenerate bioavailable Fe (Hutchins et al. 1995; Barbeau et al. 1996; Dalbec and Twining 2009). Strzepek et al. (2005) calculated that microzooplankton grazing led to the regeneration of 30% to > 100% of the Fe necessary to support bacterial and phytoplankton growth observed during the FeCycle experiment. Likewise, meso-zooplankton-mediated iron regeneration accounted for 50% of total Fe demand during a natural fertilization study at the Kerguelen Plateau (Sarhou et al. 2008). These natural ecosystem observations are consistent with laboratory studies of Fe regeneration by mesozooplankton (Hutchins et al. 1993, 1995; Hutchins and Bruland 1994) and microzooplankton (Barbeau et al. 1996, 2001; Dalbec and Twining 2009).

The chemical speciation of regenerated Fe is likely affected by the chemical and biological conditions inside the different digestive systems of meso- and microzooplankton. The activity and type of digestive enzymes are dependent on pH, temperature, and diet, which vary significantly among digestive vacuoles of microzooplankton and the gut of mesozooplankton (Fok et al. 1982; Solgaard et al. 2007; Freese et al. 2012). For example, in ciliates during the initial fusion of digestive vacuoles with lysosomes the pH is lowered to 3, killing the ingested microorganisms (Fok et al. 1982). Subsequently, the pH in the digestive vacuoles rises from 3 to 7, accompanied by acid phosphatase activity, which suggests active digestion (Fok et al. 1982). In contrast to the temporal pH changes inside digestive vacuoles, pH in the gut of copepods varies spatially between 5.4 and 9.35 and can vary with the chemical composition of the ingested prey (Pond et al. 1995; Tang et al. 2011). pH appears to have only a minor effect on the specific proteinase activity, with the highest activity at ca. pH 6 (Freese et al. 2012). However, different proteinase classes detected in the copepod *Calanus finmarchicus* can show significant pH affinities, whereby metallo- and serine-proteinases are dominant at neutral or alkaline pH, and aspartic proteinases are dominant under acidic conditions (Solgaard et al. 2007). Additionally, some copepod gut regions are characterized by low oxygen saturation during digestion, which is also affected by the chemical composition of the prey (Tang et al. 2011). Consequently, the existence of different chemical and biological conditions in grazers may lead to the degradation of different proteins and subsequently to differences in the bioavailability of the regenerated Fe.

Organic ligands released by zooplankton contribute to the dissolved (and biologically available) Fe pool in the ocean (Hutchins et al. 1999). The equilibrium binding

\* Corresponding author: jnuester@bigelow.org

constants of natural Fe(III)-binding ligands match those of known siderophores and tetrapyrrole compounds (Hutchins et al. 1999; Witter et al. 2000), and a covariance between organic ligands and phytoplankton biomass implies a biological source (Boye et al. 2006; Gerringa et al. 2006, 2008). However, the presence of ligands related to specific cell compounds (e.g., porphyrin complexes) has not been directly measured in the open ocean, although Vong et al. (2007) measured porphyrin-like complexes in coastal seawater. Sato et al. (2007) quantified the production of organic Fe(III)-binding ligands during meso- and microzooplankton grazing experiments on natural phytoplankton populations, but the existence of Fe(II)-binding ligands has only been hypothesized (Hopkinson and Barbeau 2007; Roy et al. 2008). Yet recent measurements of Fe(II) in seawater—despite rapid oxidation to Fe(III)—point to the stabilization of Fe(II) by organic ligands and/or photochemical reduction (Croot et al. 2008; Breitbarth et al. 2009; Hansard et al. 2009).

Prior studies have used uptake of Fe and phytoplankton growth to assess the bioavailability of regenerated Fe. Hutchins et al. (1999) used model compounds to show that the uptake of iron is a function of both the nature of the complexing ligand and the phytoplankton species. Sato et al. (2007) found that ligands released during microzooplankton grazing potentially restricted the bioavailability of regenerated Fe for some autotrophs such as *Synechococcus* sp. and *Micromonas pusilla* while enhancing the growth of others (e.g., *Thalassiosira weissflogii*). Additionally, Dalbec and Twining (2009) found that the growth of *T. weissflogii*, *Emiliania huxleyi*, and *Nannochloris* sp. was similar or slightly elevated in flasks containing Fe regenerated by microzooplankton grazing than in grazer-free control flasks. These results suggest that the extent to which grazing enhances or reduces the bioavailability of Fe may depend on the prey, the grazers, and ultimately the end user of the regenerated iron.

Given the chemical and biological differences in the digestive systems of meso- and microzooplankton, this study examined whether different grazer and prey taxa affect the biological availability of regenerated iron. We performed grazing experiments using the copepod *Acartia tonsa* and the heterotrophic dinoflagellate *Oxyrrhis marina* as representatives of meso- and microzooplankton grazers, and the diatom *Thalassiosira pseudonana* and the coccolithophore *E. huxleyi* as examples of prey with distinct chemical composition. The partitioning of the radiotracer  $^{55}\text{Fe}$  into cellular, adsorbed, and regenerated fractions was followed during grazing, and the bioavailability of regenerated iron to *T. pseudonana* was compared to inorganic Fe in short-term uptake experiments.

## Methods

Grazing-induced Fe regeneration and bioavailability was assessed in a three-step experiment consisting of: (1) radiolabeling either the diatom *T. pseudonana* (CCMP 1335) or the coccolithophore *E. huxleyi* (CCMP 2668) with  $^{55}\text{Fe}$ ; (2) grazing of radiolabeled *T. pseudonana* or *E. huxleyi* by the heterotrophic dinoflagellate *O. marina* (CCMP 605)

or the copepod *A. tonsa*; and (3) uptake of regenerated and inorganic Fe by the diatom *T. pseudonana* to assess the bioavailability of remineralized Fe (Fig. 1). Radiolabeling of phytoplankton occurred in a single flask, while grazing and uptake steps were carried out in triplicate flasks to assess biological variability of these processes. All experiments were carried out in filtered seawater (FSW; seawater collected in the western North Atlantic at 25°38.023'N, 77°26.804'W) using trace-metal clean sampling techniques. The background Fe concentration in this seawater was previously determined to be  $0.87 \pm 0.07 \text{ nmol L}^{-1}$  using competitive ligand equilibrium cathodic stripping voltammetry (Dalbec and Twining 2009). Four separate grazing and remineralization experiments were conducted, and they are labeled according to the combination of grazer and prey used in the grazing experiments: I, *A. tonsa* and *T. pseudonana*; II, *A. tonsa* and *E. huxleyi*; III, *O. marina* and *T. pseudonana*; and IV, *O. marina* and *E. huxleyi*.

**Radiolabeling step**—*T. pseudonana* or *E. huxleyi* were grown in FSW amended with macronutrients, trace metals, and vitamins. Final macronutrient concentrations for *T. pseudonana* were  $100 \mu\text{mol L}^{-1}$  nitrate,  $10 \mu\text{mol L}^{-1}$  phosphate, and  $100 \mu\text{mol L}^{-1}$  silicate. Macronutrient concentrations were lowered to  $35 \mu\text{mol L}^{-1}$  nitrate and  $1.5 \mu\text{mol L}^{-1}$  phosphate for *E. huxleyi* to retain cells in a calcified state (J. Nuester unpubl. observation). Trace elements were added at final concentrations of  $2.7 \text{ nmol L}^{-1}$   $\text{ZnSO}_4$ ,  $13 \text{ nmol L}^{-1}$   $\text{MnCl}_2$ ,  $4.2 \text{ nmol L}^{-1}$   $\text{CoCl}_2$ ,  $1.1 \text{ nmol L}^{-1}$   $\text{CuSO}_4$ ,  $0.1 \mu\text{mol L}^{-1}$   $\text{Na}_2\text{MoO}_4$ , and  $10 \text{ nmol L}^{-1}$   $\text{Na}_2\text{SeO}_3$ . The final concentration of Fe ranged from  $190$  to  $217 \text{ nmol L}^{-1}$  and consisted of approximately equal proportions of stable  $\text{FeCl}_3$  and  $^{55}\text{FeCl}_3$ . The speciation of the added trace metals was buffered by  $100 \mu\text{mol L}^{-1}$  ethylenediaminetetraacetic acid (EDTA). Vitamin stocks were added to achieve final concentrations of  $29.7 \mu\text{mol L}^{-1}$  thiamine,  $2.25 \text{ nmol L}^{-1}$  biotin, and  $0.37 \text{ nmol L}^{-1}$  cyanocobalamin. The culture media were then filter-sterilized using  $0.2 \mu\text{m}$  polycarbonate filter membranes and allowed to equilibrate overnight. A sample for total radioactivity was taken prior to inoculation with either *T. pseudonana* or *E. huxleyi*.

Cells were radiolabeled with  $^{55}\text{Fe}$  in 1 liter polycarbonate bottles over 5 d at  $16^\circ\text{C}$  under a 14:10 light:dark (LD) cycle at  $91 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ . Cell counts were taken daily using a hemacytometer. At the end of the radiolabeling period, samples were taken for total particulate Fe ( $\text{Fe}_{\text{part}}$ ) and intracellular Fe ( $\text{Fe}_{\text{cell}}$ ) measurements, in addition to samples for cell counts.  $\text{Fe}_{\text{part}}$  was measured by filtering cells in 10 mL of media onto  $1 \mu\text{m}$  polycarbonate filter membranes and rinsing three times with FSW. For measurements of  $\text{Fe}_{\text{cell}}$ , cells were filtered onto  $1 \mu\text{m}$  polycarbonate filter membranes that were subsequently soaked for 10 min in an oxalate-EDTA reagent to remove extracellular adsorbed Fe prior to the rinses with FSW (Tovar-Sanchez et al. 2003). In preparation for the grazing step, phytoplankton cells from 400–800 mL of culture media were filtered at  $< 100 \text{ mm Hg}$  onto  $1 \mu\text{m}$  polycarbonate filter membranes, soaked for 10 min in the oxalate-EDTA reagent, rinsed three times with FSW, and

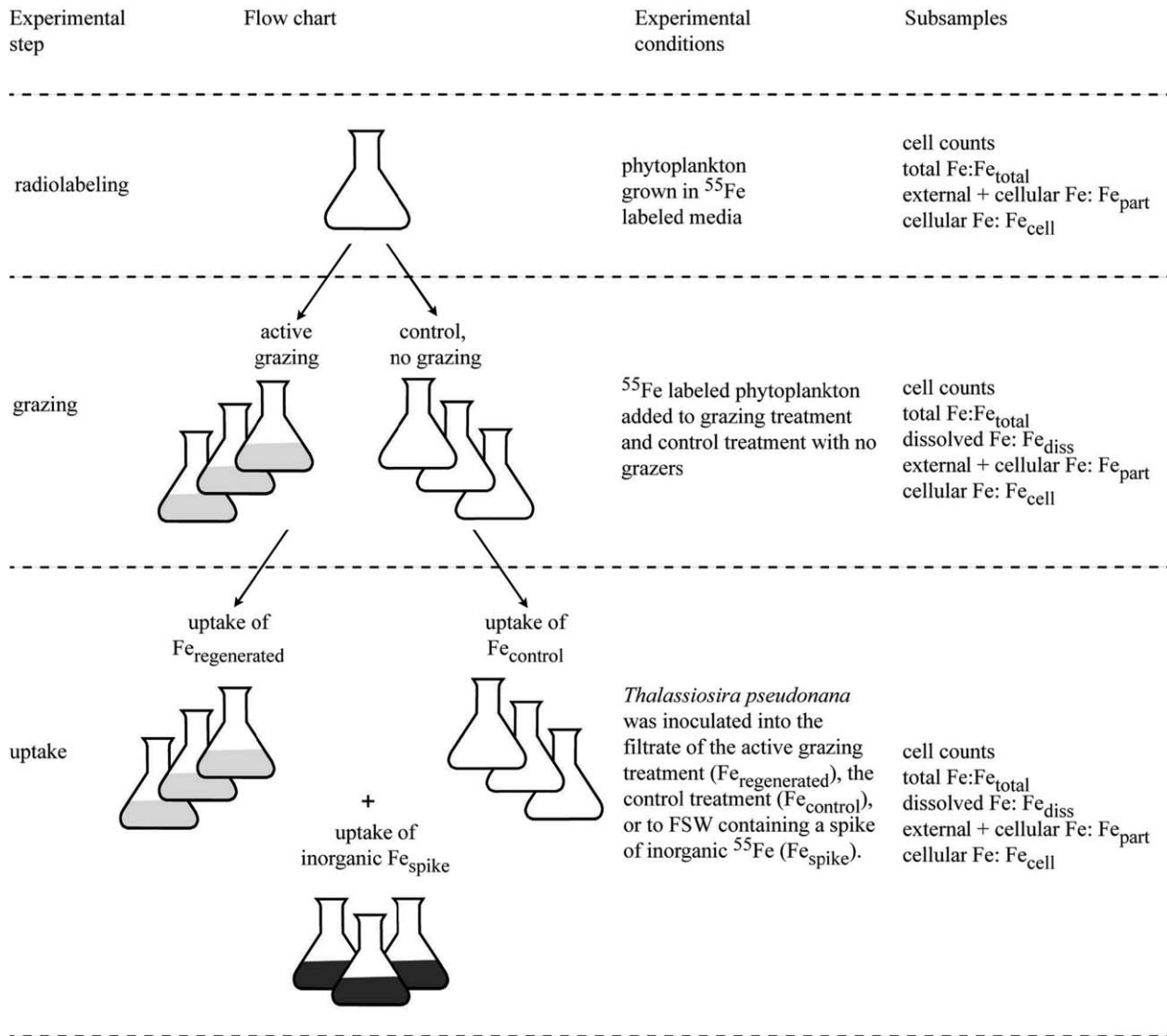


Fig. 1. Schematic outline of the experimental setup. The radiolabeling step occurred in a single flask, while the treatments of the grazing and uptake steps have been measured in triplicate.

subsequently resuspended in 50 mL of FSW. The growth conditions for each experimental treatment are summarized in Table 1.

*Grazing step*—For microzooplankton grazing experiments, *O. marina* were concentrated by centrifugation at  $700 \times g$  for 20 min. Protists were resuspended in 50 mL

FSW and added to each grazing flask to achieve a final concentration of ca. 3000 cells  $\text{mL}^{-1}$ . For mesozooplankton grazing experiments, *A. tonsa* were individually picked from laboratory cultures, transferred to FSW, and allowed to clear their guts for 1 h. *A. tonsa* were then re-picked, washed, and placed into grazing treatment flasks filled with FSW at a final concentration of 60 animals in 100 mL.

Table 1. Experimental conditions during the radiolabeling step of phytoplankton. Intracellular and total cellular Fe were measured after 5 d of labeling with  $^{55}\text{Fe}$ . Intracellular Fe was measured on cells soaked in an oxalate solution to remove externally bound Fe prior to counting. Total cellular Fe was measured on cells rinsed with seawater to remove any remaining dissolved Fe on the filter. All experiments were carried out in single flasks. nd—not determined.

Expt	Phytoplankton	Stable Fe (nmol $\text{L}^{-1}$ )	$^{55}\text{Fe}$ (nmol $\text{L}^{-1}$ )	$\text{Fe}_{\text{total}}$ (nmol $\text{L}^{-1}$ )	Growth rate ( $\text{d}^{-1}$ )	Intracellular Fe ( $\text{Fe}_{\text{cell}}$ ; amol $\text{cell}^{-1}$ )	Total cellular Fe ( $\text{Fe}_{\text{part}}$ ; amol $\text{cell}^{-1}$ )
I	<i>T. pseudonana</i>	100	117	217	0.92	22.2	81.9
II	<i>E. huxleyi</i>	100	104	204	0.38	11.1	nd
III	<i>T. pseudonana</i>	100	90	190	1.01	17.2	81.2
IV	<i>E. huxleyi</i>	100	104	204	0.36	10.5	40.0



Regeneration of cellular Fe was initiated by adding resuspended  $^{55}\text{Fe}$ -labeled *T. pseudonana* or *E. huxleyi* cells to each grazing flask at cell concentrations between 50,000 and 120,000 cells  $\text{mL}^{-1}$ . Phytoplankton cell counts, and total ( $\text{Fe}_{\text{total}}$ ) and dissolved Fe ( $\text{Fe}_{\text{diss}}$ ) were taken immediately after addition of phytoplankton. Flasks were incubated for 24 h in the dark at  $16^\circ\text{C}$  and sampled again for cell concentration,  $\text{Fe}_{\text{total}}$ , and  $\text{Fe}_{\text{diss}}$ . As no death of either *O. marina* or *A. tonsa* was observed during the grazing period, we assume that the grazing conditions did not induce stress. Grazing rates were calculated both based on the number of cells consumed (cell concentration-based grazing rates [CGR]: cells grazer $^{-1}$  d $^{-1}$ ) and biovolume consumed (biovolume-based grazing rates [BGR]:  $\mu\text{m}^3$  grazer $^{-1}$  d $^{-1}$ ), based on cell volume measurements from a Beckman Multisizer 3 Coulter counter.

Dissolved Fe ( $\text{Fe}_{\text{diss}}$ ) within the grazing media was measured on 500  $\mu\text{L}$  passed through a 0.2  $\mu\text{m}$  syringe filter (Acrodisk with Supor membrane). Total Fe ( $\text{Fe}_{\text{total}}$ ) within the grazing media was measured on 500  $\mu\text{L}$  unfiltered grazing media. The sample was added to 3 mL of scintillation cocktail and counted using a liquid scintillation counter. Parallel control flasks with similar phytoplankton cell density but excluding grazers were also sampled. Cells were then filtered out of each treatment and control flask at  $< 100$  mm Hg ( $< 2$  min) using 0.2  $\mu\text{m}$  polycarbonate filter membranes. Filtrates of triplicate treatments and control flasks were combined and homogenized separately prior to being used in the uptake step. The pathway through which dissolved Fe is regenerated may include direct egestion of dissolved Fe species, egestion of particulate matter from which dissolved Fe is subsequently released, or even sloppy feeding. The experiment design used here cannot distinguish between these potential pathways.

**Uptake step**—*T. pseudonana* cells used in the uptake step were pre-acclimated to the same conditions used in the labeling phase, but Fe was lowered to 100 nmol  $\text{L}^{-1}$  and no  $^{55}\text{Fe}$  was added. Cells were grown axenically for 6 d, preconcentrated by filtering 500 mL of culture onto 1  $\mu\text{m}$  polycarbonate filter membranes, and resuspended in 50 mL FSW. An aliquot (5 mL) was then added to flasks containing either the filtrate of the grazing treatment ( $\text{Fe}_{\text{regenerated}}$ ), the filtrate of the grazing control ( $\text{Fe}_{\text{control}}$ ), or FSW to which inorganic  $^{55}\text{Fe}$  (in 0.01 mol  $\text{L}^{-1}$  HCl) was added (1–3 nmol  $^{55}\text{Fe}$ ;  $\text{Fe}_{\text{spike}}$ ). Initial concentrations of *T. pseudonana* in the treatments were between 200,000 and 400,000 cells  $\text{mL}^{-1}$ . Cells were incubated for up to 20 h at  $16^\circ\text{C}$  under a 14:10 LD cycle at 91  $\mu\text{mol}$  quanta  $\text{m}^{-2}$   $\text{s}^{-1}$ . Samples were collected for  $\text{Fe}_{\text{total}}$ ,  $\text{Fe}_{\text{diss}}$ ,  $\text{Fe}_{\text{part}}$ , and  $\text{Fe}_{\text{cell}}$  measurements at discrete time points between 10 min and 20 h. To minimize any effects of regenerated macronutrients, the uptake rates were calculated only for the first 30 min.

**Sample analysis**— $^{55}\text{Fe}$  was quantified in 5 mL scintillation vials containing 3 mL of scintillation cocktail (Ecolume) using a Perkin-Elmer Tri-Carb 2900. Samples were counted for 5 min, and counting errors were typically  $< 5\%$ . Both radioactive and stable Fe in the different media were subsequently used to calculate the specific activity (Bq

$\text{mol}^{-1}$ ) of each sample. The specific activities were used in each step of the experiment. Cell concentrations of *T. pseudonana* and *E. huxleyi* were determined with a hemacytometer using light microscopy. To minimize counting error at least 50 cells were counted, but at cell concentrations below 50,000 cells  $\text{mL}^{-1}$  the precision dropped to  $\pm 40\%$ . Mean cell concentrations, extracellular adsorption, intracellular Fe accumulation, and Fe uptake rates were compared using a one-way ANOVA in combination with a Student *t*-test between treatments. Tests of equal variance were performed (Levene test), and in cases of unequal variance a Welch ANOVA (which does not assume equal variance) was used. Temporal changes in extracellular adsorption were analyzed statistically by linear regression. All tests were performed using the software JMP (SAS).

## Results

During the labeling phase *T. pseudonana* and *E. huxleyi* were radiolabeled with  $^{55}\text{Fe}$  and grown for 5 d to concentrations of ca.  $10^6$  and  $2 \times 10^5$  cells  $\text{mL}^{-1}$ , respectively. The growth rates of *E. huxleyi* were 0.38 d $^{-1}$  (experiment [expt] II) and 0.36 d $^{-1}$  (expt IV), ca. 2.5 times lower than the growth rates of *T. pseudonana* (0.92 d $^{-1}$  [expt I] and 1.01 d $^{-1}$  [expt III]; Table 1). During the growth period *E. huxleyi* and *T. pseudonana* accumulated 1% and 10% of the total Fe, respectively. At the end of the uptake period, oxalate-rinsed intracellular Fe ( $\text{Fe}_{\text{cell}}$ ) was 21–27% of the total cell-associated iron ( $\text{Fe}_{\text{part}}$ ).  $\text{Fe}_{\text{cell}}$  for *E. huxleyi* and *T. pseudonana* were 10.5 or 11.2 amol cell $^{-1}$  and 17.2 or 22.2 amol cell $^{-1}$ , respectively (Table 1).

During the grazing step, *O. marina* and *A. tonsa* actively ingested and remineralized Fe associated with prey cells. In control treatments without grazers, *T. pseudonana* grew while *E. huxleyi* cell concentrations decreased (Fig. 2). Observed cell loss or gain in the control treatments was used to correct the grazing rates. Grazing rates (CGR) on *T. pseudonana* were 3 and 6 times the grazing rates on *E. huxleyi* (Table 2) for *O. marina* and *A. tonsa*, respectively. *A. tonsa* ingested  $107,000 \pm 14,600$  *T. pseudonana* cells grazer $^{-1}$  d $^{-1}$  and  $17,400 \pm 9620$  *E. huxleyi* cells grazer $^{-1}$  d $^{-1}$ . The CGR for *O. marina* were lower, ca.  $30 \pm 8$  and  $11 \pm 2$  cells grazer $^{-1}$  d $^{-1}$  for *T. pseudonana* and *E. huxleyi*, respectively (Table 2). However, grazing rates of *O. marina* on the two prey were indistinguishable when calculated on the basis of prey biovolume (BGR) rather than cell number ( $p = 0.193$ ). In contrast, *A. tonsa* ingested significantly less *E. huxleyi* biovolume than *T. pseudonana* biovolume ( $p = 0.010$ ; Table 2; Fig. 3).

Iron concentrations were higher in grazing experiments containing *T. pseudonana* than in those with *E. huxleyi*. *A. tonsa* regenerated  $2.68 \pm 0.87$  or  $0.48 \pm 0.03$  nmol Fe  $\text{L}^{-1}$  when grazing on *T. pseudonana* or *E. huxleyi*, while *O. marina* grazing released  $1.21 \pm 0.10$  or  $0.87 \pm 0.10$  nmol Fe  $\text{L}^{-1}$  from *T. pseudonana* and *E. huxleyi* (Table 2). In grazer-free control treatments only  $0.19 \pm 0.003$  nmol Fe  $\text{L}^{-1}$  (expt I) and  $0.055 \pm 0.013$  nmol Fe  $\text{L}^{-1}$  (expt III) was released from *T. pseudonana*. In contrast,  $0.30 \pm 0.1$  nmol Fe  $\text{L}^{-1}$  was released in control treatments containing *E. huxleyi* (expts II, IV). The proportion of Fe released in

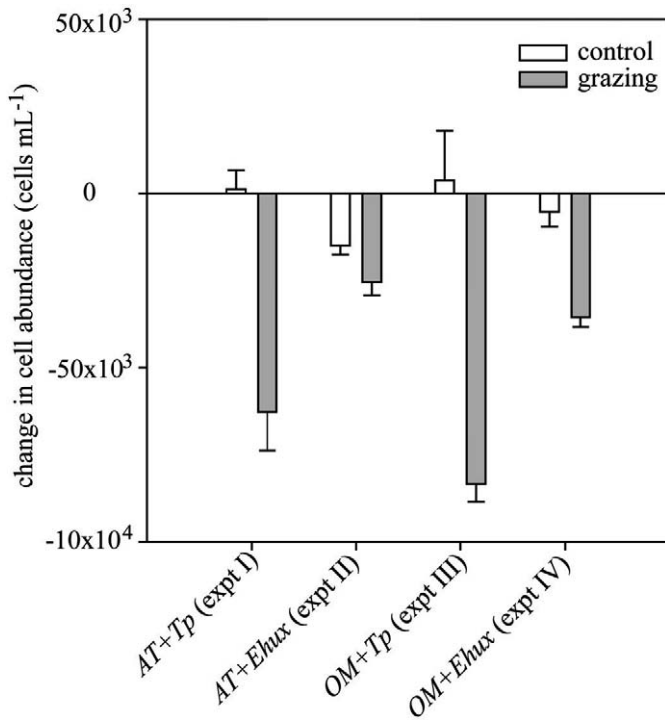


Fig. 2. Calculation of the change in cell abundance in grazing and control treatments after 24 h. Negative numbers indicate cell loss in grazing treatments of expts I-IV and control treatments of experiments with *Emiliania huxleyi* (expts II, IV). Positive numbers indicate cell growth in control treatments of experiments with *Thalassiosira pseudonana* (expts I, III). Bars are means ( $\pm$  SD) of triplicate flasks. Abbreviations: *Tp*, *T. pseudonana*; *Ehux*, *E. huxleyi*; *AT*, *Acartia tonsa*; *OM*, *Oxyrrhis marina*.

control relative to grazing treatments was 5% to 7% for experiments with *T. pseudonana*, but 32% to 63% for experiments with *E. huxleyi*. Relatively higher Fe release from *E. huxleyi* in control treatments corresponds with the observed decrease in cell concentration during the control treatment duration (Fig. 2). The ca. 40% increase in Fe regeneration by *O. marina* when feeding on *T. pseudonana* than on *E. huxleyi* can be explained by differences in BGR (Table 2). In contrast, the ca. 5.8-fold higher regeneration of Fe in experiments of *A. tonsa* feeding on *T. pseudonana* rather than on *E. huxleyi* may be explained by the ca. 3-times difference of BGRs and 2-fold difference in Fe quotas between the two prey organisms (Tables 1, 2).

The bioavailability of remineralized Fe was assessed via uptake by *T. pseudonana*. Clear differences in the initial uptake of various forms of Fe were observed (Fig. 4). The initial uptake rate of Fe<sub>regenerated</sub> was highest in experiments where Fe was regenerated from *T. pseudonana* cells ( $7.59 \pm 1.34$  amol cell<sup>-1</sup> h<sup>-1</sup> [expt I] and  $2.91 \pm 0.58$  amol cell<sup>-1</sup> h<sup>-1</sup> [expt III]) and notably lower when Fe was regenerated from *E. huxleyi* ( $0.99 \pm 0.03$  amol cell<sup>-1</sup> h<sup>-1</sup> [expt II]; Table 3). The uptake rate of Fe released to the filtrate in grazer-free control experiments was even lower (0.15–0.3 amol cell<sup>-1</sup> h<sup>-1</sup>). The uptake rate of Fe<sub>spike</sub> varied between  $0.79 \pm 0.16$  to  $2.45 \pm 0.37$  amol cell<sup>-1</sup> h<sup>-1</sup> and was significantly higher than the uptake rate of Fe<sub>control</sub> in expts

Table 2. Summary of the grazing step. Cellular grazing rates were calculated taking into account growth and cell loss in control treatments. Conversion of cellular grazing rate (CGR) to biovolume grazing rate (BGR) was based on size measurements of unlabeled *T. pseudonana* and *E. huxleyi* cells using a Coulter counter cell counter. The upper and lower numbers provided for each parameter are the mean and standard deviation (SD), respectively.

Expt	Grazer	Phytoplankton (prey)		Grazing rate (CGR) (cells grazer <sup>-1</sup> d <sup>-1</sup> )		Grazing rate (BGR) ( $\mu$ m <sup>3</sup> grazer <sup>-1</sup> d <sup>-1</sup> )		Regenerated Fe (nmol L <sup>-1</sup> )		Fe release in control experiments (nmol L <sup>-1</sup> )	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
I	<i>A. tonsa</i>	0.60	17,900	107,000	14,600	9,395,000	2,593,000	2.68	0.87	0.19	0.003
II	<i>A. tonsa</i>	0.60	6170	17,400	9620	2,337,000	1,295,000	0.48	0.03	0.30	0.10
III	<i>O. marina</i>	2940	3820	30.0	8.30	2081	575	1.21	0.10	0.055	0.013
IV	<i>O. marina</i>	2769	477	11.2	2.12	1502	285	0.87	0.10	0.30	0.14

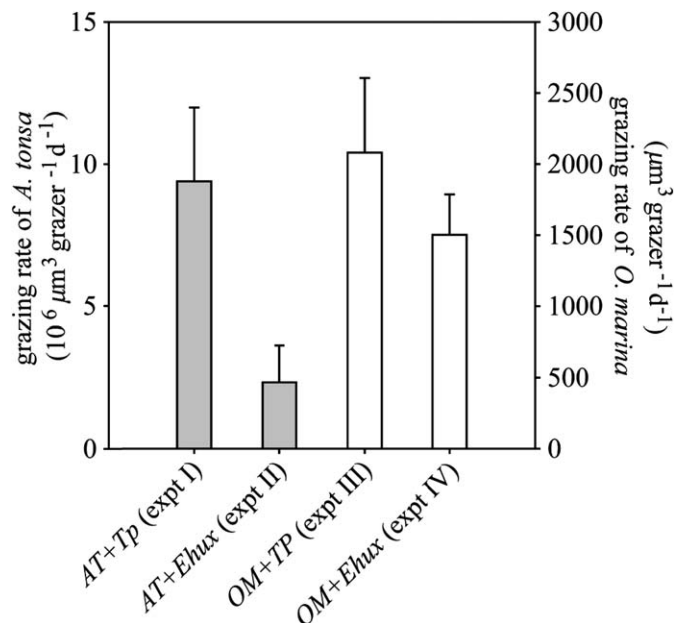


Fig. 3. Comparison of biovolume grazing rates (BGR) between *A. tonsa* and *O. marina*. Bars are means ( $\pm$  SD) of triplicate flasks.

II and III ( $p < 0.01$ ), but undistinguishable from the uptake rate of  $\text{Fe}_{\text{control}}$  in expt I ( $p = 0.31$ ). The uptake rates of  $\text{Fe}_{\text{spike}}$  and  $\text{Fe}_{\text{regenerated}}$  were undistinguishable in expt III ( $p = 0.2$ ), but the uptake rate of  $\text{Fe}_{\text{spike}}$  was significantly lower than that of  $\text{Fe}_{\text{regenerated}}$  in expt I ( $p < 0.001$ ) and expt II ( $p = 0.045$ ). Fe regenerated from grazing on *T. pseudonana* (expts I, III) was taken up faster than Fe regenerated from grazing on *E. huxleyi* (expt II;  $p < 0.032$ ).

The effects of the grazer and prey combinations on the bioavailability of Fe was further assessed after normalizing initial uptake rates to ambient Fe concentrations in the uptake experiments (Fig. 5). The mean specific uptake rates of Fe regenerated by *A. tonsa* were 7.3- and 4.1-fold faster than uptake of inorganic Fe ( $p < 0.001$ ). In contrast, the specific uptake rates of regenerated Fe produced by *O. marina* feeding on *T. pseudonana* were not significantly different from the uptake rate of inorganic Fe ( $p = 0.65$ ; Table 3). Fe released in grazer-free controls was taken up 3.5-fold slower than Fe regenerated by *A. tonsa* ( $p = 0.001$ ). The specific uptake rate of Fe released in the control treatments of expt III was higher than  $\text{Fe}_{\text{regenerated}}$  or  $\text{Fe}_{\text{spike}}$  ( $p < 0.02$ ), but this was driven by extremely low ambient Fe in this treatment (Fig. 5). Additionally, a comparison of the ratio of the specific uptake rates of regenerated to inorganic Fe between expt I and expt II revealed that the digestion of the diatom *T. pseudonana* by *A. tonsa* produced regenerated Fe that was taken up  $1.8 \pm 0.5$ -fold faster than Fe released during grazing of *A. tonsa* on the coccolithophore *E. huxleyi* ( $p = 0.035$ ; Fig. 5). Similarly, the ratio of the specific uptake rates of regenerated to inorganic Fe revealed that Fe produced during grazing of *A. tonsa* on *T. pseudonana* was taken up  $5.8 \pm 1.8$ -fold faster than Fe released during grazing of *O. marina* on *T. pseudonana* ( $p = 0.003$ ).

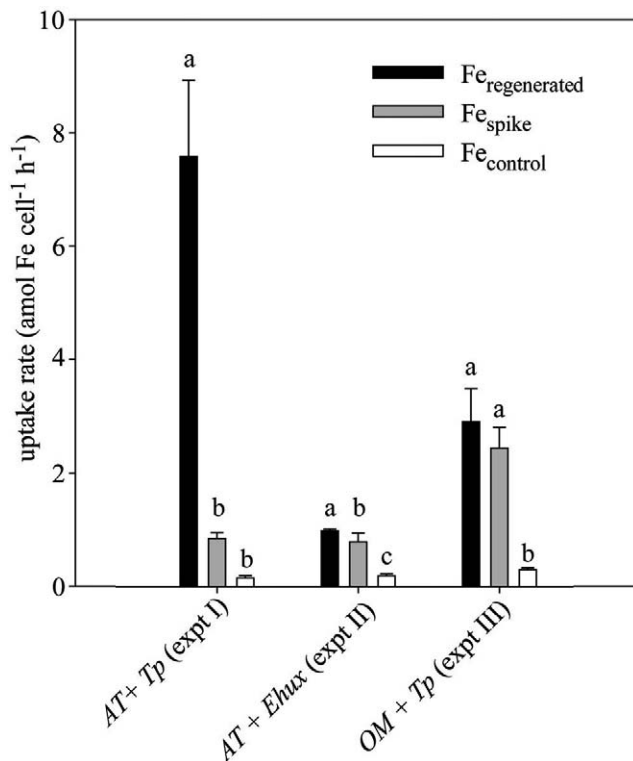


Fig. 4. Comparison of intracellular uptake rates of Fe to *T. pseudonana* during the uptake step within the first 30 min after inoculation. Intracellular Fe was measured after removal of extracellular adsorbed Fe using an oxalate wash treatment. Bars are means ( $\pm$  SD) of triplicate samples. a, b, c are used to describe statistical significance between treatments. Treatments connected by the same letter are not significantly different. Welch ANOVA for all three experiments is  $< 0.05$ .

Fe regenerated during grazing was also less likely to adsorb to phytoplankton cell surfaces than added inorganic Fe (Fig. 6). Independent of the grazer–prey combination, a significantly higher proportion of total cellular Fe was found to be adsorbed instead of internally accumulated when Fe was provided as inorganic Fe ( $\text{Fe}_{\text{spike}}$ ) than when Fe had been biologically regenerated ( $p < 0.036$ ). The exceptions were Fe regenerated by *A. tonsa* and *O. marina* grazing on *T. pseudonana*, where undistinguishable proportions of  $\text{Fe}_{\text{regenerated}}$  and  $\text{Fe}_{\text{spike}}$  were adsorbed at 0.17 h ( $p = 0.57$ ) and 4 h ( $p = 0.67$ ), respectively. The fraction of  $\text{Fe}_{\text{regenerated}}$  adsorbed to cells decreased over time in expts II and III, although the decrease was only slightly significant in expt II ( $p = 0.067$ ). In contrast, no temporal change in the fraction of  $\text{Fe}_{\text{regenerated}}$  adsorbed to cells was observed in expt I ( $p = 0.20$ ). The fraction of adsorbed  $\text{Fe}_{\text{spike}}$  decreased only slightly over time in all experiments ( $p < 0.058$ ).

## Discussion

These experiments demonstrate that phytoplankton grazing by both meso- and microzooplankton produces regenerated Fe that is readily available to phytoplankton. Our findings complement and extend previous work

Table 3. Summary of the uptake step. Specific uptake rates were calculated by dividing the uptake rate by ambient dissolved Fe concentrations at  $t_0$  of the uptake step. The upper and lower numbers provided for each parameter are the mean and standard deviation (SD), respectively.

Expt	Grazer–prey combination	Experimental treatment	Dissolved Fe at $t_0$ (nmol L <sup>-1</sup> )		Uptake rate (amol cell <sup>-1</sup> h <sup>-1</sup> )		Specific uptake rate (nmol Fe L cell <sup>-1</sup> h <sup>-1</sup> )	
			Mean	SD	Mean	SD	Mean	SD
I	<i>A. tonsa</i> <i>T. pseudonana</i>	Fe <sub>regenerated</sub>	2.68	0.87	7.59	1.34	2.83	0.45
		Fe <sub>spike</sub>	2.20	0.11	0.85	0.11	0.39	0.06
		Fe <sub>control</sub>	0.187	0.003	0.15	0.04	0.81	0.24
II	<i>A. tonsa</i> <i>E. huxleyi</i>	Fe <sub>regenerated</sub>	0.48	0.03	0.99	0.03	2.04	0.14
		Fe <sub>spike</sub>	1.55	0.12	0.79	0.16	0.5	0.08
		Fe <sub>control</sub>	0.30	0.10	0.19	0.04	0.63	0.10
III	<i>O. marina</i> <i>T. pseudonana</i>	Fe <sub>regenerated</sub>	1.21	0.10	2.91	0.58	2.38	0.39
		Fe <sub>spike</sub>	1.30	0.02	2.44	0.37	1.89	0.30
		Fe <sub>control</sub>	0.06	0.012	0.30	0.03	5.68	2.14

describing Fe regeneration by meso- (Hutchins et al. 1993, 1995; Hutchins and Bruland 1994) and microzooplankton (Barbeau et al. 1996, 2001; Dalbec and Twining 2009). Overall, Fe regenerated by the copepod *A. tonsa* was more bioavailable to phytoplankton than inorganic Fe. In contrast, the availability of Fe<sub>regenerated</sub> produced by the protist grazer was not enhanced relative to inorganic Fe. Further, prey type affected Fe availability, with Fe regenerated by copepod grazing on *T. pseudonana* taken up faster than Fe released during copepod feeding on *E. huxleyi*. These findings suggest that ecosystem structure (i.e., predator–prey combinations) plays a significant role in regulating the availability of regenerated Fe.

The rate of Fe uptake by phytoplankton is partly a function of chemical speciation. Most available data suggest that organic chelation of Fe(III) lowers the rate of cellular Fe uptake in comparison to inorganic Fe (Shaked et al. 2005; Sato et al. 2007). However, dissolved Fe in seawater is almost entirely (> 99.9%) chelated by organic ligands (Gledhill and Buck 2012). The exact nature of these ligands is still unknown but includes high-affinity siderophores and weaker ligands such as humic acids, extracellular polymeric acids, and transparent copolymer-like compounds (Gledhill and Buck 2012). Additionally, multiple lines of evidence are emerging that link biological activity to ligand production (Hunter and Boyd 2007; Gledhill and Buck 2012). Shipboard experiments during Subarctic Ecosystem Response to Iron Enrichment Study II (SEEDS II) revealed that Fe ligand concentrations increased during meso- and microzooplankton grazing while chlorophyll biomass was consumed (Sato et al. 2007). The conditional stability constants of these ligands were intermediate between the values of the strong and weak ligands detected by Rue and Bruland (1995). Bioavailability experiments suggested that ligands released during microzooplankton grazing experiments limited the availability of Fe, resulting in lower growth of the cyanobacterium *Synechococcus* sp. and the prasinophyte *Micromonas pusilla* (Sato et al. 2007). However, the growth of the centric diatom *Thalassiosira weissflogii* was similar in media containing the natural ligands, inorganic Fe, or added

protoporphyrin IX (Sato et al. 2007). This result is comparable to our own results that did not show differences between uptake of inorganic and microzooplankton-generated Fe species by the centric diatom *T. pseudonana*. The data of Sato et al. (2007) suggest that Fe regenerated during grazing is at least partially bound to

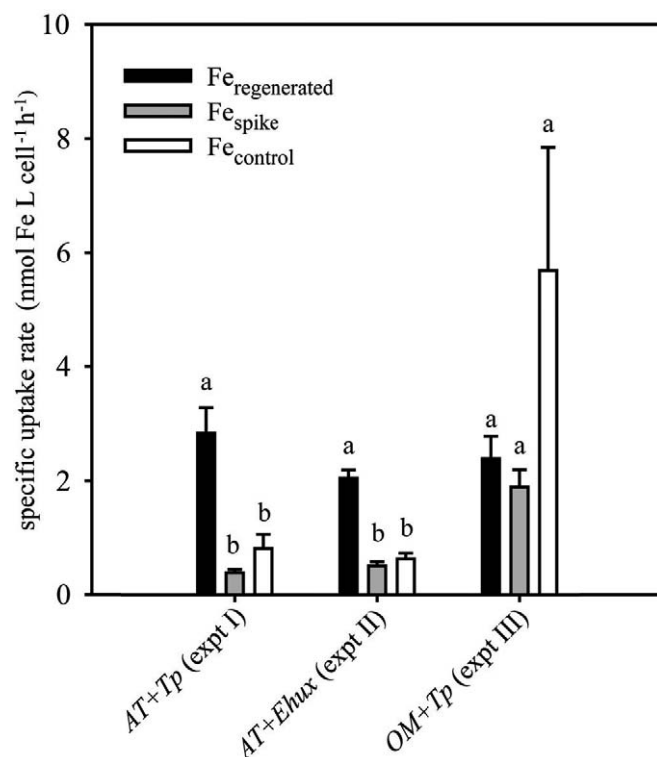


Fig. 5. Comparison between specific uptake rates of *T. pseudonana* during the uptake step for regenerated Fe (Fe<sub>regenerated</sub>), inorganic Fe (Fe<sub>spike</sub>), and Fe released during control treatments with no grazers (Fe<sub>control</sub>). Specific uptake rates have been normalized to ambient Fe of the uptake step. Bars are means ( $\pm$  SD) of triplicate flasks. a, b, c are used to describe statistical significance between treatments. Treatments connected by the same letter are not significantly different. Welch ANOVA for all three experiments is  $< 0.05$ .



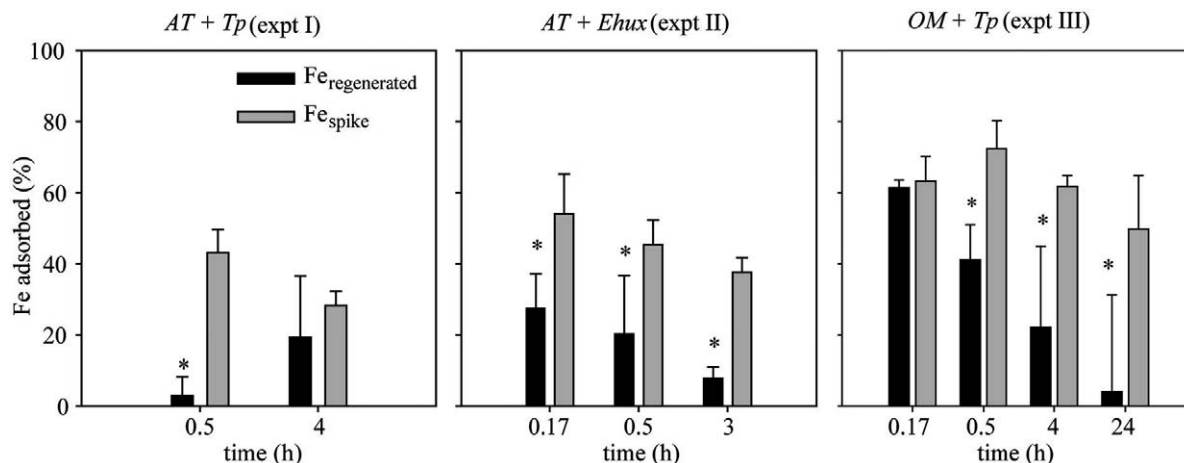


Fig. 6. Comparison of extracellular Fe adsorption to *T. pseudonana* cells over time during the uptake step between regenerated ( $\text{Fe}_{\text{regenerated}}$ ) and inorganic Fe ( $\text{Fe}_{\text{spike}}$ ). Adsorbed Fe has been calculated as the difference between total particulate Fe and intracellular Fe. Bars are means ( $\pm$  SD) of triplicate flasks. An asterisk indicates significance between treatments.

organic ligands, which may be expected to reduce biological availability. Hence, it is surprising that our results indicate that regenerated Fe is taken up in some instances several times faster than inorganic Fe.

Differences in speciation between inorganic and regenerated Fe were also suggested by the quantity of adsorbed Fe. With the exception of two time points, the fraction of cellular Fe adsorbed to the cell surface was higher for inorganic than regenerated Fe. These data support the conclusion that the chemical speciation of regenerated Fe is more favorable for internal uptake by *T. pseudonana* than inorganic Fe.

A potential class of ligands regenerated during grazing are tetrapyrroles (Hutchins et al. 1999; Mochizuki et al. 2010). Tetrapyrroles include porphyrin compounds (e.g., chlorophyll, protoporphyrin IX, phaeophorbide, hemes, and phaeophytin) that include some of the strongest Fe chelators within cells. Hutchins et al. (1999) used commercially available molecules of phaeophytin, phaeophorbide, and protoporphyrin IX to show that eukaryotic phytoplankton might be able to take up Fe(III) bound to dissolved grazing products at least as fast as inorganic Fe. Our observations of faster Fe uptake of naturally derived grazing compounds are consistent with the results of Hutchins et al. (1999). However, others have found that protoporphyrin IX is a good chelator for Fe(II) but hardly binds Fe(III) in seawater at pH 8 (Rijkenberg et al. 2006), calling into question the use of protoporphyrin IX as a model compound for regenerated Fe(III). Nevertheless, measurements of Fe(II) in certain regimes (Gledhill and Van Den Berg 1995; Blain et al. 2008; Hansard et al. 2009) have stimulated the discussion of Fe(II)-binding ligands (Breitbarth et al. 2009; Statham et al. 2012). If present, such organic ligands could inhibit the oxidation of Fe(II) (Theis and Singer 1974; Santana-Casiano et al. 2000). The nature of Fe(II)-binding ligands in the ocean is not yet known, but we speculate that protoporphyrin IX or other tetrapyrroles released during grazing may stabilize Fe(II) in seawater.

The release of ferrous Fe from phytoplankton cells during zooplankton grazing is potentially a contributing

factor for enhanced bioavailability of regenerated Fe. Such release of Fe(II) been hypothesized (Hutchins and Bruland 1994) but has not yet been verified. Most Fe in prey cells is bound to numerous metalloproteins, heme compounds, or Fe-S molecules and can exist in either ferrous or ferric states (Raven 1988; Fraústo Da Silva and Williams 2001). An increase in the regeneration of ferrous over ferric Fe would lead to more rapid uptake of Fe. For example, Sutak et al. (2012) showed that several phytoplankton species (including *T. pseudonana*) can use both ferrous and ferric species as an Fe source, although ferrous Fe was always taken up more rapidly. The suggestion that grazing may lead to the release of more Fe(II) relative to Fe(III) would be consistent with the observed faster uptake rates of regenerated iron in comparison to inorganic Fe(III) in some experiments.

Differences in the availability of Fe regenerated by unique grazer-prey combinations suggest differing speciation. The speciation of regenerated Fe in meso- and microzooplankton is most likely affected by chemical conditions inside the respective digestive systems. Copepod guts and protist digestive vacuoles differ in pH and oxygen saturation. The overall gut pH of copepods appears to be less acidic than the digestive vacuoles of protists (Fok et al. 1982; Pond et al. 1995; Tang et al. 2011). Although a pH as low as 3, as observed during the fusion of digestive vacuoles with lysosomes, could contribute potentially to the stabilization and release cellular ferrous Fe, the subsequent pH rise to 7 during the active digestion stage, which is characterized by the release of digestive enzymes (Fok et al. 1982), makes the egestion of Fe(II) less likely. In contrast, there is a higher probability of egestion of Fe(II) from copepod guts because the urosome-metasome transition zone of the copepod gut becomes anoxic during the digestion process (Tang et al. 2011). Even though pH in the copepod guts does not decrease to levels observed in digestive vacuoles, pH in the urosome of the copepod gut has been observed to be as low as 5.4 (Tang et al. 2011), which would prolong the residence time of ferrous Fe released under anoxic conditions even as the dissolved



oxygen concentration rises towards the anal opening of the copepod gut. These considerations provide evidence that chemical variations within digestive systems may contribute to the redox speciation of egested Fe.

The chemical composition of prey may additionally affect conditions within the gut and likely influences the speciation of remineralized Fe. For example, cells with calcium carbonate shells may buffer pH changes within copepod guts. Previous studies have shown that the chemical composition of ingested prey can lead to changes in the gut pH of copepods (Pond et al. 1995; Tang et al. 2011). Ingestion of *T. weissflogii* by *Calanus* species led to a lower gut pH (pH = 5.44–6.14: Tang et al. [2011]; pH = 6.46–8.10: Pond et al. [1995]) than ingestion of *E. huxleyi* (pH = 7.46–8.93: Pond et al. [1995]). These observations suggest that ingestion of prey with a different chemical composition may lead to alterations of the acidity in the guts of copepods, and consequently may affect the activity of different digestive enzymes. In general, calcium carbonate coccoliths of coccolithophores dissolve under acidic conditions and may subsequently act as a pH buffer, whereas the silicate frustule of diatoms does not dissolve at pH < 7 (Lewin 1961; Schlüter and Rickert 1998), and therefore would not affect pH. However, minute silica dissolution cannot be ruled out because slightly alkaline conditions can also be found within copepod guts (Pond et al. 1995). Additionally, the ingestion of different prey also affects the degree of oxygen saturation within different parts of the copepod gut. Feeding a copepod with the diatom *T. weissflogii* did not only decrease the pH, but also led to a stronger oxygen gradient than feeding with a cryptophyte (Tang et al. 2011). The combination of lower pH and extended oxygen undersaturation inside the gut, and also a short gut passage time when feeding on a diatom prey (Besiktepe and Dam 2002), may cause copepod grazers to release more digestive fluid of a lower pH, resulting in a higher Fe(II):Fe(III) ratio, potentially increasing the residence time of egested ferrous Fe into the surrounding seawater.

Mechanical degradation prior to chemical digestion may further affect Fe speciation and resulting availability. Copepods may crush their phytoplankton diet prior to chemical digestion (Michels et al. 2012). Crushed phytoplankton material may subsequently be more prone to chemical digestion than if the digestion is solely carried out chemically as in digestive vacuoles of microzooplankton. Matching this, the specific uptake ratio of regenerated to inorganic Fe was 5.76-fold lower when Fe was regenerated by *O. marina* grazing on *T. pseudonana* than when Fe was regenerated by grazing of *A. tonsa* on *T. pseudonana*. Similar, if mechanical predigestion makes prey material more reactive by increasing the specific surface area, it is also conceivable that some calcium carbonate of crushed coccoliths will dissolve in the gut of copepods and therefore contribute to the pH buffering of copepod guts as hypothesized above. This potential buffering of the gut pH during grazing of coccolithophores may lead to differences in Fe speciation and lower bioavailability when compared to grazing of diatoms. Overall, the differences in the specific uptake rates are a strong indication of

differences in Fe speciation that lead to differences in bioavailability.

This study shows that community composition can play a pivotal role in Fe regeneration. Since up to 90% of Fe used by phytoplankton may be regenerated through grazing processes (Boyd et al. 2005), the physiologies of the grazers and prey are important factors determining the degree of bioavailability of recycled iron. Taxonomic differences in uptake of regenerated Fe were not addressed in this study, but previous work (Sato et al. 2007; Dalbec and Twining 2009) suggests differences in uptake of naturally produced regenerated Fe among eukaryotic and prokaryotic phytoplankton. These differences in bioavailability are likely due to the fact that different phytoplankton phyla may access different Fe species using different cellular pathways that might include a prerequisite reduction step (Maldonado and Price 1999; Shaked et al. 2005; Sutak et al. 2012). Future Fe regeneration studies will provide detailed information about how digestive physiologies of different zooplankton affect organic and redox speciation of regenerated Fe.

#### Acknowledgments

This work was supported by a grant from the National Science Foundation (chemical oceanography 1061545) to D.M.F. and B.S.T. We thank the editor and the two anonymous reviewers for their comments that improved the manuscript.

#### References

- BARBEAU, K., E. B. KUJAWINSKI, AND J. W. MOFFETT. 2001. Remineralization and recycling of iron, thorium and organic carbon by heterotrophic marine protists in culture. *Aquat. Microb. Ecol.* **24**: 69–81, doi:10.3354/ame024069
- , J. MOFFETT, D. CARON, P. CROOT, AND D. ERDNER. 1996. Role of protozoan grazing in relieving iron limitation of phytoplankton. *Nature* **380**: 61–64, doi:10.1038/380061a0
- BESIKTEPE, S., AND H. G. DAM. 2002. Coupling of ingestion and defecation as a function of diet in the calanoid copepod *Acartia tonsa*. *Mar. Ecol. Prog. Ser.* **229**: 151–164, doi:10.3354/meps229151
- BLAIN, S., A. LAES, E. ACHTERBERG, P. WORSFOLD, S. USSHER, P. LAAN, AND H. BAAR. 2008. Distribution and redox speciation of dissolved iron on the European continental margin. *Limnol. Oceanogr.* **52**: 2530–2539.
- BOYD, P. W., AND OTHERS. 2005. FeCycle: Attempting an iron biogeochemical budget from a mesoscale SF6 tracer experiment in unperturbed low iron waters. *Global Biogeochem. Cycles* **19**: GB4S20, doi:10.1029/2005GB002494
- BOYE, M., AND OTHERS. 2006. The chemical speciation of iron in the north-east Atlantic Ocean. *Deep-Sea Res. I* **53**: 667–683, doi:10.1016/j.dsr.2005.12.015
- BREITBARTH, E., J. GELTING, J. WALVE, L. HOFFMANN, D. TURNER, M. HASSELLÖV, AND J. INGRI. 2009. Dissolved iron (II) in the Baltic Sea surface water and implications for cyanobacterial bloom development. *Biogeosciences* **6**: 2397–2420, doi:10.5194/bg-6-2397-2009
- CROOT, P. L., K. BLUHM, C. SCHLOSSER, P. STREU, E. BREITBARTH, R. FREW, AND M. VAN ARDELAN. 2008. Regeneration of Fe(II) during EIFeX and SOFeX. *Geophys. Res. Lett.* **35**: L19606, doi:10.1029/2008GL035063
- DALBEC, A. A., AND B. S. TWINING. 2009. Remineralization of bioavailable iron by a heterotrophic dinoflagellate. *Aquat. Microb. Ecol.* **54**: 279–290, doi:10.3354/ame01270

- DE BAAR, H. J. W., AND OTHERS. 2005. Synthesis of iron fertilization experiments: From the iron age in the age of enlightenment. *J. Geophys. Res. C: Oceans* **110**: 1–24, doi:10.1029/2004JC002601
- FOK, A., Y. LEE, AND R. ALLEN. 1982. The correlation of digestive vacuole pH and size with the digestive cycle in *Paramecium caudatum*. *J. Eukaryot. Microbiol.* **29**: 409–414, doi:10.1111/j.1550-7408.1982.tb05423.x
- FRAÚSTO DA SILVA, J. J. R., AND R. J. P. WILLIAMS. 2001. The biological chemistry of the elements, 2nd ed. Oxford Univ. Press.
- FREESE, D., T. KREIBICH, AND B. NIEHOFF. 2012. Characteristics of digestive enzymes of calanoid copepod species from different latitudes in relation to temperature, pH and food. *Comp. Biochem. Physiol. Part B Biochem. Mol. Biol.* **162**: 66–72, doi:10.1016/j.cbpb.2012.04.007
- , M. VELDHUIS, K. TIMMERMANS, G. SARTHOU, AND H. DEBAAR. 2006. Co-variance of dissolved Fe-binding ligands with phytoplankton characteristics in the Canary Basin. *Mar. Chem.* **102**: 276–290, doi:10.1016/j.marchem.2006.05.004
- GERRINGA, L., AND OTHERS. 2008. Fe-binding dissolved organic ligands near the Kerguelen Archipelago in the Southern Ocean (Indian sector). *Deep-Sea Res. II* **55**: 606–621, doi:10.1016/j.dsr2.2007.12.007
- GLEDHILL, M., AND K. N. BUCK. 2012. The organic complexation of iron in the marine environment: A review. *Front. Microbiol.* **3**: 1–17, doi:10.3389/fmicb.2012.00069
- , AND C. M. G. VAN DEN BERG. 1995. Measurement of the redox speciation of iron in seawater by catalytic cathodic stripping voltammetry. *Mar. Chem.* **50**: 51–61, doi:10.1016/0304-4203(95)00026-N
- HANSARD, S. P., W. M. LANDING, C. I. MEASURES, AND B. M. VOELKER. 2009. Dissolved iron(II) in the Pacific Ocean: Measurements from the PO2 and P16N CLIVAR/CO2 repeat hydrography expeditions. *Deep-Sea Res. I* **56**: 1117–1129, doi:10.1016/j.dsr.2009.03.006
- HOPKINSON, B., AND K. BARBEAU. 2007. Organic and redox speciation of iron in the eastern tropical North Pacific suboxic zone. *Mar. Chem.* **106**: 2–17, doi:10.1016/j.marchem.2006.02.008
- HUNTER, K., AND P. BOYD. 2007. Iron-binding ligands and their role in the ocean biogeochemistry of iron. *Environ. Chem.* **4**: 221–232, doi:10.1071/EN07012
- HUTCHINS, D., K. BRULAND, W. X. WANG, AND N. S. FISHER. 1995. Copepod grazing and the biogeochemical fate of diatom iron. *Limnol. Oceanogr.* **40**: 989–994, doi:10.4319/lo.1995.40.5.0989
- , G. DITULLIO, AND K. BRULAND. 1993. Iron and regenerated production: Evidence for biological iron recycling in two marine environments. *Limnol. Oceanogr.* **38**: 1242–1255, doi:10.4319/lo.1993.38.6.1242
- , A. WITTER, A. BUTLER, AND G. LUTHER. 1999. Competition among marine phytoplankton for different chelated iron species. *Nature* **400**: 858–861, doi:10.1038/23680
- HUTCHINS, D. A., AND K. W. BRULAND. 1994. Grazer-mediated regeneration and assimilation of Fe, Zn and Mn from planktonic prey. *Mar. Ecol. Prog. Ser.* **110**: 259–269, doi:10.3354/meps110259
- LEWIN, J. C. 1961. The dissolution of silica from diatom walls. *Geochim. Cosmochim. Acta* **21**: 182–198, doi:10.1016/S0016-7037(61)80054-9
- MALDONADO, M. T., AND N. M. PRICE. 1999. Utilization of iron bound to strong organic ligands by plankton communities in the subarctic Pacific Ocean. *Deep-Sea Res. II* **46**: 2447–2473, doi:10.1016/S0967-0645(99)00071-5
- MARTIN, J. H. 1990. Glacial-interglacial CO<sub>2</sub> change: The iron hypothesis. *Paleoceanography* **5**: 1–13, doi:10.1029/PA005i001p00001
- MICHELS, J., J. VOGT, AND S. N. GORB. 2012. Tools for crushing diatoms—opal teeth in copepods feature a rubber-like bearing composed of resilin. *Sci. Rep.* **2**: 1–6, doi:10.1038/srep00465
- MOCHIZUKI, N., AND OTHERS. 2010. The cell biology of tetrapyrroles: A life and death struggle. *Trends Plant Sci.* **15**: 488–498, doi:10.1016/j.tplants.2010.05.012
- POND, D., R. HARRIS, AND C. BROWNLEE. 1995. A microinjection technique using a pH-sensitive dye to determine the gut pH of *Calanus helgolandicus*. *Mar. Biol.* **123**: 75–79, doi:10.1007/BF00350325
- RAVEN, J. A. 1988. The iron and molybdenum use efficiencies of plant growth with different energy, carbon and nitrogen sources. *New Phytol.* **109**: 279–287, doi:10.1111/j.1469-8137.1988.tb04196.x
- RIJKENBERG, M., L. GERRINGA, V. CAROLUS, I. VELZEBOER, AND H. DEBAAR. 2006. Enhancement and inhibition of iron photoreduction by individual ligands in open ocean seawater. *Geochim. Cosmochim. Acta* **70**: 2790–2805, doi:10.1016/j.gca.2006.03.004
- ROY, E. G., M. L. WELLS, AND D. W. KING. 2008. Persistence of iron(II) in surface waters of the western subarctic Pacific. *Limnol. Oceanogr.* **53**: 89–98, doi:10.4319/lo.2008.53.1.0089
- RUE, E. L., AND K. W. BRULAND. 1995. Complexation of iron(III) by natural organic ligands in the Central North Pacific as determined by a new competitive ligand equilibration/adsorptive cathodic stripping voltammetric method. *Mar. Chem.* **50**: 117–138, doi:10.1016/0304-4203(95)00031-L
- SANTANA-CASIANO, J. M., M. GONZÁLEZ-DÁVILA, M. J. RODRÍGUEZ, AND F. J. MILLERO. 2000. The effect of organic compounds in the oxidation kinetics of Fe (II). *Mar. Chem.* **70**: 211–222, doi:10.1016/S0304-4203(00)00027-X
- SARTHOU, G., D. VINCENT, U. CHRISTAKI, I. OBERNOSTERER, K. TIMMERMANS, AND C. BRUSSAARD. 2008. The fate of biogenic iron during a phytoplankton bloom induced by natural fertilisation: Impact of copepod grazing. *Deep-Sea Res. II* **55**: 734–751, doi:10.1016/j.dsr2.2007.12.033
- SATO, M., S. TAKEDA, AND K. FURUYA. 2007. Iron regeneration and organic iron(III)-binding ligand production during in situ zooplankton grazing experiment. *Mar. Chem.* **106**: 471–488, doi:10.1016/j.marchem.2007.05.001
- SCHLÜTER, M., AND D. RICKERT. 1998. Effect of pH on the measurement of biogenic silica. *Mar. Chem.* **63**: 81–92, doi:10.1016/S0304-4203(98)00052-8
- SHAKED, Y., A. B. KUSTKA, AND F. M. M. MOREL. 2005. A general kinetic model for iron acquisition by eukaryotic phytoplankton. *Limnol. Oceanogr.* **50**: 872–882, doi:10.4319/lo.2005.50.3.0872
- SOLGAARD, G., I. STANDAL, AND K. DRAGET. 2007. Proteolytic activity and protease classes in the zooplankton species *Calanus finmarchicus*. *Comp. Biochem. Physiol. Part B Biochem. Mol. Biol.* **147**: 475–481, doi:10.1016/j.cbpb.2007.02.014
- STATHAM, P., Y. JACOBSON, AND C. VAN DEN BERG. 2012. The measurement of organically complexed FeII in natural waters using competitive ligand reverse titration. *Anal. Chim. Acta* **743**: 111–116, doi:10.1016/j.aca.2012.07.014
- STRZEPEK, R. F., M. T. MALDONADO, J. L. HIGGINS, J. HALL, K. SAFI, S. W. WILHELM, AND P. W. BOYD. 2005. Spinning the “Ferrous Wheel”: The importance of the microbial community in an iron budget during the FeCycle experiment. *Global Biogeochem. Cycles* **19**: GB4S26, doi:10.1029/2005GB002490
- SUTAK, R., H. BOTEBOLO, P.-L. BLAISEAU, T. LÉGER, F.-Y. BOUGET, J.-M. CAMADRO, AND E. LESUISSE. 2012. A comparative study of iron uptake mechanisms in marine microalgae: Iron binding at the cell surface is a critical step. *Plant Physiol.* **160**: 2271–2284, doi:10.1104/pp.112.204156

- TANG, K. W., R. N. GLUD, A. GLUD, S. RYSGAARD, AND T. G. NIELSEN. 2011. Copepod guts as biogeochemical hotspots in the sea: Evidence from microelectrode profiling of *Calanus* spp. *Limnol. Oceanogr.* **56**: 666–672, doi:10.4319/lo.2011.56.2.0666
- THEIS, T. L., AND P. C. SINGER. 1974. Complexation of iron(II) by organic matter and its effect on iron(II) oxygenation. *Environ. Sci. Technol.* **8**: 569–573, doi:10.1021/es60091a008
- TOVAR-SANCHEZ, A., S. A. SAÑUDO-WILHELMY, M. GARCIA-VARGAS, R. S. WEAVER, L. C. POPELS, AND D. A. HUTCHINS. 2003. A trace metal clean reagent to remove surface-bound iron from marine phytoplankton. *Mar. Chem.* **82**: 91–99, doi:10.1016/S0304-4203(03)00054-9
- VONG, L., A. LAES, AND S. BLAIN. 2007. Determination of iron–porphyrin-like complexes at nanomolar levels in seawater. *Anal. Chim. Acta* **588**: 237–244, doi:10.1016/j.aca.2007.02.007
- WITTER, A., D. HUTCHINS, A. BUTLER, AND G. LUTHER. 2000. Determination of conditional stability constants and kinetic constants for strong model Fe-binding ligands in seawater. *Mar. Chem.* **69**: 1–17, doi:10.1016/S0304-4203(99)00087-0

*Associate editor: Thomas Kjørboe*

*Received: 30 October 2013*

*Accepted: 16 April 2014*

*Amended: 30 April 2014*