Metabolic consequences of iron deficiency in heterotrophic marine protozoa

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Abstract
Iron is recognized as a key element regulating primary production in large regions of the ocean, but nothing is known of its direct effect on higher trophic levels. Two species of heterotrophic protozoa were thus fed iron-rich and iron-poor bacterial prey and their growth and metabolism examined. Maximum growth rates of Paraphysomonas imperforata and Paraphysomonas butcheri were observed only when iron quotas of bacterial prey were >70 μmol Fe mol C⁻¹. At lower Fe:C ratios, but at constant prey biomass, both species grew significantly slower. Iron quotas of the flagellates at these slow growth rates (~10 μmol Fe mol C⁻¹) were similar to those of iron-limited phytoplankton and bacteria. Growth rate reduction was likely the result of direct, elemental limitation by iron, judging from the positive response of the protozoa to iron addition and their biochemical characteristics. Filtration and carbon ingestion rates increased under iron limitation, but gross carbon growth efficiency (GCGE) decreased when P. imperforata consumed iron poor bacteria. Ammonium regeneration efficiency was also reduced. The decrease in GCGE was a consequence of reduced activity of the iron-dependent electron transport system, greater dissolved organic carbon excretion, and greater CO₂ evolution by iron-limited protozoa. P. imperforata excreted iron, even when limited by this element, and retained less of the ingested ration than when consuming iron-rich bacteria. Coupled with recent measurements of biogenic Fe:C in the subarctic Pacific, our results suggest that heterotrophic bacterivorous flagellates may experience iron limitation in remote oceanic regions.

Several regions of the ocean are known to be iron-limited because phytoplankton biomass increases upon addition of iron in bottle and in mesoscale fertilization experiments (e.g. Martin and Fitzwater 1988; Price et al. 1991; Martin et al. 1994; Coale et al. 1996). Research into the physiological and ecological implications of low iron availability has focused almost exclusively on the photoautotrophs. This bias reflects both the role of iron in photosynthesis and in nitrate assimilation and the importance of oceanic primary productivity in the global carbon cycle. Yet, iron is an indispensable element for growth of all aerobic organisms, and autotrophic and heterotrophic processes are tightly coupled in these iron-limited systems. Efficient grazing by microzooplankton maintains constant low chlorophyll levels (Cullen et al. 1992; Price et al. 1994) and in general dictates the extent to which new production (Fe- or N-based) is exported and sequestered (Michaels and Silver 1988). Furthermore, grazer-mediated regeneration of iron is thought to be an important vector for the supply of dissolved Fe to phytoplankton (Barbeau et al. 1996; Hutchins et al. 1993; Hutchins and Bruland 1994). For the major nutrients, such regeneration depends on the elemental requirements of the grazers relative to the composition of their prey (Olsen et al. 1986; Caron and Goldman 1990; Sterner et al. 1992), so the same might be expected for iron. Iron limitation of secondary production could clearly have important ecological and biogeochemical consequences.

Heterotrophic metabolism depends on a respiratory electron transport system (ETS) that contains many iron-dependent redox proteins. The respiratory ETS contains ~60% more iron per mol of cytochrome-c than does the photosynthetic ETS (Raven 1988), suggesting that heterotrophs could require even more iron for growth than do phototrophs. Recently, Tortell et al. (1996) demonstrated that iron limitation of marine heterotrophic bacteria is mediated by decreased activity of the respiratory ETS. Enrichment bioassays in the Southern Ocean showed stimulation of bacterial growth, so low iron concentrations may indeed limit some heterotrophs in the sea (Pakulski et al. 1996). Because phytoplankton and bacterioplankton in iron-poor, oceanic waters contain very little iron (Sunda et al. 1991; Maldonado and Price 1996; Tortell et al. 1996), grazers in these regions may also have trouble acquiring sufficient iron for growth. Elemental limitation of heterotrophic eukaryotes (mainly freshwater zooplankton) has been considered in the cases of N and P (Hessen 1992; Urabe and Wanatabe 1992; Müller-Navarra 1995; Rothhaupt 1995), but the effect of iron content of prey on consumer metabolism has not been addressed. An increase in abundance of microheterotrophs following iron addition (Buma et al. 1991; Chavez et al. 1991; Martin et al. 1994) could represent a direct stimulation of these consumers by iron or an indirect effect caused by an increase in prey density.

Possibly the most important consumers to evaluate in the context of iron limitation are members of the protozoa. These small grazers are particularly significant in iron-limited oceanic regions (Strun and Welschmeyer 1991) where large amounts of production cycle through the microbial loop (Chavez et al. 1991). Iron in the heterotrophic bacterial biomass represents up to 50% of the total biogenic pool in

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the subarctic Pacific Ocean (Tortell et al. 1996) and presumably in other iron-limited waters. As the most important consumers of bacterial biomass, the protozoa thus represent a potentially significant pathway from particulate to dissolved Fe via excretion. Here we present the results of a series of laboratory experiments that demonstrate iron limitation of bactiovorous flagellates and document its metabolic consequences. A simple extrapolation of these results suggests heterotrophic protists, like photoautotrophs and bacteria, may experience iron limitation in some regions of the sea.

Methods

Study organisms—Two chrysomonad microflagellates of the genus Paraphysomonas were examined: P. imperforata (clone VSI), a coastal species isolated from Vineyard Sound (Massachusetts), and P. butcheri (clone SS), an oceanic species from the Sargasso Sea. P. imperforata (4–6 µm in diam) is omnivorous and ubiquitous in marine waters (Goldman and Caron 1985). P. butcheri is slightly smaller (3–5 µm), but is otherwise morphologically similar to P. imperforata. Bacterial strains used as prey, Tef2 and Ju188, were both isolated from the Sargasso Sea on nutrient-enriched agar plates. They were maintained in batch cultures that were periodically renewed from stocks frozen in liquid nitrogen. Protozoan cultures were rendered unpolluted by repeated transfer (>10 times) into stationary-phase cultures of the prey strains (verified by microscopy).

Growth conditions and medium—The artificial seawater medium Aquil (Price et al. 1988–1989), containing the full complement of inorganic nutrients (N, P, Si), was used for all culturing and experiments. EDTA (100 µM) was used to buffer trace metals, with Cu, Mn, Zn and Co additions adjusted to achieve free-ion concentrations of 10^{-13.79}, 10^{-8.27}, 10^{-10.88} and 10^{-10.88} M, respectively. A total of 8.4 µM or 12.5 nm Fe was added separately as a premixed Fe-EDTA (1:1) complex, resulting in a pFe(-log[Fe'] +1) of 18.18 and 21.00, respectively (hereafter referred to as pFe18 and pFe21). Free metal ion concentrations were calculated by using the chemical equilibrium model MINEQL (Westall et al. 1976). Sterile medium was enriched with filter-sterilized glucose (acid-washed, 0.2-µm Acrodisc filters), which was purified of trace metal contaminants with Chelex 100 ion-exchange resin. Because carbon growth efficiency of bacteria is iron-dependent (Tortell et al. 1996), 50 and 100 mg liter^{-1} glucose were added in the pFe18 and pFe21 treatments, respectively. Such unequal concentrations yielded similar bacterial biomass in both iron treatments. At pFe18, bacteria grew at maximum rates and had high intracellular Fe quotas (iron-rich prey), whereas at pFe21, the bacteria grew slower and had low intracellular Fe quotas (iron-poor prey) (Tortell et al. 1996). When the bacteria were harvested in early stationary phase, they had consumed all of the glucose.

To minimize metal contamination, plasticware was acid-washed and then rinsed repeatedly with ultrapure water (Millipore, Q-H2O), and all manipulations were performed in a laminar flow hood. Experimental and stock cultures of protozoa were grown at 20°C under continuous light (150 µmol quanta m^{-2} s^{-1}). Inoculations for experiments were taken from acclimated, exponentially growing stock cultures, which were transferred weekly into fresh, stationary-phase bacterial cultures (either pFe18- or pFe21-grown). Prior to the experiments with heat-killed bacteria, protozoa were repeatedly transferred into heat-killed bacterial cultures.

Overview of experiments—Initial experiments simultaneously measured the growth rate and Fe quota (µmol Fe mol C^{-1}) of P. imperforata fed iron-rich and iron-poor bacterial prey (strain Ju188). These experiments were repeated three times for the high-iron treatment and five times for the low-iron treatment. The quotas and growth rates of an oceanic flagellate, P. butcheri, were then determined under the same conditions: three times for the high-iron treatment and once for the low-iron treatment. We also examined growth of P. imperforata by using a second bacterial prey (Tef2), with a lower iron quota than Ju188 (Tortell et al. 1996), and tested the growth of P. imperforata when fed very iron-rich bacteria (strain Ju188). These last two experiments were performed only once.

Subsequent experiments focused on metabolism, using the grazer P. imperforata and prey Ju188. In separate experiments we measured grazer Fe and C metabolism, CO2 evolution, ETS activity, and NH4+ excretion under high- and low-Fe conditions. A final experiment using “iron-coated” bacteria was designed to test biochemical vs. elemental limitation by iron. A total of five experiments were thus conducted using P. imperforata and Ju188, in addition to the growth and quota experiments described above.

The basic experimental approach was to grow bacterial cultures to stationary phase in pFe18 and pFe21 medium, harvest them by centrifugation (10,400 x g, 40 min.), and then rinse and resuspend them in a small volume of Aquil containing no trace metals, no glucose, and 100 µM EDTA as a “dissolved Fe trap” (Hutchins and Bruland 1994). This resuspension medium was referred to as Aquil-TMS (Aquil minus trace metals). Appropriate volumes of resuspended bacteria were transferred to four 250-ml polycarbonate bottles containing 200 ml of Aquil-TMS in order to achieve an initial particulate C concentration of ~2 x 10^{-7} mol ml^{-1} (~10^2 bacterial ml^{-1}). Acclimated P. imperforata (~10^6 cells ml^{-1}) were inoculated into two of the bottles, and the remaining bottles served as controls. Over the course of 5 d of protozoan growth, samples were periodically removed aseptically and analyzed as described below for the various experiments. Protozoa were preserved in Lugol’s solution and their density measured by light microscopy in a Palmer-Maloney chamber. Deviations from this protocol are noted below.

Iron quota measurements—Doubly-labeled bacterial prey were used to measure Fe quotas (µmol Fe mol C^{-1}) of the flagellates. Bacteria were grown in the medium described above containing uniformly labeled [14C]glucose (Amersham, 3 mCi mmol^{-1}) at a final activity of 2.5 µCi liter^{-1}, with either 1% (pFe18) or 10% (pFe21) of the iron as 55FeCl3 (25–40 mCi mg^{-1}, DuPont). There was no rinsing and resuspension of prey in this experiment. When the bacteria reached stationary phase (~10^7 cells ml^{-1}), the culture was split into four bottles. Three of these were inoculated with protozoa (~10^6 cells ml^{-1}) and one served as a control. Sam-
samples for protozoan enumeration were taken every 12 h. At several intervals during middle-to-late exponential phase, 10–20 ml of culture were filtered onto 2-µm polycarbonate filters, rinsed with Ti(III) EDTA-citrate solution to remove Fe hydroxides and extracellularly bound Fe (Hudson and Morel 1989), and then rinsed twice with 5 ml of synthetic ocean water (SOW; Price et al. 1988–1989). To avoid damage to the flagellates in this and other experiments, the vacuum pressure was maintained below ±0 mm Hg and the filters were not allowed to run dry prior to rinsing.

Because we used small inocula and allowed at least eight cell divisions in the labeling medium, the bacteria and protozoa had reached isotopic equilibrium at the time of sampling. Quotas were measured during that part of the protozoan growth curve where protozoan abundance was maximal and bacterial abundance minimal. At this time, bacteria represented <0.5% of the total biomass on the 2-µm filters, and protozoan retention was >95%, as determined by microscopy. Protozoan quotas were thus calculated by converting the ratio of $^{55}$Fe dpm : $^{14}$C dpm on the 2.0-µm filters to pmol Fe mol C$^{-1}$ using the specific activity of C and Fe in the bacterial growth medium, after correcting for background and decay. Bacterial quotas in the control flasks were obtained similarly using 0.2-µm filters.

We tested the sensitivity of bacteria and flagellates to the Ti rinse by comparing $^{14}$C activity on filters rinsed with Ti to $^{14}$C activity on a subset of filters that were rinsed with an equivalent volume of SOW. We found less particulate $^{14}$C activity on Ti-rinsed filters than on those that were rinsed only with SOW. The Ti rinse thus caused a small loss of labeled cellular constituents (<10% compared to SOW), but should not affect quotas measured by a dual-labeling technique.

Carbon and iron metabolism—The same doubly labeled bacterial prey described for the quota experiments were used to examine C and Fe metabolism in P. imperforata. Experimental and control cultures were run in duplicate. In this experiment, bacterial and protozoan densities, as well as particulate and dissolved $^{14}$C and $^{55}$Fe activities, were monitored at 12-h intervals over a 110-h period.

Samples for bacterial enumeration were preserved in 3.6% formalin in filtered SOW and then counted by epifluorescence microscopy (Porter and Feig 1980). Total particulate activity was determined by filtering 10–20 ml of culture onto a 0.2-µm polycarbonate filter, and then rinsing the filter twice with 5 ml of SOW. The Ti rinse was not used in this experiment because we suspected that it caused some loss of radioactivity (see above). Dissolved $^{55}$Fe activity and dissolved organic carbon (DOC) activity were determined in 1-ml samples of 0.2-µm filtrate (Aerocide filter with syringe) transferred to glass scintillation vials containing 100 µl of 1 N HCl. Because the medium contained high concentrations of EDTA, all of the reactive, regenerated Fe is likely to have remained in solution and not sorbed to cell surfaces and the container walls. Calculated Fe regeneration efficiencies thus represent an upper limit (Hutchins and Bruland 1994). CO$_2$ was allowed to purge for 24 h. During filtration, some culture was always left in the syringe so that the cells were never dry. Fluor (Ecolite) was added to all samples, and $^{14}$C and $^{55}$Fe activity measured by dual label liquid scintillation counting.

CO$_2$ evolution—An additional experiment examined C metabolism more thoroughly. Duplicate experimental cultures and one bacterial control were used. Bacteria were grown in the [14C]glucose medium described above (without $^{55}$Fe), harvested, rinsed and resuspended in Aquil-TMS, and then heat-killed at 70°C for 30 min. This procedure yielded nonaggregated prey suspensions. Clumping occurred if the bacteria were heat-killed in the glucose-containing medium. The efficacy of the heat treatment was verified by inoculating aliquots of the bacterial suspension into organic-enriched seawater medium. No growth was observed. Bacterial cultures were then allowed to sit for 16 h before they were recentrifuged, resuspended in Aquil-TMS, and inoculated with P. imperforata as described above. In addition to measurements of DOC and particulate organic carbon (POC), CO$_2$ evolution was monitored directly over the time course by trapping $^{12}$CO$_2$ from acidified samples as described by Price and Harrison (1987). Recovery of $^{12}$CO$_2$ was 100%.

Calculation of metabolic parameters—Protozoan growth rate for all experiments was calculated during the exponential period by linear regression of ln(cell density) vs. time. In the C and Fe metabolism and CO$_2$ evolution experiments, gross growth efficiencies (GGE) for C and Fe were calculated for a time interval $t$, for each replicate, following Geller and Leaderbeater (1988):

$$GGE_x = F_{st}/I_{st},$$

where GGE$_x$ is the gross growth efficiency for element $x$, $F_{st}$ is the activity associated with the flagellates at time $t$ (dpm ml$^{-1}$), and $I_{st}$ is the activity ingested by the flagellates during the interval $t$ (dpm ml$^{-1}$).

More explicitly, $F_{st}$ was obtained after correction for bacterial biomass remaining in the bottles and caught on the 0.2-µm filter:

$$F_{st} = P_{st} - B_{st},$$

where $P_{st}$ is the total particulate (0.2-µm filter) activity (dpm ml$^{-1}$) and $B_{st}$ is the activity associated with bacteria (dpm ml$^{-1}$); $B_{st} = (\text{bacteria ml}^{-1}) \times (\text{14C dpm or } ^{55}\text{Fe dpm bacterium}^{-1}).$ Activity per bacterium was determined from the controls. Ingestion was calculated as

$$I_{st} = (R_{st} - B_{st}) - C_{st},$$

where $B_{st}$ and $B_{st}$ represent the bacterial activity ($^{14}$C or $^{55}$Fe) in the flagellate bottle at the beginning and end of the interval, respectively, and $C_{st}$ is a correction for loss of particulate activity in the controls (see below). Time $t$ was taken from the beginning of exponential growth of the flagellates until their peak in biomass, as determined from inspection of the growth curve. Because the flagellates had essentially not consumed any bacteria at the beginning of this interval, all the activity at this time was associated with the bacteria, and $B_{st}$ was taken to be the total initial particulate activity. Release of an element to the dissolved phase, $E_{st}$, was calculated as

$$E_{st} = (D_{st} - D_{st}) - C_{st},$$

where $D_{st}$ and $D_{st}$ represent dissolved activity in the flagellate bottle at the end and beginning of the interval, respectively.
Where necessary, a correction \((C_r)\) was applied to account for directional changes in control cultures. This was obtained by multiplying the change in dissolved activity/bacterium in the controls \((con)\) by the logarithmic mean number of bacteria in the flagellate bottles \((exp)\) during interval \(t\). \(C_r\) was calculated similarly from particulate activity in the controls; that is,

\[
C_{r,t} = (A_t - A_0)/(\text{bacteria ml }^{-1})_\text{con} \times (\text{bacteria ml }^{-1})_\text{exp}
\]

where \(A\) refers to dissolved or particulate activity in the controls, yielding \(C_r\) and \(C_p\), respectively. For comparative purposes, \(F_v\) \((\text{growth})\), \(I_v\) \((\text{ingestion})\), and \(E_v\) \((\text{excretion})\) were converted to mol protozoan \(^{-1} \text{ d}^{-1}\) by dividing by the specific activity \((\text{SA})\) of the bacteria \((\text{dpm mol }^{-1} \text{ Fe or C})\), the logarithmic mean number of protozoa during the interval, and the duration of the interval. Rates were converted to regeneration efficiencies \((\text{RE})\) by normalizing to the quantity of the element ingested during the same period:

\[
\text{RE}_v = E_v/I_v
\]

Elemental content of the flagellates \((\text{mol protozoan }^{-1})\) was obtained by dividing \(F_v/\text{SA}\) by the number of flagellates \(\text{ml }^{-1}\) at time \(t\). Unless otherwise noted, all the data reported represent means \(\pm 1 \text{ SD}\).

**Electron transport system (ETS) activity**—Heat-killed, iron-rich and iron-poor prey were prepared as described above. Duplicate experimental cultures and one bacterial control were used. During the exponential phase, duplicate samples of 35 ml were gently filtered onto combusted \((425^\circ \text{C for 4 h})\) Whatman GF/C filters. ETS activity was measured according to Packard and Williams \((1981)\), except samples were homogenized with a Mini Bead Beater \((\text{Xymo-tech})\). Tetrazolium \((\text{INT})\) reduction rates were converted to rates of O\(_2\) consumption by using the extinction coefficient for INT-formazan reported by Kenner and Ahmed \((1975)\). We normalized ETS activity to cell density and to protein concentration in each sample, determined using the fluorescamine method of Udeh-friend et al. \((1972)\).

**NH\(_4\)\(^+\) excretion**—Ammonium excretion by \(P. \text{ imperforata}\) was monitored in a grazing experiment with iron-rich and iron-poor \(^{14}\text{C}\)-labeled prey. Duplicate experimental cultures and one bacterial control were used. In this experiment, bacteria were inhibited by UV irradiation. This treatment was used initially in our study before we had overcome the problem of clumping in heat-killed cultures \((\text{see above})\). Bacterial cultures were transferred to plastic beakers covered with cellulophane and irradiated with a 30 W germicidal bulb four times for 5 min, with gentle mixing between exposures. Cultures were then centrifuged and treated in the same manner as for the other experiments. UV irradiation inhibited bacterial replication, but not respiration. Ammonium concentrations in 0.2-\(\mu\)m filtrate were measured by the method described by Parsons et al. \((1984)\). In control cultures, which contained UV-irradiated bacteria without flagellates, \(\text{NH}_4\)\(^+\) concentration did not change over time. Experimental bottles were corrected for this constant background concentration. Carbon ingestion rates were calculated by the disappearance of \(^{14}\text{C}\) activity, as described above. Ammonium excretion rates were calculated by dividing the slope of the excretion curve \((\text{mol ml }^{-1} \text{ h}^{-1})\) by the logarithmic mean number of protozoa. Rates were expressed as regeneration efficiencies \(-\bigl(\text{NH}_4\)\(^+\) excreted/N ingested\bigr) \times 100\) by converting C ingested to N ingested using the previously published C:N ratio for strain Ju88. This ratio was the same in iron-sufficient and iron-deficient bacteria \((3.4 \pm 0.7 \text{ and } 3.9 \pm 0.3 \text{ mol C mol N}^{-1}, \text{respectively}; \text{Tortell et al. } 1996)\).

**Biochemical vs. elemental limitation**—A culture of Ju88 was grown to stationary phase in pFe21 medium, heat-killed, rinsed once, and resuspended in Aquil-TMS. The culture was then divided equally into two centrifuge bottles, one of which was enriched with 8.4 \(\mu\text{M Fe-EDTA}\). After 2 h, both cultures were harvested, resuspended in Aquil-TMS, split in two, and inoculated with acclimated \(P. \text{ imperforata}\). A preliminary investigation of the kinetics of \(^{55}\text{Fe}\) adsorption by dead bacteria demonstrated that the added iron was rapidly scavenged onto the cell surfaces. By comparing the \(^{55}\text{Fe}\) activity of Ti-rinsed and SOW-rinsed bacteria in this experiment, we determined that about 20 amol Fe cell \(^{-1}\) was bound extracellularly, roughly 10 times more than the intracellular iron content of iron-deplete bacteria.

**Results**

**Growth and quotas**—\(P. \text{ imperforata}\) grew significantly slower \((P < 0.005)\) and achieved lower levels of biomass when consuming iron-poor as opposed to iron-rich prey \((\mu = 2.52 \pm 0.08 \text{ d}^{-1}, n = 3 \text{ for pFe18}; \mu = 1.4 \pm 0.1 \text{ d}^{-1}, n = 5 \text{ for pFe21})\). The high- and low-iron bacterial quotas that elicited this growth response are reported in Table 1. Indeed, over a wider range of bacterial Fe:C and initial particulate Fe in the medium, protozoan growth increased with the iron content of prey items \((\text{Fig. 1})\). Reduction in growth was observed for both species of protozoa and for \(P. \text{ imperforata}\) fed Te2 or Ju88 \((\text{Fig. 1})\). Small differences in initial bacterial C biomass in these experiments could not account for the growth response \((\text{Pearson product-moment correlation, } P = 0.241)\), nor was bacterial Fe:C related to total particulate C \((\text{mol C ml}^{-1})\) \((\text{Pearson product-moment correlation, } P = 0.226)\). Maximum growth rates of the protozoa were observed only at bacterial Fe:C ratios \(>70 \text{ \mumol mol}^{-1}\), and growth rates at low and high

### Table 1. Iron quotas of *Paraphysomonas* spp. grown with bacterial prey that were cultured under high- and low-iron conditions (*pFe18* and *pFe2* medium, respectively). In each experiment, protozoan quotas were obtained from triplicate cultures and bacterial quotas were obtained from one control culture. Values represent means of all experiments \(\pm \text{ SE}\), calculated by error propagation, and \(n\) refers to the number of independent experiments.

<table>
<thead>
<tr>
<th>Species</th>
<th>High iron</th>
<th>Low iron</th>
</tr>
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<tbody>
<tr>
<td>Fe:C ((\text{\mumol Fe mol C}^{-1}))</td>
<td></td>
<td></td>
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<tr>
<td><em>P. butcheri</em></td>
<td>101±3</td>
<td>12.5±0.2</td>
</tr>
<tr>
<td><em>P. imperforata</em></td>
<td>98±3</td>
<td>8.2±0.1</td>
</tr>
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</table>

where \(A\) refers to dissolved or particulate activity in the controls, yielding \(C_r\) and \(C_p\), respectively. For comparative purposes, \(F_v\) \((\text{growth})\), \(I_v\) \((\text{ingestion})\), and \(E_v\) \((\text{excretion})\) were converted to mol protozoan \(^{-1} \text{ d}^{-1}\) by dividing by the specific activity \((\text{SA})\) of the bacteria \((\text{dpm mol }^{-1} \text{ Fe or C})\), the logarithmic mean number of protozoa during the interval, and the duration of the interval. Rates were converted to regeneration efficiencies \((\text{RE})\) by normalizing to the quantity of the element ingested during the same period:

\[
\text{RE}_v = E_v/I_v
\]
Fe limitation of protozoa

Fig. 1. Protozoan growth rates (d⁻¹) in batch culture as a function of (A) prey iron quota (µmol Fe mol C⁻¹) and (B) the initial concentration of particulate Fe (µmol ml⁻¹). The high cell densities are for Tcf2, which has a C quota of -7 x 10⁻¹⁵ mol Fe cell⁻¹ (P. Tortell unpubl.), and was independent of the Fe:C ratio. The curves were fitted to the Michaelis–Menton equation by nonlinear regression. A. \( \mu_{max} = 2.48 \text{ d}^{-1} \) and \( K_m = 4.60 \mu\text{mol Fe mol C}^{-1} \), \( r^2 = 0.82 \). B. \( \mu_{max} = 2.50 \text{ d}^{-1} \) and \( K_m = 1.16 \text{ pmol Fe ml}^{-1} \), \( r^2 = 0.73 \).

The reduced growth rate of protozoa fed the iron-poor diet (Fig. 1) was not a consequence of their slower ingestion rates, as they consumed more bacterial C than did those fed iron-rich prey (Table 2). The ingestion rates corresponded to filtration rates of 26.41 ± 0.09 and 60.0 ± 0.6 nl protozoan⁻¹ d⁻¹ for pFe18 and pFe21, respectively, similar to maximum rates observed for other bacterivorous flagellates (Caron et al. 1985). Initial bacterial density was comparable in the two iron treatments (Fig. 2; 5.9 ± 0.9 and 6.85 ± 0.07 x 10⁷ cells ml⁻¹ for pFe18 and pFe21, respectively). The calculated bacterial C quota, based on this initial sampling point, was slightly higher in the iron-
Gross carbon growth efficiency (GCGE) of the flagellates was also lower in the low-iron treatment. This decrease in GCGE, however, could not be accounted for solely by the difference in CAE. Mass-balance calculations for the experiment with live prey suggested the decrease in GCGE was also due to greater CO₂ evolution by the iron-limited protozoa (Table 2). This result was confirmed in a separate experiment using heat-killed bacterial prey where ¹⁴C CO₂ evolution was measured directly. Thus, in the low-iron treatment, P. imperfecta released significantly more ingested bacterial C as CO₂ (Table 2; P < 0.025). In both replicates of both iron treatments there was no change in the total amount of C measured over the course of the experiment, despite major shifts in the relative contribution of each pool. Thus, all C pools were quantitatively recovered by our methods, with no systematic losses.

Initial prey C biomass was lower in the CO₂ experiment than in the C and Fe metabolism experiment using doubly labeled prey, but the calculated metabolic parameters from both experiments were similar (Table 2). Because the bacterial densities were high relative to natural abundances and were likely above saturation (e.g. Fenchel 1982), we think protozoan metabolic parameters and growth were insensitive to the relatively small differences in initial prey density among experiments and between iron treatments.

Iron-limited protozoa released more CO₂, but they did not have greater respiration rates (O₂ consumption), according to the measurements of ETS activity. In fact, the activity of the iron-dependent ETS decreased 10-fold when P. imperfecta was fed iron-poor compared to iron-rich prey (from 4 ± 1 to 0.36 ± 0.06 pmol O₂ protozoan⁻¹ d⁻¹ in pFe18 and pFe21 cultures). This result was not due to differential retention of flagellates on GF/C filters, as the same trend was observed when ETS activity was normalized to the total protein of each sample (182 ± 5 1 and 39 ± 10 mmol O₂ g protein⁻¹ d⁻¹ in pFe18 and pFe21 cultures).

Nitrogen metabolism—Absolute rates of NH₄⁺ excretion by exponential-phase P. imperfecta were not affected by iron nutritional status (0.24 ± 0.01 and 0.233 ± 0.005 pmol NH₄⁺ flagellate⁻¹ d⁻¹ for iron-sufficient and iron-deficient cells, respectively), but NH₄⁺ regeneration efficiency of iron-sufficient protozoa (24.4 ± 0.8%) was significantly higher than that of iron-deficient cells (15.3 ± 0.8%; t-test, P < 0.05).

Iron metabolism—We calculated an iron budget (Table 3) for P. imperfecta using the iron data from the dual-label experiment (Fig. 4). Surprisingly, the iron limited protozoa consumed more of the ingested iron than did protozoa consuming iron-rich prey, and thus they had a lower gross Fe growth efficiency (GFGE) (Table 3). In the low-iron experiment, the amount of iron excreted and sequestered (14 ± 2 amol Fe protozoan⁻¹ d⁻¹) during protozoan growth equaled the amount of iron ingested (16 ± 2 amol Fe protozoan⁻¹ d⁻¹), as it should for mass balance (t-test, P > 0.05). In the high-iron experiment, slightly more iron was ingested on average (110 ± 7 amol Fe protozoan⁻¹ d⁻¹) than normalized to their faster ingestion rates, resulting in a lower C assimilation efficiency (CAF) (P < 0.025) (Table 2).
Fe limitation of protozoa

Fig. 3. Time course of carbon dynamics in cultures with bacteria and Paraphysomonas imperforata (■; replicates shown) and control cultures with bacteria alone (○; replicates shown). Particulate (POC) (A) and dissolved (DOC) (B) organic carbon in the high-iron (Fe-rich bacteria) treatment. POC (C) and DOC (D) in the low-iron (Fe-poor bacteria) treatment.

could be accounted for by growth and excretion (92 ± 6.3 amol Fe protozoan⁻¹ d⁻¹), but the discrepancy was small and not statistically significant (t-test, P > 0.05). As observed for C, the total amount of iron (particulate + dissolved) recovered over the course of the experiment was constant, so we are fairly confident that all iron pools were recovered quantitatively. Furthermore, although wall loss was not assessed directly in these experiments, we assume it was minimal in the presence of EDTA, which served as a “dissolved trap” for regenerated Fe and prevented adsorption to cell surfaces and the sides of incubation bottles (Hutchins and Bruland 1994). When the same experiment was performed without the addition of 100 μM EDTA, iron regeneration efficiencies for both treatments were ~80% less than those observed in the presence of EDTA, suggesting that a large amount of the regenerated Fe was reactive toward cell and bottle surfaces. Samples used to calculate the iron metabolic parameters were rinsed only with SOW, not with Ti. The ingested fraction thus included any Ti-labile Fe on the surface of the bacterial prey. However, comparison of Fe:C ratios on a subset of the control and experimental samples washed with both Ti-citrate reagent and SOW revealed that very little iron (~10%, pFe18; <1%, pFe21) was bound to cell-surface ligands. The process of rinsing and resuspending the prey cells in Aquil containing 100 μM EDTA (Aquil-TMS, see methods) was apparently sufficient to remove what little surface-bound Fe was present.

Discussion

Growth rate—P. imperforata and P. butcheri were unable to achieve maximum growth rates when consuming iron-poor bacteria, and P. imperforata was inhibited regardless of which bacterial stain was offered as prey (see Fig. 1). Although these data form a rather piecemeal functional response with a composite half-saturation constant of 4.6 μmol Fe mol C⁻¹, they show clearly that protozoa grow submaximally at prey Fe:C ratios <15 μmol mol⁻¹ (Fig. 1). Such
slow growth rates were obtained solely by variation in prey iron content, because prey biomass (C) was constant over the range of iron treatments. Growth limitation by iron can thus be induced in phagotrophic protozoa. An important issue, however, is whether this limitation is the direct result of iron deficiency in the protozoa or an indirect effect of low iron on their bacterial prey.

In the pFe21 growth medium used to cultivate iron-poor bacteria, both strains were growth-rate limited, whereas iron-rich bacteria grew at maximum rates at pFe18 (Tortell et al. 1996). Thus, if iron limitation altered the bacteria in other ways, iron content might not be the only difference between the two types of prey. Morphological and structural changes to algae and bacteria, for example, are known to be induced by nutrient deficiency and to reduce their digestibility (González et al. 1993; van Donk and Hessen 1993). Although they might influence consumer growth, such indirect effects of iron are not likely to have been important in our experiments. We observed elongation of some bacteria in the Jul88 cultures maintained in low-iron medium, but no morphological changes were observed in Tef2, which elicited the same iron-induced growth reduction in the protozoa (Fig. 1). Neither strain showed any obvious difference in aggregation under low-iron conditions, but this was not assessed quantitatively.

Biochemical changes that accompany nutrient limitation may also influence prey nutritional quality. Indeed, what seems to be phosphorus limitation of Daphnia is more a consequence of the low levels of eicosapentaenoic acid than of low P content in P-limited algal prey (Müller-Navarra 1995). Although we know the bulk chemical content of the prey bacteria (C:N) was not affected by iron, a large and unspecified list of potential differences exists between iron-sufficient and iron-deficient bacteria. We thus addressed the issue of mineral vs. biochemical limitation by comparing growth of protozoa fed iron-poor prey with and without added surface-bound iron. Because the results showed that growth of P. imperforata was significantly faster in the for-
Poor prey seems to be caused by direct elemental limitation mer case, growth rate reduction in the protozoa fed iron-poor prey seems to be caused by direct elemental limitation by iron.

The metabolic signature of the slow-growing protozoa provided further support for direct iron limitation. Protozoan ingestion rates would be expected to decrease if iron-poor bacteria were indigestible or difficult to capture. However, ingestion rates increased, and slower growth was a result of lower growth efficiency (Table 2). The ~50% decrease in GCGE of Paraphysomonas in low-iron cultures was reflected in a dramatic decline in the activity of the ETS. Because it contains the bulk of the iron in a heterotrophic cell, reduced ETS activity under low-iron conditions was anticipated. Electron transport system reduction seems to be a specific consequence of iron deficiency, because the metabolic reactions that maintain filtration rates, for example, were not similarly downregulated.

Taken together, the three lines of evidence presented above—the relationship between protozoan growth rate and bacterial Fe:C, the stimulation of growth by iron-coated bacteria, and the biochemistry of growth limitation—suggest the marine protozoa in our experiments experienced elemental iron limitation. The question naturally arises of whether this is simply an interesting laboratory result or whether heterotrophic bacteria (Tortell et al. 1996). This result is perhaps surprising, because a heterotrophic mode of existence lacks the iron-dependent photosynthetic reactions that are known to incur a high demand for iron in photoautotrophs. However, the ETS redox proteins needed to maintain active heterotrophic metabolism can impart a substantial iron requirement. Indeed, in iron-limited bacteria, where most cellular Fe is associated with the ETS (Righelato 1969; Light and Clegg 1974; Granger and Price in prep.), iron quotas are relatively high (Tortell et al. 1996). Assuming that all heterotrophs have similar respiratory pathways, we expected protozoa to have minimum iron quotas similar to or less than those of bacteria. Their quotas would most likely be lower, because the specific respiration rates (respiration/biomass), and hence ETS/biomass, of heterotrophs decrease with increasing size (Peters 1983). Iron should thus be "demagnified" as it passes up the food chain. Our results, however, seem to run counter to this allometric argument, because the flagellates in the low-iron experiments had higher iron quotas than did their prey. This may be a consequence of sample size, since only two species of a single genus were examined, or a consequence of scale, since the allometric relationship holds best when considering organisms that differ in size by many orders of magnitude. From a compilation of early measurements (Vinogradov 1953) we find a mean Fe:C in marine fish of 4.5 μmol Fe: mol C (median = 3, n = 72; assuming wet wt = 5 x dry wt). This lends support to the idea of iron demagnification. The data are difficult to interpret, however, because the iron nutritional status of the large organisms is not known. An alternative explanation for the high quotas is that eukaryotic metabolism is relatively more iron-costly than prokaryotic metabolism, or it uses large amounts of iron not associated with the ETS. Indeed, when we consider bacteria, protozoa, and zooplankton, we

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Prey</th>
<th>Prey</th>
<th>Prey</th>
<th>Prey</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter</td>
<td>Live killed</td>
<td>Live killed</td>
<td>Heat- killed</td>
<td>Heat- killed</td>
</tr>
<tr>
<td>Initial bacterial C (mM)</td>
<td>0.19</td>
<td>0.14</td>
<td>0.29</td>
<td>0.19</td>
</tr>
<tr>
<td>Ingestion (pmol C prot-1 d-1)</td>
<td>1.4</td>
<td>1.3</td>
<td>2.1</td>
<td>2.0</td>
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<tr>
<td>Growth (pmol C prot-1 d-1)</td>
<td>0.51</td>
<td>0.41</td>
<td>0.39</td>
<td>0.26</td>
</tr>
<tr>
<td>CO2 evolution (pmol C prot-1 d-1)</td>
<td>0.81</td>
<td>0.80</td>
<td>1.41</td>
<td>1.50</td>
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<tr>
<td>Excretion (pmol C prot-1 d-1)</td>
<td>0.11</td>
<td>0.11</td>
<td>0.34</td>
<td>0.53</td>
</tr>
<tr>
<td>GCGE (%)</td>
<td>36</td>
<td>33</td>
<td>18</td>
<td>13</td>
</tr>
<tr>
<td>CAE (%)</td>
<td>92</td>
<td>91</td>
<td>84</td>
<td>73</td>
</tr>
<tr>
<td>CO2/I (%)</td>
<td>56</td>
<td>63</td>
<td>66</td>
<td>79</td>
</tr>
</tbody>
</table>

*Calculated by difference.

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<table>
<thead>
<tr>
<th>Parameter</th>
<th>High iron</th>
<th>Low iron</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial bacterial Fe (nM)</td>
<td>17.7</td>
<td>1.8</td>
</tr>
<tr>
<td>Ingestion (amol Fe prot-1 d-1)</td>
<td>110</td>
<td>16</td>
</tr>
<tr>
<td>Growth (amol Fe prot-1 d-1)</td>
<td>28</td>
<td>2.0</td>
</tr>
<tr>
<td>Excretion (amol Fe prot-1 d-1)</td>
<td>64</td>
<td>12</td>
</tr>
<tr>
<td>GFeGE (%)</td>
<td>26</td>
<td>14</td>
</tr>
<tr>
<td>FeRE (%)</td>
<td>58</td>
<td>84</td>
</tr>
</tbody>
</table>

The types of prey items naturally consumed by protozoa, it offers another explanation for the observation that microheterotroph biomass increases following iron addition in bottle incubations (Buma et al. 1991; Chavez et al. 1991) and mesoscale fertilization experiments (Martin et al. 1994).

Iron quotas—Iron quotas of the protozoa measured under low-iron conditions were higher than those of phytoplankton (Maldonado and Price 1996) and a number of marine heterotrophic bacteria (Tortell et al. 1996). This result is perhaps surprising, because a heterotrophic mode of existence lacks the iron-dependent photosynthetic reactions that are known to incur a high demand for iron in photoautotrophs. However, the ETS redox proteins needed to maintain active heterotrophic metabolism can impart a substantial iron requirement. Indeed, in iron-limited bacteria, where most cellular Fe is associated with the ETS (Righelato 1969; Light and Clegg 1974; Granger and Price in prep.), iron quotas are relatively high (Tortell et al. 1996). Assuming that all heterotrophs have similar respiratory pathways, we expected protozoa to have minimum iron quotas similar to or less than those of bacteria. Their quotas would most likely be lower, because the specific respiration rates (respiration/biomass), and hence ETS/biomass, of heterotrophs decrease with increasing size (Peters 1983). Iron should thus be "demagnified" as it passes up the food chain. Our results, however, seem to run counter to this allometric argument, because the flagellates in the low-iron experiments had higher iron quotas than did their prey. This may be a consequence of sample size, since only two species of a single genus were examined, or a consequence of scale, since the allometric relationship holds best when considering organisms that differ in size by many orders of magnitude. From a compilation of early measurements (Vinogradov 1953) we find a mean Fe:C in marine fish of 4.5 μmol Fe: mol C (median = 3, n = 72; assuming wet wt = 5 x dry wt). This lends support to the idea of iron demagnification. The data are difficult to interpret, however, because the iron nutritional status of the large organisms is not known. An alternative explanation for the high quotas is that eukaryotic metabolism is relatively more iron-costly than prokaryotic metabolism, or it uses large amounts of iron not associated with the ETS. Indeed, when we consider bacteria, protozoa, and zooplankton, we
find a dramatic decrease in the ratio of ETS to Fe (Table 4), suggesting a greater importance of non-ETS iron in larger organisms.

Carbon and nitrogen metabolism—Iron limitation profoundly affected carbon metabolism in *P. imperforata*. These protozoa converted less prey C into biomass and released more ingested C as DOC and CO₂, compared to those consuming iron-rich prey. The validity of these results rests on the equal fulfilment in both iron treatments of assumptions made in calculating GCGE. Most important among these, we assumed that at the end of the flagellate growth interval POC consisted entirely of protozoa and uneaten bacteria. GGEs determined from measurements of protozoan volume are ~12% lower than those measured by the method we used (Fenchel 1982, Caron et al. 1985, Geider and Leadbeater 1988). Presumably the discrepancy exists because some ingested C is egested as unincorporated POC (see Stoecker 1984). If this egested C is retained by the filters, it would be counted as protozoan biomass and would thus increase the measured G, so that CO₂ evolution calculated by difference (CO₂ = I - G - E) would be lower than CO₂ evolution measured directly. Because the ratio of CO₂ measured to CO₂ calculated in the CO₂ evolution experiment was 1.08 and 1.27 for the iron-rich and iron-poor treatments, overestimation of GCGE was, if anything, more severe in the low-iron treatment. Thus, we think that the observed differences in C metabolism between Fe treatments are real.

The ammonium excretion results showed that N metabolism was also affected by iron status. Lower NH₄⁺ regeneration efficiencies of iron-limited protozoa implies that they excreted more of their ingested N as DON or urea, or accumulated more N in biomass. An increased N content of the protozoa seems unlikely, however, because C per protzoan was the same in both iron treatments, and C:N ratios of other plankton are known to be independent of iron (Mal-
An alternative way to evaluate the validity of the iron results is to look at their internal consistency. It should be that
\[
\text{Fe:Fe}_\text{flagellate} = \text{Fe:Fe}_\text{toxin} \times \frac{\text{Fe:Fe}_\text{GC}}{\text{Fe:Fe}_\text{Tune}},
\]
where \(\text{Fe:Fe}_\text{GC}\) and \(\text{Fe:Fe}_\text{Tune}\) refer to the flagellate. We find that the right-hand side of this relationship exceeds the left-hand side by \(\sim 14\%\) for both low- and high-iron treatments. This suggests that while there are some methodological inconsistencies, they act equally in both treatments. We are thus confident that the problems of reassimilation and particulate egestion do not confound our interpretation of the data.

A biological explanation for the paradoxical result of lower \(\text{Fe:Fe}_\text{GC}\) from iron-poor bacteria may be that these prey contain relatively large pools of nonassimilable Fe. Price (1968) proposed that the order in which cellular iron pools are lost as iron limitation proceeds reflects the strength of cellular iron chelates, so that severely iron-limited plant cells retain only that which is most tightly bound. Although this model has yet to be confirmed, bacteria grown with excess iron-limited cells. Such chemical forms may be less available for biological assimilation. During preliminary investigations into iron partitioning in strain Jull88, we found that the iron of iron-replete cells was more reactive toward glass beads than was that of iron-deplete cells (data not shown).

Cellular partitioning of iron within prey cells might also affect protozoan assimilation, as does with metazoan grazers (Reinfelder and Fisher 1991; Huchins et al. 1995). This may explain the difference between the pattern of regeneration observed here for iron (greater efficiency when prey are iron-poor) and the results obtained for N and P regeneration (Goldman and Caron 1985; Goldman et al. 1987). Perhaps the relative distribution of trace metals within prey cells is more sensitive to prey nutritional status than is major nutrient distribution, or, alternatively, the assimilation of trace metals by grazers may be more dependent on the cellular forms of the nutrient. Another possibility is that under iron stress the ability of protozoans to transport all materials across the food vacuole membranes is decreased, leading to high Fe regeneration efficiencies. Clearly, the factors that regulate metal assimilation by protozoa in general, and their possible interaction with prey nutritional status in particular, deserve further attention. For example, phytoplankton, although iron-poor, might provide a good source of dietary Fe if their cellular pools are more easily assimilated than are those of bacteria.

Remineralization of cellular Fe is thought to be a key process in the surface-water biogeochemistry of iron (Bruland et al. 1991; Huchins et al. 1993). We observed very high maximum iron regeneration efficiencies for \(P. \text{imperforata}\) consuming bacterial prey (59 and 84% for iron-replete and iron-deficient prey). Coupled with the large amount of iron in the bacterial community (Tortell et al. 1996), this result suggests that protozoan bactivory is a potentially important source of regenerated Fe. However, the chemical nature and reactivity of this iron, and hence its availability to phytoplankton and bacterioplankton, is unknown. Some regenerated metals may not be immediately available for phytoplankton use (Twiss and Campbell 1995). Indeed, the transience of the biological (phytoplankton) response to large-scale iron addition (Martin et al. 1994; Coale et al. 1996) speaks against rapid and efficient recycling of cellular Fe in these parts of the sea.

This paper documents the first investigation of iron limitation in heterotrophic marine protozoa. \(P. \text{imperforata}\) and \(P. \text{butcheri}\) have relatively high cellular iron requirements and grow slowly when fed iron-poor bacterial prey. Under low-iron conditions, these protozoa have lower GCGEs, excrete more of their ingested ration as DOC and DON, and regenerate less NH\(_4\). Protozoa in iron-poor waters may thus be less efficient at converting bacterial prey into biomass, thereby affecting the role of the microbial loop as a remineralization pathway vs. a link to higher trophic levels. Collectively, the results suggest that the concept of iron limitation can be extended to heterotrophic grazers, and imply that the entire ecology of oceanic regions could be modulated by the supply and availability of iron.

References


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