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Dual-labeling techniques for trace metal biogeochemical investigations in aquatic plankton communities

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ABSTRACT: Recent work demonstrating the ecological importance of trace metals in aquatic plankton communities has stimulated interest in biological metal cycling and transfer in natural waters. Like major nutrients, sources of metals to phytoplankton can include both exogenous (new) and endogenous (recycled) supplies. Indirect evidence suggests that the subcellular location of metals in plankton cells---surface-adsorbed versus intracellular—is a primary factor controlling their biogeochemical fate after processing by zooplankton grazers. However, there are no methods presently available to directly measure the turnover of intra- and extracellular metal pools, or the uptake of new and regenerated metal sources by phytoplankton. We present here new techniques that use dual radioisotopes of the same trace element to make the first direct measurements of these biogeochemically important processes in marine and freshwater plankton communities. Since our methods depend on quantitative removal of surface-bound isotopes from plankton cells, we first compared the efficiency with which a variety of published surface-wash techniques remove adsorbed trace metals from phytoplankton cells. A widely used Fe surface-wash method that employs a Ti(III)/citrate/EDTA reagent was the most effective technique examined for removing a variety of extracellular trace metals, including Co, Cd, Zn and Mn. We then demonstrated the use of the dual radiolabels ^{59}Fe and ^{55}Fe to compare new and regenerated Fe uptake by marine phytoplankton in laboratory and field experiments. Results indicated that new (dissolved) Fe was utilized more readily by phytoplankton than Fe regenerated from the intracellular pools of other plankton, but transfer of intracellular Fe was substantial. This dual isotope pair was also used to examine the fate of diatom intracellular ^{55}Fe and surface-adsorbed ^{59}Fe after grazing by copepods. Similar freshwater experiments examined the cycling of diatom intracellular ^{60}Co and extracellular ^{57}Co after grazing by cladocerans. Both grazing experiments showed that intracellular metals are most efficiently assimilated by zooplankton, while extracellular Fe and Co are preferentially regenerated to dissolved forms during grazing. Dual-labeling techniques promise to allow direct, unambiguous characterization of difficult-to-resolve portions of the biogeochemical cycles of many biologically important trace elements, including Fe, Co, Mn, Ni, Ag, Cd and Se.

KEY WORDS: Iron · Trace metals · Plankton · Biogeochemistry

INTRODUCTION

It is now widely accepted that trace metals can play an important role in controlling primary productivity and major nutrient cycling. Fe availability can limit phytoplankton production in remote open ocean regimes (Martin et al. 1991, Coale et al. 1996), in coastal upwelling zones (Hutchins & Bruland 1998, Hutchins et al. 1998), and even in some lakes (Auclair 1995). Other metals including Co, Cd, Mn and Zn may also have stimulatory or inhibitory effects on plankton growth (Bruland et al. 1991). Despite this recent interest in metals as potentially limiting micronutrients and contaminants, our knowledge of the routes by which trace elements cycle within the plankton community is still limited.

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Hutchins et al. (1993) demonstrated that Fe is recycled within plankton assemblages in much the same manner as major nutrients. Dugdale & Goering's (1967) new versus regenerated nitrogen cycling model has been applied to Fe, leading to suggestions that most primary productivity in low Fe regimes is supported by recycled biological Fe rather than by new inputs (Hutchins 1995). Like major nutrients, processes such as zooplankton grazing and viral lysis appear to drive the turnover and recycling of cellular Fe and other trace metals in the ocean (Hutchins & Bruland 1994, Lee & Fisher 1994, Hutchins et al. 1995, Chase & Price 1997, Gobler et al. 1997, Wang & Fisher 1998). Similarly, grazing is a dominant force controlling the rates at which elements like Cd and Zn are biologically regenerated and recycled in lakes (Twiss & Campbell 1995, Twiss et al. 1996). However, there are no techniques presently available to make direct measurements of new and regenerated trace metal uptake by phytoplankton.

Recent work has given us a better understanding of some of the factors that influence metal cycling by zooplankton. Reinfelder & Fisher (1991) demonstrated that the fraction of ingested trace metals assimilated by copepods can be closely predicted by the amount in the cytoplasm of the phytoplankton food. Metals bound to cell walls and membranes appear to be released in fecal pellets. Grazer assimilation of Fe also correlates with algal cytoplasmic content, as well as being linearly related to total intracellular content (including both cytoplasmic and membrane-bound fractions; Hutchins et al. 1995). It can be inferred from these investigations that extracellular surface-adsorbed pools are more likely regenerated to the dissolved phase, but methods for directly measuring the fate of specific subcellular metal pools following ingestion by grazers have been lacking.

To address this problem, we developed a new dual-labeling technique in which intracellular and extracellular metal pools in prey cells are separately labeled with 2 isotopes of a single trace metal. Dual labeling also enables a comparison of the bioavailability of one isotope originating from intracellular pools, with that of another isotope of the same element from the dissolved phase. These types of experiments require accurate determination and controlled manipulations of cellular surface-bound and internal metal pools.

A number of published techniques are available which remove surface-adsorbed metals from plankton cells, allowing measurements of intracellular pools. The amount of metal in surface-bound pools is calculated as the difference between samples with and without surface washing. Careful application of these techniques is the key to successfully carrying out dual-labeling experiments. However, a variety of surface washes have been employed in various investigations. Early marine work used washes with the synthetic chelator EDTA (Davies 1970) or ascorbate (Anderson & Morel 1982, Harrison & Morel 1986). More recently, a reagent that contains Ti(III)/citrate/EDTA has been widely used in radioisotope studies of phytoplankton Fe uptake (Hudson & Morel 1989). Other published techniques include diethylenetriaminepentaacetic acid (DTPA) for 'soft' metals such as Cd (Lee et al. 1995), and 8-hydroxyquinoline-5-sulfonate (sulfoxine), which has been used for Cd, Co, Ni and Zn (Price & Morel 1990, 1991).

This diversity of techniques makes it difficult to determine the comparability of various measurements of intra- and extracellular metals, and to select an appropriate method for removing surface-bound metals for dual-labeling investigations. Accordingly, as a first step in this work we compared the effectiveness of Ti reagent, DTPA and sulfoxine in removing surface-adsorbed metals. We compared these wash methods using diatom cultures pulse-labeled with ^{109}Cd, ^{57}Co, ^{59}Fe, ^{54}Mn and ^{65}Zn. All but Zn have multiple radioisotopes and are therefore useful in dual-labeling work (see 'Discussion').

We present here a series of experiments in which we directly measure grazer-mediated cycling of intra- and extracellular metals by using 2 different radioisotopes of the same metal. We also demonstrate the use of dual-labeling techniques to examine new versus regenerated trace metal availability to marine phytoplankton. These experiments use the electron-capture isotope ^{55}Fe (beta peak energy at 232 keV) and the gamma- and beta-emitting isotope ^{59}Fe (gamma peak energies at 1099 and 1292 keV). Other experiments use the gamma emitters ^{57}Co (122 keV) and ^{60}Co (1173, 1332 keV) to examine the fate of surface-bound and internal phytoplankton pools of this important micronutrient after grazing by freshwater cladocerans. These experiments illustrate some of the many possible ways in which dual-labeling methods can be used to investigate biological trace element cycling in aquatic plankton communities.

MATERIALS AND METHODS

Measurements of subcellular metal pools. Cultures of the diatom *Thalassiosira weissflogii* were grown in Aquil medium (Price et al. 1989), harvested by filtration, and resuspended to a density of 10^4 ml^{-1} in 5 polycarbonate bottles each containing 50 ml UV-photooxidized, 0.2 µm filtered seawater (UVSW). For each radioisotope (either ^{59}Fe, ^{57}Co, ^{109}Cd, ^{65}Zn or ^{54}Mn), 111 kBq was added to each of the 5 bottles; molar additions were 4.3×10^{-8} M Fe, 4×10^{-9} M Co, 5.9×10^{-7} M

Cd, 4.86×10^{-7} M Zn, and 4.0×10^{-7} M Mn. The diatoms were exposed to a radioisotope for 10 min, filtered onto a 1 µm polycarbonate membrane, rinsed 3 times with UVSW, and resuspended in 65 ml UVSW. From each resuspended sample six 5 ml aliquots were immediately removed to be washed using each surface-wash technique (either Ti reagent, DTPA or sulfoxine). These methods were compared with 6 samples washed only with three 5 ml rinses of UVSW.

An assumption in this pulse-labeling method is that the cells have little time to internalize the metal isotopes during the brief exposure period, and therefore nearly all of the radioisotope should be located on external surfaces. Growing the inoculum under metal-replete conditions to reduce cellular demand and therefore uptake rates also helps to reduce internalization of radiolabel during the external pulse-label period.

Surface wash protocols included Ti reagent, 1, 50, and 100 mM DTPA, and 1 mM sulfoxine, all at pH 8. All treatments used a 10 min exposure to the surface-wash medium, except the Ti reagent which was 3 min (Hudson & Morel 1989). After this exposure period, the samples were filtered onto 1 µm polycarbonate membranes and rinsed 3 times with 5 ml UVSW. Gamma activities of the filters were measured with background correction on a Wallac 1480 gamma counter at 1292 keV (^{59}Fe), 122 keV (^{57}Co), 88 keV (^{109}Cd), 1115 keV (^{65}Zn) and 834 keV (^{54}Mn). The fraction of each metal removed was calculated as: $[1-(A_r/A_0)] \times 100$, where A_r is the radioactivity remaining after washing and A_0 is the activity of the samples rinsed only with seawater.

Marine grazing experiments. Intracellular pools of *Thalassiosira weissflogii* were radiolabeled by growing cells in 300 ml Aquil medium without added stable Fe but containing 7.96 MBq of ^{55}Fe EDTA (0.5 µM Fe) for 3 d. The cells were harvested by filtration and surface-adsorbed ^{55}Fe was removed with Ti reagent. Rewashing an aliquot of the radiolabeled cells demonstrated that the first wash removed >95% of the Ti-labile ^{55}Fe. Pulse labeling of extracellular pools was accomplished by resuspending the cells in 50 ml UVSW containing 185 kBq of ^{59}Fe for 15 min, followed by 3 UVSW rinses. A Ti-washed sample showed that >93% of the ^{59}Fe could be removed after the pulse-labeling period. The dual-labeled cells (>95% of the ^{55}Fe intracellular, >93% of the ^{59}Fe surface-adsorbed) were immediately resuspended in UVSW at a density of 10^5 ml^{-1} in three 150 ml containers containing a natural copepod assemblage (mostly *Acartia tonsa*) collected from Stony Brook Harbor, Long Island, New York, using net tows. The copepod density was approximately 1 ml^{-1}. Grazers were allowed to feed for 1 h in the dark and were then removed from the feeding containers and placed in depuration bottles containing 150 ml UVSW with unlabeled *T. weissflogii* at the same density as in the feeding containers. The experimental design is depicted in Fig. 1.

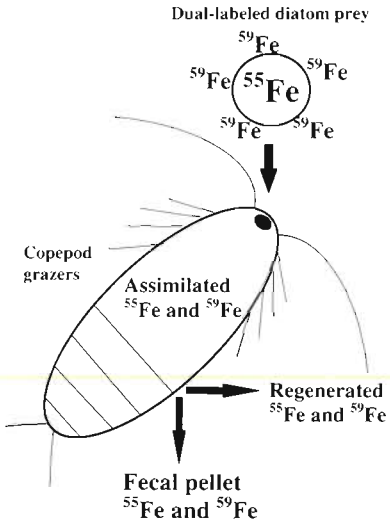

Fig. 1. Schematic diagram of the methods used in the dual-labeling grazing experiments. Diatom prey (*Thalassiosira weissflogii*) were grown in medium containing ^{55}Fe to label intracellular pools. Ti-washing was then used to remove surface-bound ^{55}Fe and the cells were pulse-labeled with ^{59}Fe, producing dual-labeled cells with nearly all their ^{55}Fe in intracellular pools and all their ^{59}Fe in surface-adsorbed pools. The dual-labeled cells were then fed to copepod grazers, and amounts of each isotope ending up in assimilated, fecal pellet and dissolved pools were measured. Similar protocols were used for the freshwater ^{57}Co/^{60}Co experiments using cladoceran grazers

After a 4.5 h depuration period, the distribution of ^{55}Fe and ^{59}Fe was measured by size fractionating the grazers (200 µm Nitex), fecal pellets (20 µm Nitex), and originally unlabeled diatom cells (1 µm). Grazers and fecal pellets were gravity filtered, and cells were gently vacuum filtered (<10 kPa); all 3 fractions were rinsed 3 times with UVSW to remove adhering liquid before counting. Dissolved activity was measured by concentrating the 1 µm filtrate by evaporation on a warm hot plate until dry before adding LSC cocktail to the remaining salts. ^{59}Fe gamma activity was measured immediately on an LKB gamma counter at 1292 keV with background correction. ^{55}Fe beta radioactivity was measured by LSC after storing the samples for 193 d to allow ~95% of the interfering ^{59}Fe beta activity ($t_{1/2}$ = 45 d) to decay away. The ^{55}Fe activity was corrected for quenching and for decay ($t_{1/2}$ = 2.7 yr) during the storage period. Amounts of ^{55}Fe and ^{59}Fe regenerated to the dissolved phase by the grazers were calculated by summing the activity in the evaporated filtrate fraction and the 1 µm filter fraction. Radioactivity associated with this 1 µm fraction (con-

taining the originally unlabeled diatom cells from the depuration containers) could only have originated by adsorption or uptake of an isotope that had first been regenerated to the dissolved phase by the grazers.

Freshwater grazing experiments. Freshwater grazing experiments were carried out using similar techniques to those outlined above, except that internal diatom pools were labeled with ^{60}Co and external pools were labeled with ^{57}Co. In seawater, Co is not particularly particle-reactive, due in part to its complexation by chloride ions (Turner et al. 1981), but in freshwater where chloro-complexation is low this metal adsorbs readily to cell surfaces. Cultures of the freshwater diatom *Cyclotella* sp., strain 1020, were labeled in WCL-1 medium (Guillard 1975) with 37 kBq of ^{60}Co for 7 d. Cells were then filtered onto 3 µm polycarbonate membranes for removal of surface-bound ^{60}Co. Because effects of Ti reagent on freshwater phytoplankton have not been investigated, we used a 10 min exposure to 10^{-3} M EDTA (pH 7) as a surface wash in these experiments. EDTA is a much more effective metal chelator in freshwater than it is in seawater, because levels of competing cations (especially Ca^{2+} and Mg^{2+}) are much lower in freshwater systems.

External pulse labeling was carried out using 111 kBq of ^{57}Co in 30 ml pond water for 10 min followed by 3 pond water rinses. Rewashing a subsample with the 10^{-3} M EDTA removed >85% of the total cellular ^{57}Co activity. The dual-labeled diatom cells were then resuspended in triplicate bottles containing 120 ml 0.2 µm of filtered pond water (10^5 cells ml^{-1}) with a natural cladoceran assemblage (*Simocephalus* sp.) collected from a pond on the SUNY (State University of New York) Stony Brook campus. The cladoceran density in the grazing bottles was ~1 ml^{-1}, and grazers were fed for 2 h in the dark. The cladocerans were then removed and their ^{57}Co and ^{60}Co gamma radioactivities counted before being depurated for 4 h as in the marine experiments. Activities in the grazers, fecal pellets, unlabeled diatom cells from the depuration containers, and the dissolved phase (<0.2 µm) were then determined by gamma counting (^{57}Co = 122 keV, ^{60}Co = 1173, 1332 keV) with appropriate corrections for background and spillover.

Field new and regenerated Fe uptake experiments. Freshly upwelled surface seawater (34.3 µM NO_3^-, 23.1 µM H_2SiO_3, 3.5 nM Fe) was collected using trace metal clean techniques on 29 June 1996 off Point Año Nuevo, California (36° 59.6' N, 122° 12.9' W). Total reactive dissolved Fe measurements were provided courtesy of E. Rue and K. Bruland using electrochemical measurements of UV-oxidized samples (Rue & Bruland 1997). 2.7 l of this water was cleanly filtered through 5 µm acid-washed polycarbonate filters to remove large phytoplankton, zooplankton and detritus. ^{55}Fe (5.18 MBq or 3.6×10^{-8} M Fe, chelated 1:1 with EDTA) was added to the remaining natural picoplankton community, composed largely of heterotrophic bacteria and small autotrophic flagellates. This water was incubated on deck in a spectrally corrected incubator for 3 d, then cleanly filtered again through 5 µm filters to insure that all large particles had been removed. The 5 µm filtrate containing the labeled picoplankton assemblage was collected and filtered onto a Ti-washed 0.2 µm filter using gentle vacuum (<10 kPa), and the intracellularly radiolabeled picoplankton were resuspended into 175 ml 0.2 µm filtered, chelexed seawater.

Specific activity of the radiolabeled picoplankton was calculated as the radioactivity of the ^{55}Fe added to the labeling bottle (dpm) divided by the sum of the ambient dissolved Fe and added ^{55}Fe (mols). Chlorophyll *a* biomass in the labeling bottles increased ~8.5× during the 3 d incubation (not shown), suggesting that the final community was uniformly radiolabeled and that the original particulate Fe pool (as picoplankton cells) contributed only a small fraction of the final particulate Fe concentrations in the bottles. Consequently, particulate Fe concentrations were not measured and the original particulate Fe pool was not included in the specific activity calculations.

Unfiltered surface seawater for the uptake portion of the experiment was collected on 2 July 1996 off Davenport, California (36° 59.939' N, 122° 14.000' W, 1.9 nM Fe) and dispensed into two 2.7 l acid-washed polycarbonate bottles. To each bottle was added 75 ml of the concentrated ^{55}Fe-labeled picoplankton suspension and 92.5 kBq of ^{59}Fe stock (7.0×10^{-10} M Fe) in 0.1 N Ultrex HCl. Specific activity of the dissolved Fe pool in the uptake bottles was calculated as the radioactivity of the added ^{59}Fe (dpm) divided by the sum of the ambient dissolved Fe and the added ^{59}Fe (mols). Uptake of dissolved ^{59}Fe and regenerated intracellular picoplankton ^{55}Fe into intracellular pools of the large phytoplankton size class was followed by filtering 50 to 100 ml samples from the replicate bottles onto Ti-washed 10 µM polycarbonate filters periodically during a 59 h incubation. Microscopic examination of this large size class showed that it was composed largely of typical upwelling zone chain-forming diatom species such as *Chaetoceros* spp. A schematic of the experimental setup is presented in Fig. 2.

Filters were counted immediately for ^{59}Fe gamma radioactivity on a Hewlett Packard Ge 3000 gamma counter, then stored for 183 d to allow ~94% of the ^{59}Fe beta activity to decay before counting ^{55}Fe beta activity on a Wallac 1409 liquid scintillation counter. Both measured isotope activities were corrected for background, and ^{55}Fe beta activity was corrected for quenching and for radioactive decay during the storage period.

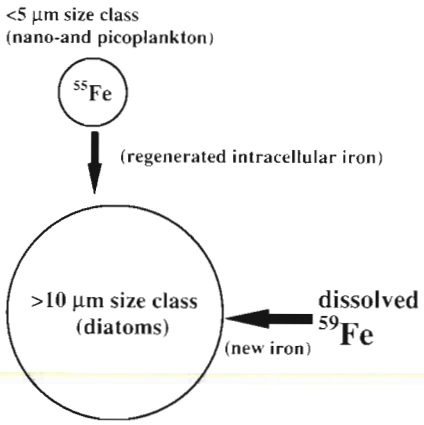

Fig. 2. Schematic diagram of the methods used in the field new and regenerated Fe uptake dual-labeling experiments. A natural community of pico- and nanoplankton (<5 µm) was labeled with ^{55}Fe for 3 d, Ti washed to remove surface-adsorbed ^{55}Fe, and added to a natural community of large diatoms (>10 µm) to simulate transfer of regenerated Fe from small to large cells. Dissolved ^{59}Fe was also added to the bottles to simulate an input of new Fe to the system, and uptake of both isotopes by the diatoms was measured over time. Similar methods were used in the laboratory new and regenerated Fe uptake experiments measuring transfer from cultured picoplankton (*Synechococcus* sp.) to diatoms (*Thalassiosira weissflogii*)

Laboratory new and regenerated Fe uptake experiments. Hutchins et al. (1993, 1995) and Hutchins & Bruland (1994) suggested that grazing is likely to be the primary means by which cellular trace metals are regenerated and recycled in plankton communities. However, other processes including viral lysis (Gobler et al. 1997), autolysis (Berges & Falkowski 1998), bacterial decay of cell debris (Lee & Fisher 1992, 1993), and release of cellular metals across the membranes of healthy cells also undoubtedly play a role in this process. The dual-labeling experiments presented here compare uptake of regenerated cellular ^{55}Fe and dissolved ^{59}Fe in a controlled laboratory culture system, without external forcing by turnover mechanisms such as grazing and viral lysis. Thus, they give an estimate of the potential importance of direct release from healthy growing cells as an Fe recycling mechanism, relative to uptake from dissolved sources.

Cultures of the unicellular coccoid cyanobacterium *Synechococcus* sp. (strain WH7803, ~1 µm diameter) were radiolabeled with ^{55}Fe (523.5 Bq ml^{-1}) in Aquil medium for 4 d. Exponential phase cells were harvested on 0.2 µm filters and washed with Ti reagent, then resuspended in 100 ml UVSW and filtered through a 5.0 µm membrane to remove cell aggregates or larger particles. These cells were added to triplicate 700 ml bottles containing an exponential phase *Thalassiosira weissflogii* culture (1.5×10^6 cells ml^{-1}), along with 51 Bq ml^{-1} dissolved ^{59}Fe. Diatom uptake of regenerated intracellular cyanobacterial ^{55}Fe and dissolved ^{59}Fe was followed for 58 h by filtration onto 5 µm filters.

Instead of counting ^{59}Fe gamma activity immediately, holding the filters for about 6 mo to allow ^{59}Fe decay, and then counting ^{55}Fe beta activity as in the experiments presented above, these filters were analyzed immediately using recently available dual-labeling LSC software (Wallac 1409-111 Dual Label DOT-DPM). When properly calibrated, this software corrects for overlap in energy detection windows, allowing simultaneous determination of ^{55}Fe and ^{59}Fe beta radioactivities in the same sample with errors of <1%. Thus, Fe dual-labeling experiments can be carried out without the necessity of a lengthy storage period, greatly facilitating the timely completion of the work.

RESULTS

Measurements of subcellular metal pools

Results of the surface-washing methods intercomparison are presented in Fig. 3. For all 5 metals, the Ti reagent technique of Hudson & Morel (1989) was most effective at removing surface-adsorbed metals, with amounts removed ranging from 81% (Cd) to 97% (Fe). 100 mM DTPA was relatively effective at removing the 'soft' metals Zn and Cd (78 and 64% removed, respec-

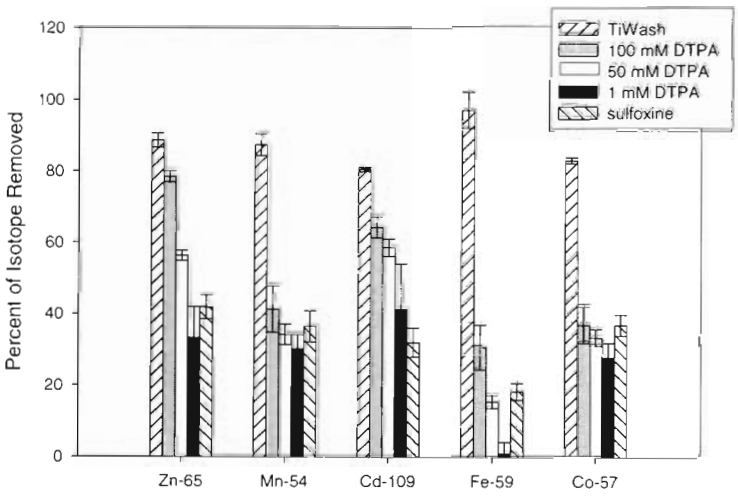

Fig. 3. Percent of surface-bound metal isotopes removed from cultured *Thalassiosira weissflogii* cells by the Ti reagent (47 mM Ti(III), citrate and EDTA), 100, 50 and 1 mM DTPA, and 1 mM sulfoxine. Bars and error bars represent the means and standard deviations of 6 replicates

tively) for which the technique was developed (Lee et al. 1995), but was less effective for Fe, Mn and Co. Reducing the concentration of the DTPA also reduced its effectiveness as a surface wash. Sulfoxine was least effective at removing surface-bound metals, with amounts removed ranging from 18% (Fe) to 42% (Zn).

Ti reagent was developed specifically for Fe and contains 0.047 M EDTA and 0.047 M citrate. These chelators form a ternary complex with titanous ion (Ti^{3+}). During the washing procedure Ti(III) donates electrons to Fe(III), thereby reducing Fe to non-particle reactive Fe(II), which is then easily washed off cell surfaces. It is thus not surprising that this reagent removed nearly 100% of surface-bound Fe. However, these results demonstrate that the Ti reagent is also very effective at removing even metals such as Zn, which do not undergo redox chemistry, probably due to the high levels of the chelators EDTA and citrate in the reagent. Ti reagent appears to be the technique of choice for effective removal of a wide range of surface-adsorbed metals, and with the exception of the freshwater Co work presented here, we use this method to remove surface-adsorbed metals in all of our dual-labeling experiments.

Marine grazing experiments

The fate of diatom extracellular ^{59}Fe and intracellular ^{55}Fe after grazing by copepods is illustrated in Fig. 4. It is apparent that the major fate of both pools was regeneration to the dissolved phase, although as predicted (Hutchins et al. 1995) extracellular ^{59}Fe was regenerated somewhat more efficiently (80%) than intracellular ^{55}Fe (60%). Intracellular ^{55}Fe was retained by the grazers twice as efficiently (30%) as was surface-adsorbed ^{59}Fe (15%), again supporting the results of earlier single isotope experiments (Reinfelder & Fisher 1991, Hutchins et al. 1995). In this experiment fecal pellets were a minor fate for both intracellular and extracellular pools (5 to 7%).

These results should not be taken to represent a complete mass balance of all the radiolabeled Fe in prey cells which was consumed during the feeding period. The size of the fecal pellet pool is undoubtedly underestimated because in this experiment we measured amounts of ^{55}Fe and ^{59}Fe produced as fecal pellets only in the depuration containers, not those produced during the 1 h feeding. Another minor source of underestimation of Fe in fecal pellets may have been from loss during the gentle seawater rinses on the 20 µm Nitex used to isolate this size fraction. Experiments showed that such losses accounted for about 20% of the fecal pellet radioactivity, and this lost isotope represents loosely adsorbed material which is easily exchangeable with dissolved pools. Although this experiment did not attempt to completely account for all ^{55}Fe and ^{59}Fe consumed by the copepods during the feeding period, it provides a good estimate of the fate of the ingested extra- and intracellular Fe contained in the digestive system of the grazers at the beginning of the depuration period.

Freshwater grazing experiments

Results of the ^{57}Co/^{60}Co freshwater cladoceran grazing experiment are presented in Fig. 5. As in the marine copepod experiment, the main fate of both pools was regeneration to dissolved forms, although again about 21% more surface-bound ^{57}Co ended up in dissolved forms (87%) than was the case for intracellular ^{60}Co (66%). Also similar to the marine experiment was the greater assimilation by the grazers of

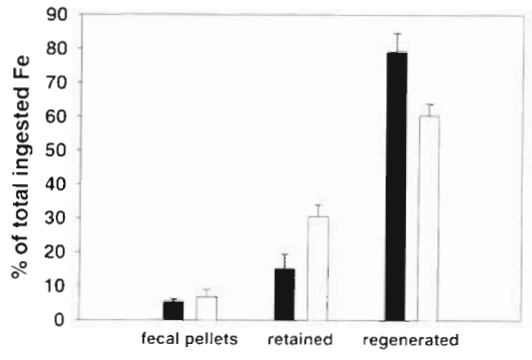

Fig. 4. Amounts of diatom prey surface-bound ^{59}Fe (black bars) and intracellular ^{55}Fe (white bars) in fecal pellets, retained (assimilated) and regenerated (dissolved) pools after 4.5 h depuration by marine copepod grazers. Bars and error bars represent the means and standard deviations of triplicate bottles

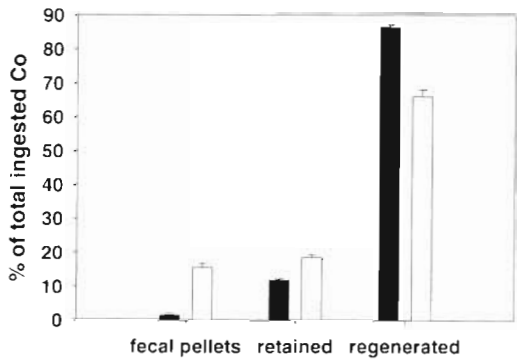

Fig. 5. Amounts of diatom prey surface-bound ^{57}Co (black bars) and intracellular ^{60}Co (white bars) in fecal pellets, retained (assimilated), and regenerated (dissolved) pools after 4 h depuration by freshwater cladocerans. Bars and error bars represent the means and standard deviations of triplicate bottles

intracellular ^{60}Co (19%) compared to surface-bound ^{57}Co (12%). Fecal pellets contained only a minor fraction of the original ingested extracellular ^{57}Co (2%) and a greater fraction of intracellular ^{60}Co (16%). As in the copepod experiment presented above, these represent only percentages of the activity present in the grazers at the beginning of depuration, and amounts produced as fecal pellets or regenerated during the 2 h grazing period are not included.

Field new and regenerated Fe uptake experiments

Fig. 6 demonstrates transfer of dissolved ^{59}Fe and ^{55}Fe regenerated from picoplankton intracellular pools, to the intracellular pool of the natural diatom assemblage (>10 µm) in the California upwelling region. As expected, transfer of 'new' dissolved Fe to large cells is faster than transfer of recycled cellular Fe in these coastal waters. Dual-labeling experiments carried out at realistic dissolved and particulate Fe concentrations in low-Fe oceanic regions would likely show the opposite trend, with recycled Fe being more important (Hutchins et al. 1993). However, uptake of recycled picoplankton Fe did represent a significant portion (~34%) of the final molar amount of Fe assimilated by the diatom assemblage, even in this neritic system.

Laboratory new and regenerated Fe uptake experiments

Transfer of extracellular ^{59}Fe and intracellular ^{55}Fe from cultured *Synechococcus* sp. to the diatom *Thalassiosira weissflogii* is illustrated in Fig. 7. The transfer of surface-bound Fe from the cyanobacteria to the diatoms was 1.6 × larger (1.3×10^{-10} mol) than the transfer of intracellular Fe (8.3×10^{-11} mol). However, the amount of intracellular Fe transferred was relatively large, suggesting that release from healthy growing cells may be an important process in Fe recycling. Exchange of Fe and other metals between intracellular and dissolved pools is likely to occur in natural waters as well, a process that is analogous to the isotope exchange measured in this experiment. One possible mechanism for the loss of intracellular Fe to the environment is release as organically bound Fe, as metals in neutrally charged organic complexes are more likely to cross hydrophobic cell membranes (Phinney & Bruland 1994, 1997) than are metals in ionic forms. Another possibility is autolysis, since nutrient- or light-limited phytoplankton may lyse spontaneously (Berges & Falkowski 1998). We did not count cells to monitor this possibility in our experiments, but autolysis seems

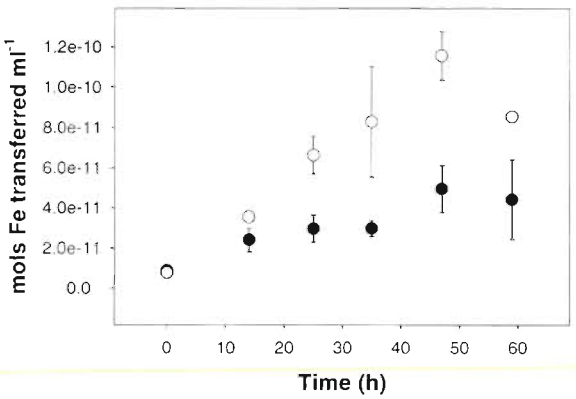

Fig. 6. Transfer of intracellular ^{55}Fe from radiolabeled natural picoplankton (●) and dissolved ^{59}Fe (○) to the intracellular pool of a natural large diatom assemblage in the California coastal upwelling region. Symbols and error bars represent the means and ranges of duplicate bottles

unlikely since our labeled cyanobacteria were healthy, nutrient-replete cultures in exponential growth phase. Regardless of the mechanisms involved, this dual-labeling experiment makes it apparent that Fe regeneration can occur independently of external cell turnover processes such as grazing and viral lysis.

DISCUSSION AND CONCLUSIONS

These experiments demonstrate several ways in which trace metal dual-labeling techniques can be used in biogeochemical investigations in both seawater and freshwater plankton communities. These results are not intended to provide final answers to questions about the roles of grazing and regeneration

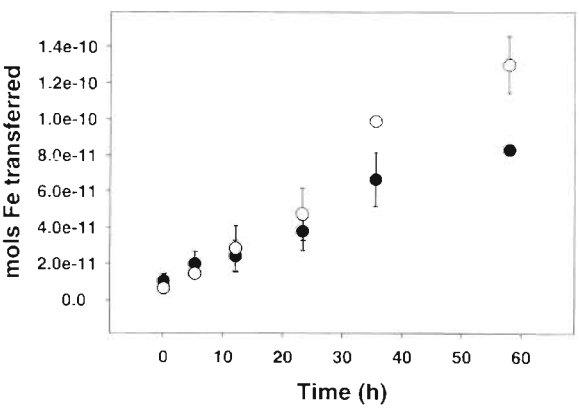

Fig. 7. Transfer of intracellular ^{55}Fe (●) and extracellular ^{59}Fe (○) from radiolabeled *Synechococcus* sp. cultures to *Thalassiosira weissflogii* cultures in the laboratory. Symbols and error bars represent the means and standard deviations of triplicate bottles

in cellular metal cycling. Obviously amounts of metals transferred from extra- and intracellular pools will be dependent on the initial conditions (dissolved and particulate metal concentrations, grazer and cell densities, etc.) which are chosen for a particular experiment. Transfer of intracellular Fe radiolabel in uptake experiments such as those presented here also undoubtedly underestimates recycled Fe fluxes in natural systems, since size fractionation techniques can only measure transfer between size classes, not recycling within size classes. Rather than providing definitive quantification of aquatic trace metal biogeochemical pathways, our intention is instead to demonstrate the advantages of dual-labeling techniques for investigations into a number of processes which are important in biota-trace metal interactions.

Effective removal of surface-bound metals is essential for dual-labeling experiments of the types presented here. Our intercomparison suggests that the Ti reagent, although developed specifically for Fe (Hudson & Morel 1989), is the best general surface-wash method for a number of metals in seawater, including Fe, Co, Cd, Zn and Mn. DTPA in high concentrations (100 mM) is also an acceptable alternative for 'soft' metals such as Cd and Zn. In general, however, we suggest the use of the Ti reagent to remove surface-bound metals from plankton cells during marine dual-labeling work. The EDTA wash employed in the freshwater experiment presented here was 80% effective at removing surface-bound Co; however, the possibility that other wash techniques might more efficiently remove extracellular metals in freshwater should also be examined.

These experiments demonstrate the utility of dual-labeling methods in investigations into grazer-mediated cycling of plankton-associated metals, and new versus regenerated metal uptake by phytoplankton. However, dual-labeling techniques can also be used to investigate other important processes, such as the bioavailability of different physiochemical and redox species of metals to aquatic organisms. As a recent example, Boisson et al. (1998) examined the relative availability of organic ^{60}Co-cobalamine (B12) and inorganic ^{57}Co to the marine macroalga *Fucus vesiculosus* using dual-labeling experiments. Dual-labeling experiments could also be used to examine the relative bioavailability of various physical species of trace metals (such as colloidal and truly dissolved forms) to aquatic organisms.

Where accurate knowledge of the short-term fate of extra- and intracellular metal pools is required, as in the grazing work presented here, experiments should be of limited duration (several hours to 1 d) to minimize exchange between isotope pools in the dual-labeled cells. For experiments examining the ultimate fate of these pools, as in our new versus regenerated Fe uptake experiments, exchange between intracellular and extracellular compartments is one of the main parameters of interest and so investigations can be carried out over longer time periods. For instance, such experiments could yield information about the long-range fluxes of cellular metals originating in extra- and intracellular pools into particulate and dissolved phases by mechanisms such as viral lysis and bacterial decay of cell debris.

For certain types of experiments, additional control treatments not included here would be desirable. Our data suggest that even healthy cells can lose substantial amounts of intracellular metals to the dissolved phase (Figs 6 & 7). Uptake of this dissolved metal by grazers during dual-labeling experiments would tend to bias measurements of elemental assimilation from food sources. Our experience suggests that uptake of Fe from dissolved sources by zooplankton is negligible compared to amounts assimilated from food (<1% of the body burden, Hutchins unpubl. results). However, for other metals (such as Cd or Ag) zooplankton dissolved uptake can be significant (Wang & Fisher 1998), and experimental designs should include no-grazer controls to quantify metal release from prey intracellular pools to the dissolved phase. Short experimental grazing periods such as those used here (1 h or less) should also be used to minimize uptake of metals from dissolved sources, since release of phytoplankton intracellular metals is relatively slow (<1% of total cellular Fe per hour in the experiment shown in Fig 7).

These experiments have focused on the use of the dual-label pairs ^{59}Fe/^{55}Fe and ^{57}Co/^{60}Co. However, a number of other trace elements have multiple isotopes with half-lives long enough for practical experimental use. These include Ni (^{56}Ni, ^{63}N), Ag (^{110m}Ag, ^{111}Ag), Cd (^{109}Cd, ^{113m}Cd, ^{115m}Cd), Mn (^{54}Mn, ^{52}Mn), and Se (^{72}Se, ^{75}Se), among others (Table 1). Although not all of these isotopes are commercially available, many can be obtained by special arrangement with isotope vendors or national laboratories. Phosphorus has 2 isotopes which are commercially available (^{32}P, ^{33}P), suggesting that dual-label techniques might be used for some investigations into major nutrient cycling as well.

In some cases, radioactive emission windows from the isotopes used in a particular dual-labeling experiment may overlap. When working with gamma-emitting radioisotopes, this can be readily overcome by using low energy germanium gamma detectors, which have much greater resolution than typical NaI(Tl) detectors. Additionally, it is often possible to take advantage of differences in radioisotope half-lives by counting emissions from the shorter-lived isotope first, then waiting an appropriate period until interference from this source has decayed enough to be negligible

Table 1. Radioisotopes which are potentially suitable for dual-labeling experiments. Included are the isotopes, their half-lives ($t_{1/2}$), daughter products, and their principal energy emission. EC: electron capture

Isotope	$t_{1/2}$	Mode of decay	Daughter	Principal gamma ray energy (keV)	Gamma ray intensity (%)	Beta particle energy (keV)
^{55}Fe	2.73 yr	EC	^{55}Mn	5		232
^{59}Fe	44.5 d	β-	^{59}Co	1110	57	
^{32}P	14.3 d	β-	^{32}Si			1710
^{33}P	25.3 d	β-	^{33}S			249
^{52}Mn	5.6 d	EC	^{52}Cr	1434	100	575
^{54}Mn	312 d	EC	^{54}Cr	835	100	
^{57}Co	271 d	EC	^{57}Fe	122	86	
^{58}Co	70.9 d	EC	^{58}Fe	811	99	2308
^{60}Co	5.27 yr	β-	^{60}Ni	1173	100	315
^{56}Ni	6.1 d	EC	^{56}Co	158	99	
^{63}Ni	100 yr	β-	^{63}Cu			65
^{72}Se	8.4 d	EC	^{72}As	46	58	
^{75}Se	120 d	EC	^{75}As	265	58	
^{110m}Ag	250 d	β-	^{110}Cd	658	96	
^{111}Ag	7.5 d	β-	^{111}Cd	342	7	1035
^{109}Cd	454 d	EC	^{109}Ag	88	4	
^{113m}Cd	13.7 yr	β-	^{113}In			590
^{115m}Cd	44.6 d	β-	^{115}In	934	2	1620

before counting the longer-lived isotope. This is the method we used for ^{55}Fe and ^{59}Fe in the experiments presented in Figs. 4 & 6. Similar methods could be used in dual-labeling experiments with other isotopes with overlapping emission windows, such as the beta energies of ^{75}Se (beta peak at 864 keV, gamma peaks at 136 and 265 keV) and ^{79}Se (beta peak at 151 keV, no gamma emissions). In spite of the problem of beta energy window overlap, these isotopes could be used in dual-labeling experiments due to their greatly differing half-lives. Such a hypothetical experiment might measure the gamma emissions of ^{75}Se, then allow this isotope to decay ($t_{1/2}$ = 119.8 d) before measuring beta emissions from ^{79}Se ($t_{1/2} = 6.5 \times 10^4$ yr). The availability of new sophisticated counting software which can quantify and correct for beta spectral overlap, such as the software used to generate the data presented in Fig. 7, will allow much wider application of dual-labeling methods within realistic experimental time frames.

Although not investigated here, the use of inductively coupled plasma mass spectrometry (ICPMS) could further extend the usefulness of dual-labeling methods. Many elements without multiple practical radioisotopes do have multiple stable isotopes that could be used in these types of investigations. For instance, Zn has only 1 useful radioisotope (^{65}Zn), but has 2 other stable isotopes with similar isotopic abundances (^{66}Zn and ^{68}Zn). Similarly, Cu (^{63}Cu, ^{65}Cu) and Pb (^{206}Pb, ^{207}Pb, ^{208}Pb) stable isotopes could also be used in dual-labeling experiments.

In recent years aquatic biogeochemists have come to appreciate that the complexity of biological trace element cycling in the ocean and in freshwater rivals that of the much better studied major nutrient elements. These experiments demonstrate that dual labeling can be used as an innovative new tool to selectively examine the multiple routes by which trace elements cycle in aquatic plankton communities, giving unique insights into the magnitude and rates of alternate biogeochemical pathways. Imaginative application of these techniques, coupled with new advances in counting techniques, should lead to other ways in which experiments using multiple isotopes of a single element can unravel the complexities of biological trace metal cycling in natural waters.

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