ORIGINAL ARTICLE

Genetic indicators of iron limitation in wild populations of *Thalassiosira oceanica* from the northeast Pacific Ocean

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Assessing the iron (Fe) nutritional status of natural diatom populations has proven challenging as physiological and molecular responses can differ in diatoms of the same genus. We evaluated expression of genes encoding flavodoxin (*FLDA1*) and an Fe-starvation induced protein (*ISIP3*) as indicators of Fe limitation in the marine diatom *Thalassiosira oceanica*. The specificity of the response to Fe limitation was tested in cultures grown under Fe- and macronutrient-deficient conditions, as well as throughout the diurnal light cycle. Both genes showed a robust and specific response to Fe limitation in laboratory cultures and were detected in small volume samples collected from the northeast Pacific, demonstrating the sensitivity of this method. Overall, *FLDA1* and *ISIP3* expression was inversely related to Fe concentrations and offered insight into the Fe nutritional health of *T. oceanica* in the field. As *T. oceanica* is a species tolerant to low Fe, indications of Fe limitation in *T. oceanica* populations may serve as a proxy for severe Fe stress in the overall diatom community. At two shallow coastal locations, *FLD1A* and *ISIP3* expression revealed Fe stress in areas where dissolved Fe concentrations were high, demonstrating that this approach may be powerful for identifying regions where Fe supply may not be biologically available.
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Introduction

Growth of primary producers in large areas of the world's oceans is predicted to be limited by low iron (Fe) concentrations (Moore *et al.*, 2004). Martin and Fitzwater (1988) demonstrated this in the northeast Pacific Ocean by showing that the growth of phytoplankton and subsequent utilization of macronutrients was promoted by Fe addition. Diatoms appear particularly responsive to influxes of Fe in low Fe waters. For example, diatom populations in both the equatorial Pacific and the Southern Ocean, regions with low Fe availability and chronically high nitrate and low chlorophyll, bloom upon the addition of Fe (Boyd *et al.*, 2007). These studies reveal that diatoms are capable of persisting in Fe-poor environments and rapidly acquiring Fe when it becomes available. Because of the importance of Fe to diatom growth and metabolism, much effort has been spent elucidating the molecular basis of Fe physiology in diatoms (Allen *et al.*, 2008; Kustka *et al.*, 2007; Lommer *et al.*, 2012; Maldonado *et al.*, 2006; Marchetti *et al.*, 2012; Nunn *et al.*, 2013; Thamatrakoln *et al.*, 2012; Whitney *et al.*, 2011) offering the potential to identify genetic indicators of Fe status in natural populations.

In Fe-limiting conditions, diatoms can lower their Fe demand by swapping the non-Fe-containing protein flavodoxin for the Fe-containing ferredoxin protein to maintain photosynthetic electron transport (Doucette *et al.*, 1996; Erdner *et al.*, 1999; LaRoche *et al.*, 1995; McKay *et al.*, 1997, 1999). Comparisons of ferredoxin and flavodoxin protein abundances in cultured diatom isolates grown in Fe-replete and Fe-limiting conditions have demonstrated that this is a strategy used by many diatoms (LaRoche *et al.*, 1995).

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Tracking these proteins in the field, however, has yielded incongruent results. Several studies measuring only flavodoxin have shown that naturally Fe-limited diatoms accumulated flavodoxin proteins and reduced expression with Fe addition in the North Pacific (LaRoche *et al.*, 1996; Maldonado *et al.*, 2001). Conversely, Fe-enrichment studies in the equatorial Pacific (Erdner and Anderson, 1999) and the subarctic Pacific (Suzuki *et al.*, 2009) detected only flavodoxin proteins and not ferredoxin proteins. It can be difficult to interpret the Fe status in natural diatom assemblages with these bulk flavodoxin and ferredoxin protein detection methods because the contribution of an individual species to these protein pools cannot be established. A related factor confounding the interpretation of flavodoxin protein abundances in natural samples is that in many diatoms, multiple genes encode flavodoxin proteins, with expression of the various gene copies showing differing sensitivities to Fe and potentially encoding for flavodoxin proteins with different functions (Lommer *et al.*, 2012; Whitney *et al.*, 2011). Because of the differences in tolerance to low Fe among diatom species (Sunda and Huntsman, 1995) and their genetic complexity, molecular markers that are not specific to an individual species or gene product may yield results that are misleading and do not accurately describe the metabolic state of the cells sampled.

We have developed species-specific molecular markers to describe the severity of Fe limitation in the diatom, *Thalassiosira oceanica*. We targeted this diatom as it is highly tolerant to low Fe, reaching near maximal growth rates in low levels of Fe where other isolates were unable to grow (Sunda and Huntsman, 1995). Therefore, as a species, an observation of Fe stress in *T. oceanica* may be a good biological indicator of Fe stress in natural diatom populations. *T. oceanica* is also an ecologically important diatom. It is found in many oceanic environments (Aizawa *et al.*, 2005; Garcia and Odebrecht, 2009; Harris *et al.*, 1995; Kaczmarska *et al.*, 2005; Tomas, 1997) including many samples from the low Fe waters of the northeast Pacific Ocean (Chappell *et al.*, 2013). The *T. oceanica* genes encoding the Fe-responsive flavodoxin (*FLDA1*) and Fe-starvation induced protein three (*ISIP3*) were selected as targets for molecular indicators of Fe status as they show a robust transcriptional response to Fe limitation (Chappell and Jenkins unpublished, Lommer *et al.*, 2012). The *FLDA1* gene is a homolog of the Fe-regulated gene identified by Whitney *et al.*, (2011) in the diatom *T. weissflogii*. The *ISIP3* gene encodes for a predicted cell surface protein with a putative role as an Fe receptor (Lommer *et al.*, 2012). It appears to be common among diatoms, with several laboratory studies demonstrating its regulation by Fe limitation (Allen *et al.*, 2008; Chappell and Jenkins, unpublished; Lommer *et al.*, 2012).

To determine the utility of *FLDA1* and *ISIP3* as biological markers of Fe stress in *T. oceanica*, we characterized their expression patterns in cells grown under a variety of environmentally relevant culture conditions, including Fe limitation, macronutrient deficiency and over diurnal cycling. *FLDA1* and *ISIP3* gene expression were also measured in *T. oceanica*-containing field samples from the northeast Pacific Ocean (Chappell *et al.*, 2013). The samples were harvested in small volumes at near-shore and offshore locations representing a wide range of Fe concentrations. These data allow us to correlate gene expression levels with total Fe concentrations and show that the use of these genes as biological indicators is both specific and sensitive.

Materials and methods

Culture conditions

T. oceanica (CCMP 1005) isolates were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (West Boothbay Harbor, ME, USA). Culture experiments were performed using a modified version of f/2 made in 0.2 µm filtered and microwave-sterilized Sargasso seawater (Guillard and Hargraves, 1993). Fe was added separately to achieve the desired concentrations. Cultures grown under replete conditions received 400 nM Fe (11.7 µM EDTA). The inoculum for the Fe-limited treatment came from replete cultures that had undergone two successive dilutions (1:10) into media without added Fe, resulting in f/2 media with < 4 nM Fe. Macronutrient-deficiency experiments were performed with 10 µM silicate for silicate limitation, 3 µM phosphate for phosphate limitation and 88 µM nitrate for nitrate limitation. Appropriate Fe/ETDA and macronutrient concentrations were determined using small volume test cultures followed by nutrient readdition experiments of the limiting nutrient to verify that it was a lack of a single nutrient-controlling growth. All macronutrient stocks were processed through a Chelex 100 ion-exchange column (Bio-Rad Laboratories Inc., Hercules, CA, USA) containing resin, prepared according to Price *et al.* (1989) and 0.2 µm Acrodisc filter-sterilized (Pall Corporation, Port Washington, NY, USA). All media preparation and culture transferring was performed in a Class-100 HEPA filtered hood. For all experiments, triplicate *T. oceanica* cultures were grown at a light level of 140 µE m^{-2} s^{-1} at 25 °C in a Percival incubator (Percival Scientific, Perry, IA, USA) and incubated gently shaking on a MaxQ 2000 orbital shaker (Thermo Fisher Scientific, Waltham, MA, USA). With the exception of one Fe limitation experiment conducted under constant light, cultures were grown on a 14:10 light:dark cycle. Growth of the cultures was monitored daily with fluorescence measurements and cell counts (data not shown). Cultures were harvested in mid-exponential phase when growth of the nutrient-deficient cultures began to decrease when compared with the replete cultures. Biomass was collected by gentle filtration onto 2 µm polyester filters (Sterlitech Corporation, Kent, WA, USA). Filters were placed in screw cap tubes containing 500 µl Qiagen Buffer RLT (Qiagen, Venlo, Netherlands), flash frozen in liquid

nitrogen and stored at $-80\,°C$ until RNA extractions were conducted.

To monitor how gene expression responds following a pulse of Fe to Fe-limited cells, cultures that had been preacclimated to growth at 4 nM added Fe (through two successive transfers from replete media, the first at a 1:10 dilution, the second by transferring enough cells to start the culture at $\sim 4 \times 10^4$ cells ml^{-1}) were transferred into media containing 4 nM Fe, 1 nM Fe and 0 nM Fe at a cell density of $\sim 4 \times 10^4$ cells ml^{-1} and growth was monitored for 4 days. On the fourth day, cultures were split and Fe:EDTA was added to half of the cultures to bring the [Fe] in the media to 400 nM. Cells were filtered for gene expression analysis before the cultures were split, 2 h after Fe was added ($+2Hr$) and 4 h after Fe was added ($+4Hr$).

To monitor gene expression changes associated with a light:dark cycle, replete and Fe-limited *T. oceanica* cells were grown as semicontinuous batch cultures. Triplicate replete (400 nM Fe) and Fe-limited (4 nM) cultures were grown at 25 °C at 150 µE m^{-2} s^{-1} on a 14:10 h light:dark cycle for 48 h. Fe-limited cultures were preacclimated to growth at 4 nM Fe before the start of the experiment. Samples were collected every 3 h by gentle filtration onto 2 µm polyester filters (Sterlitech) and flash frozen. At each time point either 140 or 115 ml of culture was removed for gene expression analysis from replete and Fe-limited cultures, respectively. An equal volume of the appropriate media was added back to ensure cell numbers were not drifting from the expected concentration. Cell counts were taken every 12 h and fluorescence measurements every 24 h (data not shown).

Phylogenetic analysis of diatom FLDA1 and ISIP3 sequences

FLDA1 and *ISIP3* sequences from *T. oceanica* were used for homology-based searches to identify diatom sequences from protein (NCBI) and transcriptome (Marine Microbial Eukaryote Transcriptome Project (MMETSP; Keeling *et al.*, 2014) databases (http://camera.calit2.net/mmetsp/). Transcript and protein sequences were trimmed to include conserved areas (161 amino acids for *FLDA1* and 320–360 amino acids for *ISIP3*) and aligned (gap open cost = 10; gap extension cost = 1) using the MUSCLE alignment algorithm (Edgar, 2004) within the CLC Workbench package (CLC Inc, Aarhus, Denmark). The resulting alignments were used to generate maximum-likelihood trees with bootstrap values calculated from 1000 replicates using PhyML (starting tree = UPGMA, substitution model = Dayhoff (Dayhoff *et al.*, 1978)) in the CLC Genomics Workbench package.

Field sample collection

Samples were collected from the northeast Pacific Ocean aboard the *R/V Thomas G Thompson* (cruise T0206) from 12 May to 8 June 2007. A rosette mounted with a conductivity/temperature/depth profiler (CTD) was used to collect seawater. Approximately 1 l of surface water was gently filtered onto 25 mm, 0.2 µm Supor filters (Pall Corporation) using a peristaltic pump. Filters were placed in screw cap tubes containing 500 µl Qiagen Buffer RLT (Qiagen), flash frozen in liquid nitrogen and stored at $-80\,°C$ until RNA extractions were conducted. Fifty milliliters of surface water was filtered onto GF/F filters (GE Healthcare Bio-Sciences, Uppsala, Sweden) for extracted chlorophyll *a* analysis. Chlorophyll *a* was extracted at sea in 90% acetone for ~ 24 h at $-20\,°C$ and measured with a calibrated Turner 10-AU fluorometer (Turner Designs, Sunnyvale, CA, USA). Environmental data, such as temperature and salinity, were obtained from the SeaBird SBE-911+ CTD system attached to the sampling rosette and have been previously reported in Chappell *et al.*, (2013). Surface (10 m) dissolved Fe (0.2-µm filtration) concentrations were determined as described in Roy *et al.*, (2008) and have also been reported in Chappell *et al.*, (2013). Briefly, total Fe concentrations were determined by chemically reducing all dissolved Fe species to Fe (II) with sulfite. The amount of Fe (II) was determined using an automated flow injection-based FeLume system that measures the luminescence associated with the reaction between Fe (II) and an alkaline luminol solution (Waterville Analytical, Waterville, ME, USA).

RNA extraction and cDNA synthesis

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol, with the following exceptions: cells were lysed using 0.5 and 0.1 mm zirconia/silica beads (BioSpec, Bartlesville, OK, USA) mixed with the lysis buffer and bead-beaten until the solution looked homogenous (~ 1 min). The lysis solution was then put over Qiashredder columns (Qiagen) to remove any large plant material that could clog the spin columns. To aid in the removal of DNA, two DNase digestions were performed. First, Qiagen's RNase-free DNase Set (an on-column treatment) was used according to the manufacturer's instructions. The second DNA removal step was conducted using the Turbo DNA-free kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. The RNA was then quantified in duplicate using the Qubit Fluorometer (Life Technologies). Following quantification, all RNA samples from culture experiments were diluted to 31.25 ng µl^{-1}; this allowed us to add equal volumes (8 µl) to the complementary DNA (cDNA) synthesis reactions. cDNA from laboratory culture experiments was synthesized from 250 ng of DNase-treated RNA using Applied Biosystems' High-Capacity cDNA Reverse Transcription kit (Life Technologies) according to the manufacturer's protocol except for the substitution of an oligo (dT) primer for the provided random hex

primer. As total RNA yield from field samples was limited, RNA samples from the field were diluted to $11 \, ng \, \mu l^{-1}$ and equal volumes ($8 \, \mu l$) were added to the complementary DNA (cDNA) synthesis reactions.

Quantitative real-time PCR (qRT-PCR)

To generate the qRT-PCR plasmid standards, transcripts of interest were isolated from *T. oceanica* using PCR amplification of *T. oceanica* cDNA. *T. oceanica* cDNA was added to PCR reactions consisting of $0.5 \, \mu M$ of each primer, $12.5 \, \mu l$ of BIO-X-ACT Short Mix (Bioline Reagents, London, UK) and $5.5 \, \mu l$ of AccuGENE Molecular Biology Grade Water (Lonza Group, Basel, Switzerland). Reactions were performed in an Eppendorf Mastercycler ep thermocycler (Eppendorf, Hamburg, Germany) using the following parameters: a denaturation step at $94 \, ^{\circ}C$ for 3 min, followed by 30 extension cycles ($94 \, ^{\circ}C$ 30 s, annealing for 30 s, $72 \, ^{\circ}C$ 1 min), a final 5 min extension step at $72 \, ^{\circ}C$ and then an incubation at $4 \, ^{\circ}C$ until analyzed. Primers used for target gene isolation in *T. oceanica* are listed in Table 1 (PCR).

After amplification, the PCR products were loaded onto a 1% agarose (wt/vol) TAE gel. Bands were purified using the QIAquick Gel Extraction Kit according to the manufacturer's protocol (Qiagen). The purified products were cloned into pGEM T vector (Promega, Fitchburg, WI, USA), transformed in α-Select Silver Efficiency (Bioline) competent cells and identified by blue-white screening. The plasmids were purified using the QIAprep Spin Miniprep kit (Qiagen) and subsequently sequenced on the Applied Biosystems 3130xl Genetic Analyzer (Life Technologies). Purified plasmids were linearized using the restriction endonuclease, SpeI (New England Biolabs, Ipswich, MA, USA), and quantified using the Qubit Fluorometer. The quantified plasmids carrying the target inserts were then used to generate standards in triplicate for qRT-PCR. Serial dilutions of the plasmids, ranging from <10 to $>10^6$ copies per reaction, were used to quantify transcript abundance of the target genes in experimental samples.

qRT-PCR was performed using the Light Cycler 480 Real-Time PCR System (Roche Applied Science, Penzberg, Germany) and the Light Cycler 480 Probes Master reaction mix (Roche Applied Science). Primers and probes were designed using the Geneious software package (Drummond *et al.*, 2010) and used at final concentrations of 200 and 100 nM, respectively (Table 1; qRT-PCR). Probes for actin were dual labeled with 5′ HEX and the 3′ Black Hole quencher BHQ1. Probes for *FLDA1* and *ISIP3* were dual labeled with 5′ FAM and the 3′ Black Hole quencher. In this study, reactions were not multiplexed but run in individual reactions. For each reaction, $2 \, \mu l$ of cDNA or plasmid standard was added to $18 \, \mu l$ of the qRT-PCR reaction mixture containing the master mix and primers/probe and set up following the manufacturer's protocol. The qRT-PCR cycling conditions were: 1 cycle at $95 \, ^{\circ}C$ for 5 min, followed by 45 cycles of $95 \, ^{\circ}C$ for 10 s and $60 \, ^{\circ}C$ for 30 s. All environmental samples were examined in qRT-PCR triplicates. For the diurnal culture experiment, single qRT-PCR reactions were run for each biological culture replicate at each time point; average qRT-PCR determinations for duplicate cultures are shown. For the macronutrient limitation experiments, duplicate qRT-PCR reactions were run for each biological replicate culture; average qRT-PCR determinations for triplicate cultures are shown. All qPCR reactions had amplification efficiencies $>95\%$.

Standards made from plasmid dilutions for actin, *FLDA1* and *ISIP3* were run in triplicate. Actin was used as a reference gene; relative transcript abundances represent *FLDA1* and *ISIP3* abundances relative to actin. In *T. pseudonana*, actin expression

Table 1 Genes and primers used to amplify targets for plasmid standards (PCR) and measure transcript abundance (qRT-PCR) in *T. oceanica*

Gene name	Reaction type	Primer type	Primer sequence (5′-3′)
Actin	PCR	Forward	AGATGACGCTCCGAGATCAGTGTT
		Reverse	AGGATCGAACCTCCAATCCAGACA
	qRT-PCR	Forward	GTGACGAGGCCCAGGCGAAG
		Reverse	CCTCGGTGAGGAGGACCGGG
		Probe[a]	TACAACGAGCTCCGCGTCGC
ISIP3	PCR	Forward	CGAGGCTACGTGCTCCAGCG
		Reverse	GAGCGCACAGTCGTCGGCTT
	qRT-PCR	Forward	ATTTCTATCCGCTTCCCAAC
		Reverse	TGTATTGATGGATGTGGCAG
		Probe[b]	TCGGACCCTTTGATGTCTACTTGAAGT
FLDA1	PCR	Forward	CGCTGATGCAGTTGGCGAGC
		Reverse	TGAGCTGTTCAACCCATGACTTCGC
	qRT-PCR	Forward	AGCAGCCGGTGCTAACATGG
		Reverse	GCCCTGTCTTCGCTGAGATCGT
		Probe[b]	GCTAACATGGTAGGGTTCAC

Abbreviation: qRT-PCR, quantitative real-time PCR
[a]Probes labeled with 5′-HEX and 3′-BHQ1.
[b]Probes labeled with 5′-FAM and 3′-BHQ1.

levels were found to be stable across a variety of experimental conditions, including Fe limitation, justifying its use as a reference gene in this study (Alexander *et al.*, 2012). We also confirmed that actin expression levels are stable across multiple experimental conditions in *T. oceanica* by qPCR as well as in high throughput sequence data sets of expressed genes (transcriptomes) (Chappell and Jenkins, unpublished data). Controls were also included from the cDNA reactions lacking reverse transcriptase as well as no-cDNA template controls.

Primer/probe specificity

A homology-based approach was used to evaluate the specificity of the primer/probe pairs used in this study. In addition to *FLDA1* and *ISIP3* sequences, actin sequences from diatoms were identified from protein (NCBI) and transcriptome (Marine Microbial Eukaryote Transcriptome Project) databases using *T. oceanica* sequences in homology-based searches; accession numbers of the sequences are listed in Supplementary Tables 1–3, respectively. The coding DNA sequence for each protein was extracted from the appropriate database and evaluated for the possibility of the probe binding to each sequence. Up to two mismatches between each sequence and the probe were allowed as long as the mismatches were less than five bases away from the 3′ end of the probe. We permitted up to two mismatches to account for potential nonspecific probe hybridization. Our experience with probe-based qPCR demonstrates that a single mismatch to the probe can result in no observable fluorescence signal. Sequences showing a probe match within our mismatch criteria were then tested for binding with the qPCR primers, allowing for up to four mismatches and requiring an exact match for at least three bases at the 3′ end of binding. No sequences outside of our intended *T. oceanica* targets matched both primers and probe of any of the three genes.

Statistical analyses

SigmaStat version 3.5 (Systat Software, San Jose, CA, USA) was used to determine statistically significant differences among samples in the diel experiment. All other statistical analyses were determined using Prism version 4.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Flavodoxin and ISIP3 expression in T. oceanica cultures

Fe limitation significantly induced both *FLDA1* (Figure 1a) and *ISIP3* (Figure 1b) expression >500-fold relative to nutrient-replete expression levels when cells were grown in conditions of both constant light and a 14:10 light:dark cycle ($P < 0.001$, one-way analysis of variance (ANOVA);
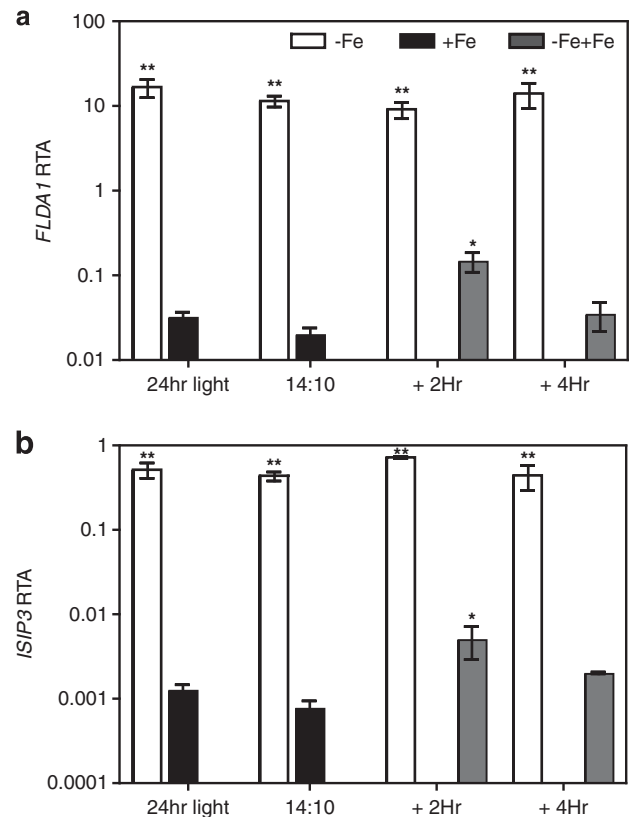

Figure 1 Relative transcript abundances (transcript abundances normalized to actin) for *FLDA1* (**a**) and *ISIP3* (**b**) in *T. oceanica* cultures grown under Fe limitation (−Fe), replete (+Fe), and following Fe readdition to Fe-limited cultures (−Fe+Fe). 14:10 indicates cultures grown on a 14:10 light:dark cycle and represents the initial time (T0) for the Fe readdition. Error bars represent s.d. for 12 biological replicates for the 14:10 replete samples, 22 biological replicates for the 14:10 Fe-limited samples and biological triplicates for both the 24 h light and Fe feedback experiments (+2Hr and +4Hr). Single asterisks (*) indicate a significant increase over replete expression ($P < 0.001$, one-way ANOVA). Double asterisks (**) indicate a significant increase over replete expression and both Fe readdition timepoints ($P < 0.001$, one-way ANOVA).

Figure 1). Two hours after the addition of Fe, both *FLDA1* (Figure 1a) and *ISIP3* (Figure 1b) expression dropped to levels that were significantly lower than Fe-limited expression levels ($P < 0.001$, one-way ANOVA), but still significantly higher than replete values ($P < 0.005$, one-way ANOVA). Four hours after Fe addition, expression levels were no longer significantly different from replete expression levels ($P > 0.05$, one-way ANOVA). Expression levels of *FLDA1* in cells grown under Fe-limiting conditions were significantly higher than those measured in macronutrient (nitrate, phosphate and silicate) deficient cultures by 100-fold or higher ($P < 0.001$, one-way ANOVA; Figure 2a). *ISIP3* expression levels in cells grown under Fe-limiting conditions were also significantly higher than those measured in macronutrient-deficient conditions by 1000-fold or greater ($P < 0.001$, one-way ANOVA; Figure 2b). Expression of *FLDA1* increased with macronutrient deficiency when compared with nutrient-replete
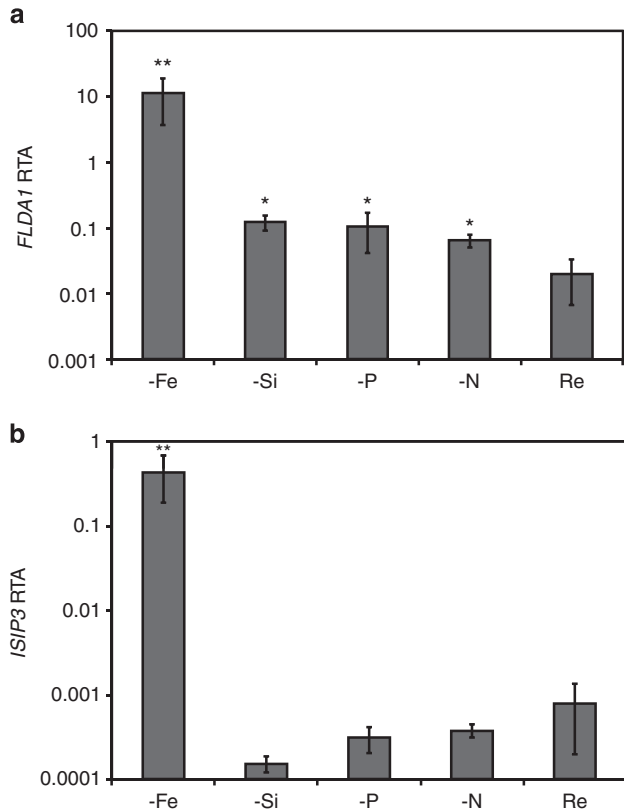
a

b

Figure 2 *FLDA1* (**a**) and *ISIP3* (**b**) RTA (normalized to actin) in *T. oceanica* cultures grown under macronutrient- and Fe-limiting conditions. Error bars represent the s.d. from biological triplicates for nitrogen (− N), phosphorus (− P) and silica (− Si) limited cultures, 22 biological replicates for Fe-limited (− Fe) cultures and 12 biological replicates for replete (Re) cultures. Double asterisks (**) indicate significant difference in expression relative to all other treatments ($P < 0.001$, one-way ANOVA). Single asterisks (*) indicate significant difference in expression relative to replete cultures ($P < 0.05$, one-way ANOVA).

a

b

Figure 3 *FLDA1* (**a**) and *ISIP3* (**b**) RTA in *T. oceanica* semicontinuous batch cultures grown in Fe replete (+ Fe) and Fe-limited (− Fe) media under a 14:10 h light:dark cycle. Error bars represent the s.d. of biological replicates. Black and white boxes along the x-axis indicate dark and light periods, respectively. Daily mean values of expression in Fe-limited cells were significantly higher than replete cells for both genes ($P < 0.001$, one-way ANOVA).

cells ($P < 0.001$, one-way ANOVA), but the expression levels in nutrient-deficient cells were significantly lower than those of cells growing in Fe-limiting conditions ($P < 0.001$, one-way ANOVA; Figure 2a). *ISIP3* expression decreased with macronutrient deficiency when compared with nutrient-replete cells, with silicate deficiency yielding the largest decrease, however even silicate deficient *ISIP3* expression was not significantly different from expression levels in nutrient-replete cells ($P > 0.05$, one-way ANOVA; Figure 2b).

FLDA1 expression was relatively constant throughout the diel cycle in both replete and Fe-limited semicontinuous batch cultures, but was expressed at significantly different levels between the Fe treatments with transcript abundance nearly two orders of magnitude higher in Fe-limited cells (comparing daily mean values $P < 0.001$, one-way ANOVA; Figure 3a). Under the influence of a light:dark cycle, *ISIP3* expression in both Fe-replete and Fe-limited cultures showed a diel rhythm, but the daily mean was significantly higher in Fe-limited cells ($P < 0.001$, one-way ANOVA; Figure 3b). In both Fe-replete and
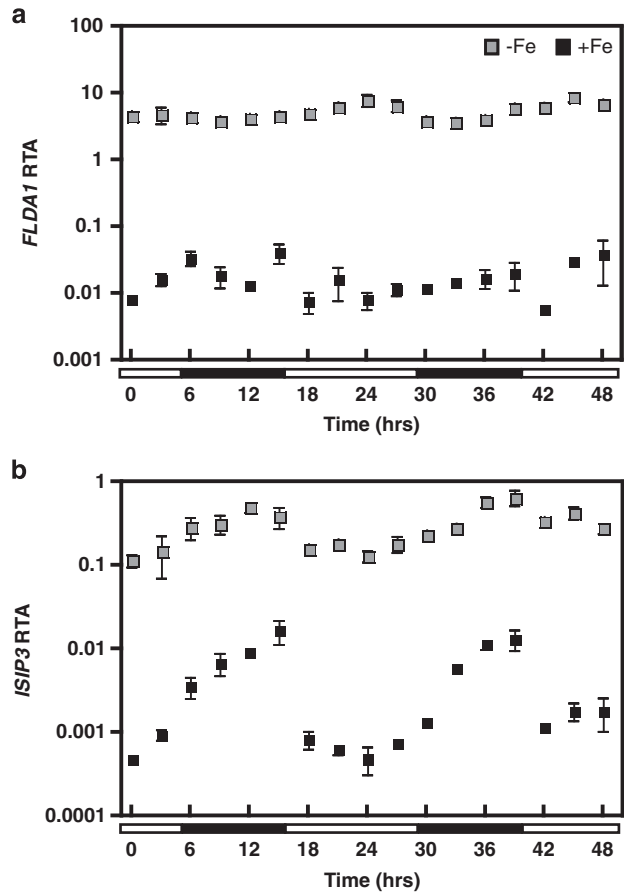
Fe-limited *T. oceanica* cells, *ISIP3* expression was relatively constant through the daylight hours, increased during the dark hours and maximum expression levels were detected near the end of the dark cycle; significant peaks in expression were measured at hours 15 and 39 in Fe replete cells and at hour 39 in Fe-limited cells ($P < 0.05$, one-way ANOVA; Figure 3b).

Comparison of FLDA1 and ISIP3 in diatoms

The applicability of *FLDA1* and *ISIP3* as molecular markers of Fe stress in diatoms was evaluated by investigating the occurrence of these genes in available diatom genomes and transcriptomes. Phylogenetic comparison of *FLDA1* (Supplementary Figure S1) shows that this gene is present in both pennate and centric diatoms. The *T. oceanica* *FLDA1* protein shares homology with another copy in *T. oceanica* (Supplementary Figure S1). The *ISIP3* protein is also widespread in both pennate and centric diatom lineages (Supplementary Figure S2).

We also used the *ISIP3* and *FLDA1* databases to evaluate the specificity of *ISIP3* and *FLDA1* probes and primers to *T. oceanica* compared with other diatoms. Our bioinformatic analysis of probe and primer specificity indicates that our probe and primer combinations for *ISIP3*, *FLDA1* and β actin are specific to *T. oceanica* (accession numbers of sequences analyzed are listed in Supplementary Tables 1–3, respectively). Our analysis is limited by the fact that the current sequence database is restricted largely to predicted transcript sequences derived from transcriptome sequencing. An absence of a homolog to either of these genes in transcriptome data does not mean that an organism lacks those genes. Most transcriptomes in the MMETSP database (Keeling *et al.*, 2014) were not generated under Fe limitation and contain transcripts expressed under nutrient-replete conditions. This is particularly relevant for *ISIP3*, which has almost no detectable expression under nutrient-replete conditions. Therefore, the specificity of our primers and probes will need to be re-evaluated as the sequence databases expand.

Sampling locations and environmental data
Samples for molecular analyses were collected from the northeast Pacific in the spring of 2007 with stations analyzed specifically for this study shown in Figure 4 (complete cruise track and information available in (Chappell *et al.*, 2013)). The study sites selected were based on the previous identification of *T. oceanica* at these locations and represent coastal stations (7, 26, 27 and 30) as well as stations that were collected along two offshore transects (stations 9, 11–15, 17 and 32–34).

These study sites also represent a range of surface-dissolved Fe concentrations, chlorophyll *a* values and bottom depths (Table 2). Surface chlorophyll *a* values were highest at the coastal stations (Figure 5a). A positive correlation was detected between Fe concentrations and chlorophyll *a* values (Spearman's correlation coefficient $r = 0.53$; $P < 0.05$), suggesting Fe availability was influencing phytoplankton abundance.
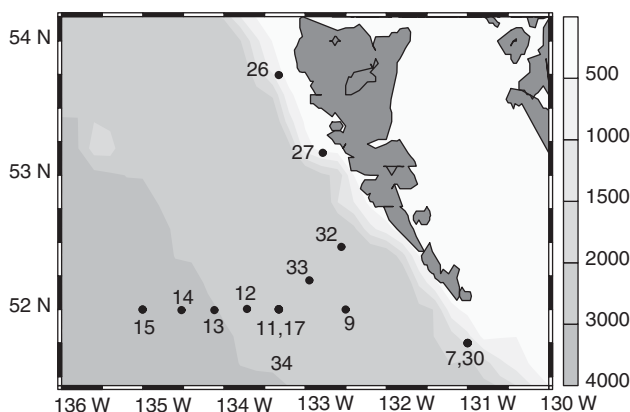

Figure 4 Northeast Pacific sample collection sites used in this study. Shading on the plot indicates bottom depth. Plot was generated using Ocean Data View (Schlitzer, 2012).

Gene expression in Northeast Pacific T. oceanica cells
T. oceanica *FLDA1* and *ISIP3* transcripts were detected in all of the samples with *FLDA1* expressed at levels 10-fold greater than *ISIP3* (Figures 5b and c). Changes in expression associated with the sampling sites were similar between the genes. Low expression levels were measured at station 7 located off the southern tip of Haida Gwaii where Fe concentrations were among the highest (Figures 5b and c). Conversely, peak expression levels for both *FLDA1* and *ISIP3* were measured in *T. oceanica* cells collected from the low Fe waters of station 13 (Figures 5b and c). Many stations had expression levels for both genes that were over an order of magnitude higher than replete levels measured in laboratory samples. The extremes in expression levels represent fold changes of ~225 and 300 for *ISIP3* and *FLDA1*, respectively. Spearman's analysis of the correlation coefficient showed negative relationships between Fe concentrations and both *FLDA1* ($r = -0.59$; $P < 0.05$) and *ISIP3* expression ($r = -0.65$; $P < 0.03$), when all of the sampling locations were included with the exception of nearshore stations 26 and 27 (Figure 4) where both high Fe concentrations and expression levels were measured (see discussion below).

Discussion

The aim of this study was to characterize expression patterns of the putative Fe-responsive genes, *FLDA1* and *ISIP3*, and evaluate them as species-specific molecular markers of Fe limitation in natural populations of *T. oceanica*. In the northeast Pacific, *T. oceanica* was described as a relatively abundant Thalassiosiroid (Chappell *et al.*, 2013). The results from this study show that *T. oceanica* cells from this region differentially regulate *FLDA1* and *ISIP3* expression in response to Fe availability, revealing the Fe nutritional status of this important diatom. These results also speak to the sensitivity of this method as changes in transcript abundance were measured in small volume samples (~1 l).

Robust transcriptional responses in Fe-limited T. oceanica cultures
Fe-limited *T. oceanica* cultures strongly enhanced *FLDA1* transcription, a molecular response that has been previously demonstrated in this diatom (Chappell and Jenkins, unpublished; Lommer *et al.*, 2012) as well as in *T. weissflogii* (Whitney *et al.*, 2011) and *Phaeodactylum tricornutum* (Allen *et al.*, 2008). The robust transcriptional response of *FLDA1* to Fe limitation, rapid downregulation of expression following a pulse of Fe and minimal transcriptional response under macronutrient-deficient conditions support the use of this gene as a marker of Fe limitation in *T. oceanica* cells found in their natural environment. Furthermore, as changes in *FLDA1*

Table 2 Location, sampling times, surface chlorophyll *a*, dissolved Fe concentrations and information on bottom depth from stations analyzed in this study

Station	Sampling date	Latitude (°N)	Longitude (°W)	Sampling time	Chlorophyll a ($\mu g \, l^{-1}$)	Dissolved Fe (nM)[a]	Bottom depth (m)[a]
7	5/17/2007	51°45.01′	130°59.92′	8:00	7.70	1.45	2260
9	5/19/2007	52°00.00′	132°54.70′	18:45	29.69	0.30	2610
11	5/20/2007	52°00.14′	133°19.45′	0:15	1.73	0.16	2692
12	5/20/2007	52°00.23′	133°42.91′	3:30	2.49	0.17	2906
13	5/20/2007	51°59.85′	134°07.06′	6:10	4.87	0.16	3005
14	5/20/2007	51°59.76′	134°31.30′	8:55	4.01	0.13	3191
15	5/20/2007	51°59.99′	135°00.07′	11:55	4.37	0.12	3379
17	5/21/2007	52°00.14′	133°19.45′	11:35	3.03	0.16	2692
26	6/1/2007	53°44.80′	133°19.71′	0:30	31.32	1.64	352
27	6/1/2007	53°10.01′	132°47.07′	7:00	13.56	1.73	768
30	6/2/2007	51°44.92′	131°00.03′	19:10	11.35	1.59	2138
32	6/3/2007	52°27.94′	132°33.27′	21:30	3.87	0.30	2926
33	6/4/2007	52°13.00′	132°33.27′	4:45	4.51	0.12	2608
34	6/4/2007	51°59.86′	133°18.90′	12:15	4.68	0.11	2691

[a]Dissolved Fe and bottom depth data previously published in Chappell *et al.* (2013).

expression did not correlate with the light:dark cycle, the time of day of sampling will not impact the assessment or interpretation of the Fe nutritional health of *T. oceanica* cells.

As not all diatoms contain an Fe-responsive copy of *FLDA1* (Lommer *et al.*, 2012; Whitney *et al.*, 2011), we also evaluated the utility of *ISIP3* as an Fe-stress marker. The strong induction of *ISIP3* with Fe limitation supports previous findings (Lommer *et al.*, 2012) and appears to be a common response among Fe-limited diatoms (Allen *et al.*, 2008). *ISIP3* also has characteristics that indicate it can be a useful marker of Fe limitation in field populations of *T. oceanica*, including the strong induction with Fe limitation, sudden decrease in expression following an Fe pulse and the reduction in expression levels in response to macronutrient-deficient conditions. An important factor to consider in field samples, where *T. oceanica* cells maybe low in abundance, is that *ISIP3* expression levels were one to two orders of magnitude less than those seen in *FLDA1*. Another factor to consider is that *ISIP3* expression was found to coordinate with the light:dark cycle, indicating transcription is regulated by diel periodicities as well as Fe limitation. Even with these changes in expression, *ISIP3* expression was significantly higher in Fe-limited cells when compared with nutrient-replete cells throughout the diel period. In severely Fe-limited cells, the relatively small increase in expression during the dark may not be an important factor in assessing the use of this gene as a marker of Fe stress as Fe limitation induces expression by up to 1000-fold. *ISIP3* has been speculated to function in Fe uptake with a proposed role as a cell surface Fe receptor (Lommer *et al.*, 2012). Thus, a possible explanation for the diel periodicity of *ISIP3* is that the transcripts may accumulate at the end of the dark period so the cell can boost Fe acquisition in preparation for Fe-demanding, daytime processes such as photosynthesis. Under low Fe conditions this response is likely enhanced to meet cellular Fe demands. Despite the caveats associated with *ISIP3*, its presence in available published and draft genomes, including those that do not have an Fe-responsive flavodoxin copy (i.e., *T. pseudonana*), highlights its utility as a molecular marker for Fe stress in diatoms.

FLDA1 and ISIP3 transcript patterns reveal differences in Fe status in T. oceanica populations of the NE Pacific

The consistent transcriptional response to Fe limitation indicated that *FLDA1* and *ISIP3* could be sensitive markers of Fe stress for *T. oceanica* cells in natural diatom populations. To test this, their expression was evaluated in samples collected from the northeast Pacific on a cruise where both the presence of *T. oceanica* had been confirmed and surface-dissolved Fe concentrations were measured (Chappell *et al.*, 2013). In general, the highest abundances of expressed genes were measured at sites that had the lowest dissolved Fe concentrations and vice versa, although when all the data were considered there was not a significant correlation between gene expression and Fe concentrations. The lack of coordination between variables can be attributed to variations in expression levels at shallow coastal stations 26 and 27 that had high dissolved Fe and to low expression levels at station 32 that had low dissolved Fe.

Samples from stations (11, 12, 17 and 34) were collected in the vicinity of a 3-month-old Haida eddy (Chappell *et al.*, 2013; Xiu *et al.*, 2011). Haida eddies have been associated with the transport of coastal water and nutrients to the open ocean (Whitney and Robert, 2002). A recent study estimated that Haida eddies may be a significant source of dissolved Fe to the open ocean, comparable to atmospheric deposition rates, fueling phytoplankton growth through dynamic upwelling of Fe from subsurface ($\sim 100 \, m$) Fe maxima (Xiu *et al.*, 2011). We hypothesize the variations seen in *FLDA1* and *ISIP3* expression levels at the eddy stations reflect *T. oceanica* cells responding to
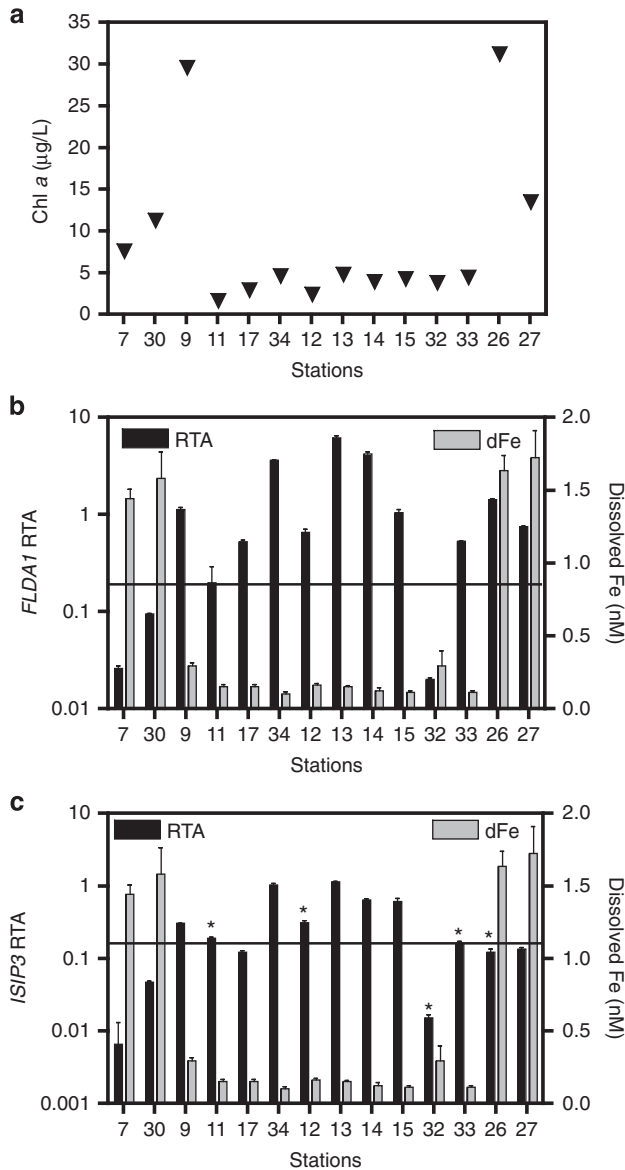
Figure 5 (a.) Surface chlorophyll *a* values at stations sampled in the northeast Pacific. (b.) *T. oceanica FLDA1* expression (RTA) and dissolved Fe (dFe) concentrations from the corresponding stations. (c.) *ISIP3* expression (RTA) and dissolved Fe (dFe) concentrations from the corresponding stations. Physically proximal stations are displayed adjacently on the X-axis. The solid lines in **b** and **c** represent an expression level one order of magnitude higher than replete values in laboratory culture experiments. The highest nighttime replete value was used for *ISIP3*. Asterisks (*) denote samples taken during nighttime hours, where *ISIP3* values may be elevated as a result of diel fluctuations. Error bars represent the s.d. of triplicate determinations. Dissolved Fe data previously presented in Chappell *et al.* (2013).

these rapid changes in Fe inputs. For example, station 11, representing the center of the eddy (52.002° N, 133.324° W), had low Fe levels and correspondingly lower *FLDA1* and *ISIP3* expression levels when compared with surrounding stations 9 and 12–15.

The eddy center was subsequently resampled 1 and 14 days later (stations 17 and 34). *FLDA1* expression increased when sampled a day later at station 17 and increased further when sampled 2 weeks later at station 34 indicating elevated Fe stress for *T. oceanica* in this location. *ISIP3* expression patterns were similar, with a large increase in expression when sampled at station 34 when compared with station 11; however, contrary to what we saw with *FLD1A* expression, *ISIP3* expression was slightly lower at station 17 than station 11. Interpreting *ISIP3* expression levels with the reoccupation of the eddy center is complicated by the diel periodicity of *ISIP3* expression. Station 11 was sampled during the night and thus likely had elevated *ISIP3* expression over what would have been measured had it been sampled during daylight hours. Stations 17 and 34 were both sampled during the daylight hours, so the increase in *ISIP3* expression at station 34 can be interpreted as an increased Fe-stress response in *T. oceanica* rather than a result of diel variability. Of the stations sampled, station 34 had some of the highest expression levels of both *FLDA1* and *ISIP3*, indicating *T. oceanica* cells were severely Fe stressed. This station also had the lowest Fe values and relatively low chlorophyll *a*, consistent with severe Fe limitation of the entire phytoplankton population.

Gene expression repressed with Fe enrichment

Stations demonstrating low dissolved Fe and elevated *FLDA1* and *ISIP3* gene expression may have experienced a recent delivery of Fe. This may have occurred in the center of the Haida eddy when it was originally sampled at station 11; this would explain both the low Fe concentrations and correspondingly lower *FLD1A* and *ISIP3* gene expression levels. The Fe feedback experiments with laboratory cultures demonstrated that *T. oceanica* rapidly downregulates *FLD1A* and *ISIP3* expression in response to inputs of Fe.

Gene expression measures may indicate when Fe is not biologically available

Shallow coastal sampling sites (stations 26 and 27) had noncorresponding gene expression and Fe values. Both *FLDA1* and *ISIP3* expression were high indicating that *T. oceanica* was stressed for Fe, despite both high Fe values and relatively high chlorophyll *a*. At these shallow coastal stations, the dissolved Fe may be terrestrially sourced and/or associated with sediment resuspension that may come in a form that is unavailable to these oceanic diatoms. Rigorous testing of this hypothesis is beyond the scope of this study; however, the methods we have developed should prove useful in evaluating its validity when combined with analyses of Fe source.

Conclusions

As the number of sequenced diatom genomes and transcriptomes increases, it is becoming increasingly clearer that diatoms differ in their gene composition and transcriptional response to Fe limitation, raising the demand for species-specific markers of Fe stress. We used the Fe-limited *T. oceanica* transcriptome (Lommer *et al.*, 2012) and a *Thalassiosira* fingerprinting method (Chappell *et al.*, 2013) to develop and test *T. oceanica*-specific markers of Fe limitation. Few studies have used a similar approach to probe the health of diatoms *in situ*; many studies have used bulk measurements to characterize the community response to Fe limitation with the pitfalls of not knowing who or how much individuals are contributing to that response. We have shown that *FLDA1* and *ISIP3* are specific and sensitive to Fe availability in *T. oceanica* with variations in expression reflecting changes in the severity of Fe stress. This was seen in the northeast Pacific Ocean, where gene expression variability in samples collected from a Haida eddy were likely caused by the influx and rapid drawdown of Fe. *T. oceanica* is a diatom with demonstrated abilities to tolerate low Fe levels (Sunda and Huntsman, 1995). An indication of Fe stress in wild populations of this diatom may serve as a proxy for Fe stress and potentially severe Fe stress in other diatoms occupying these waters. Additionally, gene expression measures can reveal when Fe may be biologically unavailable to a phytoplankton community providing a valuable investigative tool for detailed characterization of Fe supply.

Conflict of Interest

The authors declare no conflicts of interest.

Acknowledgements

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