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Citation	Biogeosciences, 11(9), 2503-2517 https://doi.org/10.5194/bg-11-2503-2014
Issue Date	2014-05-07
Doc URL	https://hdl.handle.net/2115/56745
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Type	journal article
File Information	bg-11-2503-2014.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Biogeosciences, 11, 2503–2517, 2014
www.biogeosciences.net/11/2503/2014/
doi:10.5194/bg-11-2503-2014
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Biogeosciences Open Access

Spatial variability in iron nutritional status of large diatoms in the Sea of Okhotsk with special reference to the Amur River discharge

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Received: 19 November 2013 – Published in Biogeosciences Discuss.: 8 January 2014
Revised: 17 March 2014 – Accepted: 22 March 2014 – Published: 7 May 2014

Abstract. The Sea of Okhotsk is known as one of the most biologically productive regions among the world's oceans, and its productivity is supported in part by the discharge of iron (Fe)-rich water from the Amur River. However, little is known about the effect of riverine-derived Fe input on the physiology of the large diatoms which often flourish in surface waters of the productive continental shelf region. We conducted diatom-specific immunochemical ferredoxin (Fd) and flavodoxin (Fld) assays in order to investigate the spatial variability of Fe nutritional status in the microplankton-sized (20–200 µm; hereafter micro-sized) diatoms. The Fd index, defined as the proportion of Fd to the sum of Fd plus Fld accumulations in the cells, was used to assess their Fe nutritional status. Additionally, active chlorophyll fluorescence measurements using pulse–amplitude-modulated (PAM) fluorometry were carried out to obtain the maximum photochemical quantum efficiency (F_v / F_m) of photosystem II for the total micro-sized phytoplankton assemblages including diatoms. During our observations in the summer of 2006, the micro-sized diatoms were relatively abundant (> 10 µg C L^{-1}) in the neritic region, and formed a massive bloom in Sakhalin Bay near the mouth of the Amur River. Values of the Fd index and F_v / F_m were high (> 0.9 and > 0.65, respectively) near the river mouth, indicating that Fe was sufficient for growth of the diatoms. However, in oceanic waters of the Sea of Okhotsk, the diatom Fd index declined as cellular Fld accumulation increased. These results suggest that there was a distinct gradient in Fe nutritional status in the micro-sized diatoms from near the Amur River mouth to open waters in the Sea of Okhotsk. A significant correlation between dissolved Fe (D-Fe) concentration and the Fd index was found in waters off Sakhalin Island, indicating that D-Fe was a key factor for the photophysiology of this diatom size class. In the vicinity of the Kuril Islands between the Sea of Okhotsk and the Pacific Ocean, micro-sized diatoms only accumulated Fld (i.e., Fd index = 0), despite strong vertical mixing consistent with elevated surface D-Fe levels (> 0.4 nM). Since higher Fe quotas are generally required for diatoms growing under low-light conditions, the micro-sized diatoms off the Kuril Islands possibly encountered Fe and light co-limitations. The differential expressions of Fd and Fld in micro-sized diatoms helped us to understand how these organisms respond to Fe availability in the Sea of Okhotsk in connection with the Amur River discharge.

1 Introduction

Iron (Fe) plays an important role in metabolic processes such as photosynthesis, respiration, and nitrogen assimilation for marine phytoplankton (Twining and Baines, 2013; Behrenfeld and Milligan, 2013). Over the last two decades, much attention has been focused on the availability of Fe to phytoplankton in the open ocean, especially high-nutrient, low-chlorophyll (HNLC) waters such as the Southern Ocean, the subarctic Pacific, and the eastern equatorial Pacific (de Baar

et al., 2005; Boyd et al., 2007). However, it has become evident that Fe limitation for phytoplankton growth can occur not only in offshore waters, but also in coastal upwelling regions and marginal seas (e.g., Bruland et al., 2001; Hutchins et al., 2002; Aguilar-Islas et al., 2007; Sedwick et al., 2011; Gerringa et al., 2012). In coastal waters, both riverine and sedimentary Fe can cause pronounced near-shore to offshore gradients in dissolved Fe concentrations. Riverine Fe is an important supply term at source, but much of the incoming Fe is rapidly scavenged onto sinking particles in estuaries (Boyle et al., 1977; Boyd and Ellwood, 2010). The removal of Fe can vary depending on the origin and chemical structure of the Fe provided into the coastal waters, because Fe complexes with organic ligands may act to protect Fe from removal by scavenging (Buck et al., 2007). Therefore, to determine to what extent Fe is available for phytoplankton growth in such regions is crucial (Chase et al., 2007), because this in turn can control the structure and dynamics of lower trophic level processes in the near-shore environment. Among the phytoplankton, it is well known that large diatoms often flourish in coastal waters (e.g., Orlova et al., 2004; Suzuki et al., 2011). Such large diatoms tend to be grazed by large zooplankton, resulting in shorter, simpler food webs that may result in more efficient matter and energy transfer (Ryther, 1969). However, the growth of large diatoms is expected to be more sensitive to Fe deficit than that of small cells due to their small cell surface to volume ratios (Sunda and Huntsman, 1997; Timmermans et al., 2004; Sarthou et al., 2005). To our knowledge, no literature has been published on spatiotemporal variability in the Fe nutritional status of large diatoms in coastal or marginal seas.

The Sea of Okhotsk, one of the marginal seas of the western North Pacific, is known as one of the most biologically productive regions among the world's oceans, especially along its continental shelf (Sorokin and Sorokin, 1999; Isada et al., 2013). Diatoms account for much of the production in these waters with growth supported by sea-ice melting during spring and the Amur River discharge from summer to fall (Nakatsuka et al., 2004). In winter, the Sea of Okhotsk is covered with sea ice and is the most southern extension of the region of seasonal ice formation (Kimura and Wakatsuchi, 2000). From spring to summer, increases in irradiance and temperature lead to the melting of sea ice, resulting in the formation of a pycnocline in surface waters of the Sea of the Okhotsk. Also impacting the region is the Amur River, the longest river (4350 km) in the far eastern region of Russia, supplying not only large volumes of freshwater (Ogi et al., 2001), but also high levels of nutrients including Fe and organic matter into the Sea of Okhotsk via the southward-flowing East Sakhalin Current which runs along the eastern coast of Sakhalin Island (Nishioka et al., 2007; Andreev and Pavlova, 2009; Nagao et al., 2010; Takao et al., 2014). According to Nagao et al. (2010), Amur River water contains ca. 4 µM of dissolved Fe and its annual flux from the river to the Sea of Okhotsk is estimated as $(1.1 \pm 0.7) \times 10^{11}$ g yr^{-1}. The dissolved Fe level is four orders of magnitude higher than that typically reported for low-Fe HNLC waters (< 0.2 nM; Johnson et al., 1997) and the annual flux corresponds to ca. 10 % of the global riverine value (Raiswell, 2006). The changes in physical and chemical conditions from spring to summer allow phytoplankton to remain in the sunlit surface layer and form massive diatom blooms until nutrients are depleted in the Sea of Okhotsk (Sorokin and Sorokin, 1999, 2002). Thus, the Sea of Okhotsk is an excellent natural laboratory to investigate the importance of riverine Fe to large diatoms.

Regarding the Fe dynamics in the study area, Nishioka et al. (2007, 2011, 2013, 2014) demonstrated the importance of the transport of intermediate dense shelf water (DSW) from the Sea of Okhotsk to the North Pacific via the Kuril Straits, mainly through the Bussol' Strait, the deepest channel of the Kuril Islands. In winter, large amounts of sea ice are produced along the Siberian coast on the northwestern continental shelf of the Sea of Okhotsk. The sea-ice formation generates a large volume of Fe-containing brine, which subsequently settles to the bottom of the northwestern continental shelf along the Siberian coast to form the DSW formation (Martin et al., 1998). The high levels of Fe derived from the Amur River should contribute significantly to the contents of Fe in the brine. Additionally, large amounts of dissolved and particulate Fe are introduced to the DSW by re-suspension of sediments (Nishioka et al., 2014). Since the density of DSW is relatively low (26.8–27.0 $\sigma\theta$), it tends to penetrate the upper part (250–450 m) of the Okhotsk Sea intermediate water (OSIW). The OSIW flows southward along the east coast of Sakhalin Island and is transported to the Kuril Straits. Because the tidal flow generates intense vertical mixing around the Bussol' Strait (Yagi and Yasuda, 2012), the mixing transports the deep water with high-Fe content to the surface (Nishioka et al., 2007, 2013). Although these processes should enhance the availability of Fe for phytoplankton growth, little knowledge is available on this issue.

In this study, we report on the status of Fe nutrition in microplankton-sized (i.e., 20–200 µm, hereafter micro-sized) diatoms with reference to dissolved Fe levels in surface waters of the western part of the Sea of Okhotsk during late summer 2006. Fe deficiency in the large diatoms was assessed by determining the ferredoxin (Fd) index, which is defined as the proportion of Fd to the sum of Fd plus flavodoxin (Fld) accumulations (Doucette et al., 1996). Under Fe deficient conditions, the Fe-S (iron-sulfur) protein Fd, which is located in the acceptor side of photosystem I (PSI), can be replaced by a functionally equivalent non-Fe-containing Fld in most cyanobacteria and some eukaryotic algae including diatoms (Zurbriggen et al., 2007). The small (6–15 kDa) non-heme protein Fd accepts electrons from PSI and transfers them to Fd $NADP^+$ reductase (FNR), acting in both cyclic and non-cyclic electron transport systems (LaRoche et al., 1999). By comparison, Fld, a flavoprotein ranging from 16–20 kDa (Zurbriggen et al., 2007), has an oxidation-reduction

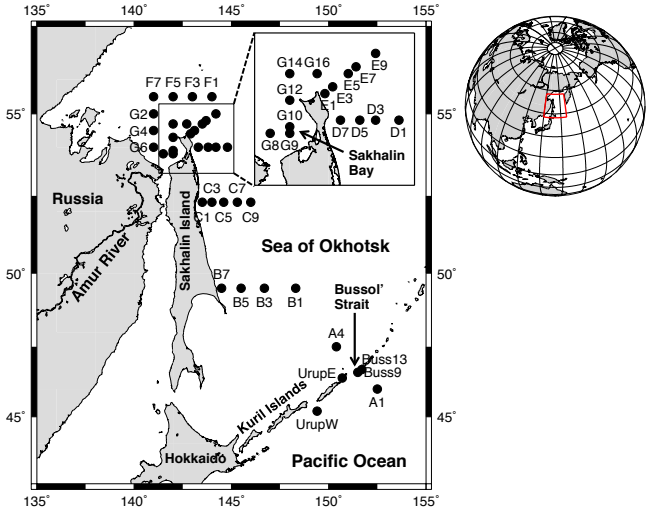

Fig. 1. Maps of the Sea of Okhotsk and its adjacent waters. Closed circles indicate our sampling stations during the R/V *Prof. Khromov* cruise between 13 August and 14 September 2006.

potential resembling that of Fd (ca. −400 mV; Medina and Gómez-Moreno, 2004), while the energetic cost of Fld synthesis could be slightly higher than that of Fd due to the larger size. The Fd index or the abundance of Fld have been used as diagnostic markers for cellular Fe status in marine diatoms in the laboratory and field (e.g., LaRoche et al., 1996; Erdner and Anderson, 1999; McKay et al., 1999; Pankowski and McMinn, 2009a; Suzuki et al., 2009; Hattori-Saito et al., 2010). The protein expression assays can obviate the weaknesses of conventional bottle incubation methods (i.e., so-called the bottle effect; Carpenter and Lively, 1980, and trace metal contamination; Fitzwater et al., 1982), because they do not require any bottle incubation. The immunochemical detection of Fd and Fld with the western blot technique used in this study are useful to estimate taxon-specific Fe nutritional status in a semi-quantitative manner. Although Fd and Fld analyses with high-performance liquid chromatography (HPLC) can yield more quantitative data (Doucette et al., 1996; Erdner and Anderson, 1999), the HPLC technique would require a large quantity of extracted protein and cannot provide taxonomic discrimination. In this study, we revealed using the immunochemical Fd and Fld assays that there was a distinct gradient of Fe nutritional status in the micro-sized diatoms from near the Amur River mouth to open waters in the Sea of Okhotsk.

2 Materials and methods

2.1 Hydrographic observations

Our observations were conducted in the Sea of Okhotsk from 13 August to 14 September 2006, on board the Russian R/V *Professor Khromov* as part of the Amur–Okhotsk project of the Research Institute of Humanity and Nature (RIHN), Japan. The sampling stations were located in the western part of the Sea of Okhotsk, which is influenced by the Amur River, the Bussol' Strait and off the strait in the western subarctic Pacific (Fig. 1). Seawater samples except for the ferredoxin (Fd) and flavodoxin (Fld) assays and F_v / F_m measurements described below were obtained from the surface (2–6 m) using a CTD carousel multi-sampler system (CTD-CMS) with acid-cleaned Teflon-coated Niskin-X bottles. Nutrients (nitrate plus nitrite, phosphate, and silicate) were determined with a segmented continuous flow autoanalyzer (QuAAtro, Bran + Luebbe) following the manufacturer's protocol. The procedure of Fe analysis is detailed in Nishioka et al. (2011, 2014). In brief, subsampling was done with 0.22 µm Durapore filters (Millipak 100, Millipore Corp.) connected to the water sampler Niskin-X spigot, and the filtrate was collected in acid-cleaned LDPE bottles under gravity pressure. The filtrate samples were maintained at pH ∼ 2 for more than one month at room temperature, and then the pH was adjusted to 3.2 immediately before analysis onshore. The dissolved Fe (leachable Fe in 0.22 µm filtrate; hereafter D-Fe) was analyzed with a FIA chemiluminescence detection system (Obata et al., 1993). For chlorophyll (Chl) *a* measurements, seawater samples were filtered onto 25 mm Whatman GF/F glass-fiber filters under gentle vacuum (< 0.013 MPa). After filtration, the filters were soaked in 6 mL *N*,*N*-dimethylformamide (DMF) in a glass cuvette at −20 °C for over a day to extract algal pigments (Suzuki and Ishimaru, 1990). The Chl *a* concentration was determined using a Turner Designs 10-AU fluorometer following the non-acidification method of Welshmeyer (1994).

2.2 Light microscopy

Seawater collected with the Niskin-X bottles was preserved using 20 % formalin buffered with sodium acetate (0.5 % final concentration; Horner, 2002) before analysis. The samples were concentrated on land using Utermöhl chambers (Hydro-Bios Apparatebau GmbH) and analyzed with a Leica DM IL inverted microscope. Identification of diatoms was carried out following Tomas (1997). Micro-sized diatoms were identified with ≥ 20 µm in apical, transapical or pervalvar axes. Cell volumes were estimated for each species by applying cellular dimensions to the formulae for solid geometric shapes most closely matching the shapes of the cells (Hillebrand et al., 1999). The carbon biomass of diatoms was calculated with the empirical equations of Menden-Deuer and Lessard (2000). To examine the dissimilarity of the diatom species detected (i.e., species richness) among the stations, a similarity matrix was obtained using the Bray–Curtis index (Bray and Curtis, 1957) and the program EstimateS (ver. 9.1.0; Colwell, 2013). For constructing the matrix, the presence or absence of diatom species in a sample was scored as 1 (present) or 0 (absent). An unweighted pair group clustering method using the arithmetic mean (UPGMA) was

applied to the similarity matrix to classify the stations into groups.

2.3 Ferredoxin (Fd) and flavodoxin (Fld) assays

Seawater was collected from the water intake (ca. 5 m) of the research vessel and filtered through a plankton net of 20 µm nylon mesh followed by passing through 200 µm nylon mesh to remove large zooplankton. After the pre-filtration, cells were collected onto 5 µm nylon mesh, rinsed from the nylon mesh with the filtered seawater prepared with Whatman GF/F filters, and concentrated into a pellet by centrifugation. The samples obtained were stored in liquid nitrogen or a deep-freezer (−80 °C) until analysis on land. Levels of diatom-specific Fd and Fld in the microplankton size-fraction (20–200 µm) were determined following Suzuki et al. (2009) with a few modifications. Single or duplicate samples per sampling station were analyzed and the duplicate data obtained were averaged. In brief, a pellet was sonicated with a Branson SONIFIER model 250 in ice-cold acetone containing 10 % trichloroacetic acid (TCA) and 0.07 % 2-mercaptoethanol, and the extracts were stored at −20 °C for 1 h. Precipitated protein was centrifuged, rinsed with acetone, suspended in 1 % SDS solution, and heated at 95 °C for 3 min. Total protein concentration in the solution was determined with BCA assay (Pierce). The protein solution was mixed with an equal volume of 2 × PAGE-reducing sample buffer with SDS-Tris-Gly buffer (Laemmli, 1970). For SDS-PAGE, samples containing 10 µg of protein and positive controls of Fd or Fld (see below) were resolved by electrophoresis at 20 mA for 3–4 h at room temperature. Gels were transferred to PVDF membranes at 10 V for 20 min at room temperature in a Tris buffer containing 20 % methanol. Membranes containing transferred proteins were blocked overnight in phosphate-buffered saline buffer containing Tween 20 (PBST) and 1 % bovine serum albumin. Blots were subsequently incubated for 1 h with primary antibody diluted (1 : 10 000) in Can Get Signal Solution 1 (Toyobo Co. Ltd.) for anti-Fd or in PBST for anti-Fld antibodies against diatoms followed by rinsing in the respective buffers (3 × 10 min). The anti-Fd antibody was raised against an antigen of the synthetic peptide, N-LVHQEDELY-C, corresponding to the C-terminal end of Fd encoded by the *petF* gene of diatoms. The anti-Fld antibody derived from diatom protein is detailed in LaRoche et al. (1995). The blots were incubated for 1 h with donkey anti-rabbit IgG-horseradish peroxidase conjugate diluted in each buffer solution (1 : 25 000). Immunoreactive proteins were detected by chemiluminescence using the ECL Plus reagent set (GE Healthcare), and the chemiluminescent signal was captured with a cooled CCD camera (Ez-Capture, Atto Co. Ltd.). The bands obtained were quantified with Gel-Pro Analyzer (ver. 4.5) software (Media Cybernetics). The intensity of each band was normalized to that of the positive controls of Fd or Fld, which were obtained from the synthetic peptide connected to a 27 kDa carrier protein (New England Bio Labs) and from an Fe-deficient culture of the centric diatom *Thalassiosira nordenskioeldii*, respectively. To evaluate the extent of Fe deficiency in micro-sized diatoms, we adopted the Fd index (i.e. Fd/(Fd + Fld)) proposed by Doucette et al. (1996). In Fe-sufficient algae, the Fd index approaches 1 (e.g., Li et al., 2004; Strzepek and Harrison, 2004), indicating that only Fd has accumulated in the cells.

2.4 HPLC pigment analysis

For estimating phytoplankton community composition in the micro-sized phytoplankton, pigment analyses were conducted with high-performance liquid chromatography (HPLC). The acetone extracts from the pellets for the Fd and Fld assays (see Sect. 2.3) were filtered through 0.45 µm PTFE filters to remove fine particles. The 250 µL filtrate was mixed with 250 µL of 28 mM tetrabutylammonium acetate (TBAA) aqueous solution, and 250 µL of sample was injected into a Shimadzu CLASS-VP HPLC system equipped with a photodiode array detector (SPD-M10A VP) and a Zorbax Eclipse XDB-C8 column (3.5 µm particle size, 4.6 × 150 mm). Following Van Heukelem and Thomas (2001), we used a binary solvent system consisting of solvent A (70 : 30 v/v methanol and 28 mM TBAA at pH 6.5) and solvent B (methanol). The flow rate was held constant at 1.2 mL min^{-1}. Pigments were separated with a linear gradient from 5 to 95 % B over the course of 22 min, followed by an isocratic hold at 95 % B for 8 min. The column temperature was kept at 60 °C. Pigment standards were obtained from DHI, Sigma-Aldrich, and Extrasynthese.

2.5 PAM fluorometry

The onboard measurements with pulse–amplitude-modulated (PAM) fluorometry were conducted in the vicinity of Sakhalin Island (i.e., Stns B1–G16). An aliquot of the pellets (20–200 µm in size) collected for the Fd and Fld assays (see Sect. 2.3) was suspended in filtered seawater, transferred to acid-cleaned amber-colored high-density polyethylene (HDPE) bottles and placed in an incubator adjusted to the in situ surface water temperature for 30 min in order to open the reaction centers of photosystem II (PSII) for the micro-sized phytoplankton. The water samples were transferred to a quartz cell under low-light conditions and analyzed in the dark using a Water-PAM fluorometer (Heinz Waltz GmbH) with red LEDs (650–650 nm in peak emission) to obtain the maximum photochemical quantum efficiency (F_v / F_m) of PSII for total micro-sized phytoplankton. The measurements were repeated at least three times and the data obtained were averaged.

Table 1. Hydrographic properties in surface waters at each sampling station.

Station	Depth (m)	Temperature (°C)	Salinity	$NO_3 + NO_2$ (μM)	PO_4 (μM)	$Si(OH)_4$ (μM)	D-Fe (nM)	Chl *a* ($\mu g L^{-1}$)
UrupW	5	5.25	33.02	17.48	1.69	28.57	0.54	2.78
UrupE	5	3.76	33.13	13.17	1.66	31.47	0.91	2.52
Buss9	6	2.67	33.10	24.44	2.13	53.18	1.33	0.63
Buss13	5	2.57	33.35	27.47	2.43	56.08	0.72	0.45
A1	5	13.87	32.76	8.77	1.12	11.30	0.43	0.43
A4	4	9.33	32.63	8.96	0.99	38.32	0.38	1.79
B1	5	13.67	32.50	0.00	0.21	0.00	0.11	0.31
B3	5	12.82	32.26	0.05	0.16	0.00	0.25	0.63
B5	5	12.66	32.41	0.04	0.23	1.05	0.82	0.44
B7	5	10.55	32.39	0.08	0.40	0.24	3.82	0.67
C1	5	2.55	31.67	9.14	1.29	12.36	10.80	2.63
C3	5	10.61	31.05	0.09	0.23	0.00	3.86	0.76
C5	5	10.98	31.45	0.20	0.30	1.21	1.23	2.05
C7	5	16.06	29.70	0.22	0.27	0.59	0.77	1.65
C9	5	15.95	31.07	0.09	0.08	0.61	0.37	0.42
D1	5	12.14	30.51	1.52	0.40	3.23	0.61	5.68
D3	5	10.47	31.01	4.01	0.56	1.45	1.21	4.99
D5	3	9.80	31.24	4.90	0.74	5.42	1.42	4.02
D7	2	13.25	28.25	0.24	0.19	5.57	1.00	3.37
E1	6	6.24	31.19	2.77	0.76	7.64	10.08	0.93
E3	4	8.49	31.21	1.68	0.54	5.57	7.69	1.80
E5	3	12.60	28.43	0.97	0.26	5.73	5.96	1.91
E7	5	13.67	27.44	0.33	0.15	5.23	4.52	1.81
E9	5	7.75	32.48	6.76	0.86	1.54	0.26	1.86
F1	2	13.09	29.72	0.00	0.15	3.41	1.16	5.58
F3	3	13.49	28.40	0.05	0.19	5.17	4.29	2.71
F5	5	12.01	30.33	0.04	0.05	5.11	2.16	2.58
F7	5	13.91	31.40	2.13	0.03	3.79	1.70	1.52
G2	6	14.10	31.58	0.01	0.07	3.98	1.50	1.17
G4	2	11.26	31.13	0.00	0.11	0.00	2.94	3.99
G6	5	12.97	31.16	0.09	0.24	2.47	3.64	3.10
G8	3	15.03	17.98	2.45	0.14	45.31	90.32	10.98
G9	2	15.86	16.73	0.05	0.00	26.00	56.09	15.16
G10	5	16.18	16.74	0.18	0.03	19.65	37.56	11.45
G12	2	13.44	26.26	0.16	0.11	11.30	45.27	5.40
G14	5	13.76	31.79	0.35	0.20	6.54	4.02	1.41
G16	2	12.59	29.00	0.10	0.15	2.82	7.55	2.18

3 Results

3.1 Hydrography

In terms of the geographic (Fig. 1) and hydrographic conditions (Table 1, Fig. 2), the study area was divided into two realms: north and east of Sakhalin Island (Stns B1–G16) and the vicinity of the Kuril Islands (Stns UrupW-A4). The upper 2–6 m layer from which we collected samples at each station was within the surface mixed layer. The lowest salinity was observed at Stn G9 with the influence of fresh water reaching to Stns C1–C9 (< 32 in salinity), consistent with high discharge of fresh water from the Amur River and distributed along the East Sakhalin coast through the southward-flowing East Sakhalin Current (Ohshima et al., 2002). Higher concentrations (> 30 nM) of D-Fe were observed at Stns G8–12 near the Amur River mouth, where Chl *a* levels were also high ($> 5\,\mu g\,L^{-1}$). Off Sakhalin Island, significant correlations between salinity and D-Fe or silicate were found (Fig. 2a; Spearman's rank correlation coefficient) ($\rho > 0.5$, $p < 0.01$). In addition, a correlation between D-Fe and silicate was also high near Sakhalin Island ($\rho = -0.73$, $p < 0.001$). These results suggest that both D-Fe and silicate were mainly derived from the Amur River discharge. Nitrate plus nitrite and phosphate levels were generally low (< 1 μM), although higher levels (> 2 μM) of nitrate plus nitrite were sometimes observed on the continental shelf along the east coast of Sakhalin Island. A negative correlation

Table 2. Dominant micro-sized diatoms off (a) Sakhalin Island and (b) the Kuril Islands in terms of carbon biomass[a].

(a) Species	Dominancy (%)	(b) Species	Dominancy (%)
Ditylum brightwellii	31.22	*Chaetoceros concavicornis*	71.34
Chaetoceros radicans	19.02	*Corethron criophilum*	8.95
Ch. debilis	17.65	*Thalassiosira* spp.	3.80
Coscinodiscus spp.	10.38	*Pseudo-nitzschia seriata* complex	3.62
Chaetoceros spp.	8.18	*Chaetoceros* spp.	3.42
Pseudo-nitzschia seriata complex	5.56	*Neodenticula seminae*	2.77
Thalassiosira spp.	2.46	*Rhizosolenia setigera*	1.98
Rhizosolenia setigera	1.62	*Fragilariopsis* spp.	1.88
Skeletonema costatum	1.31	*Proboscia alata*	0.42
Thalassionema nitzschioides	0.91	*Cylindrotheca closterium*	0.39
Minidiscus sp.	0.70	*Navicula* spp.	0.37
Nitzschia spp.	0.33	*Lioloma* spp.	0.31
Ch. concavicornis	0.21	*Bacterosira bathyomphala*	0.30
Navicula spp.	0.13	*Coscinodiscus* spp.	0.30
Fragilariopsis spp.	0.12	*Guinardia delicatula*	0.09
Corethron criophilum	0.07	Others	0.06
Neodenticula seminae	0.06		
Guinardia delicatula	0.06		
Cylindrotheca closterium	0.01		

[a] The dominancy of each species was estimated against the total carbon biomass of the micro-sized diatoms.

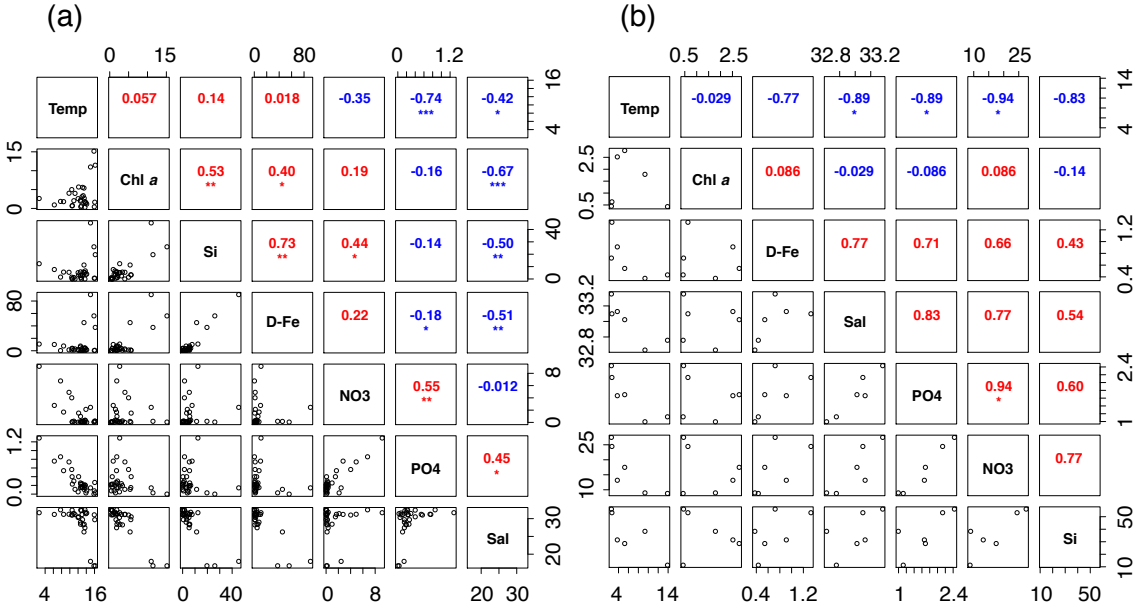

Fig. 2. Multi-panel display of pairwise relationships between the environmental variables, which are listed in Table 1: **(a)** off Sakhalin Island (Stns B1–G16) and **(b)** off the Kuril Islands (Stns UrupW–A4) with Spearman's rank correlations (ρ). The Temp, Si, NO_3, and Sal correspond to temperature, $Si(OH)_4$, NO_3+NO_2, and salinity, respectively. The symbols ***, **, and * indicate the significance levels $p < 0.001$, < 0.01, and < 0.05, respectively.

($\rho = -0.67$, $p < 0.001$) between salinity and Chl *a* biomass was observed off Sakhalin Island.

At Stns UrupW-A4 off the Kuril Islands, salinity, and macronutrient levels were substantially higher than those at the stations off Sakhalin Island (Table 1). The high-macronutrient concentrations were induced coincident with intense tidal-flow-induced vertical mixing (Yagi and Yasuda, 2012). Significant negative correlations between temperature and nitrate plus nitrite or phosphate also existed (Fig. 2b). Both D-Fe and Chl *a* concentrations were variable among stations (0.38–1.33 nM and 0.43–2.78 $\mu g\,L^{-1}$, respectively)

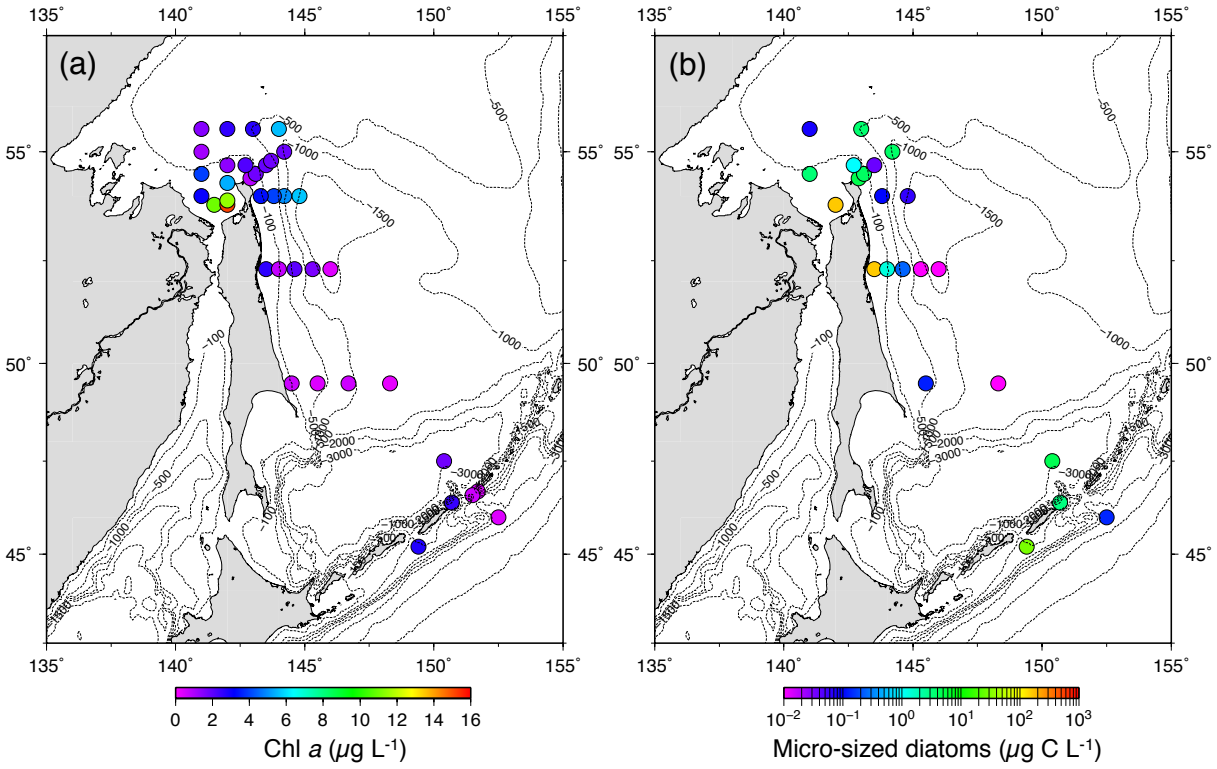

Fig. 3. Plots of **(a)** chlorophyll (Chl) *a* ($\mu g\,L^{-1}$) concentration and **(b)** micro-sized diatoms' carbon biomass ($\mu g\,C\,L^{-1}$) in surface waters at each sampling station.

and they did not show significant relationship with the other environmental parameters off the Kuril Islands.

3.2 Micro-sized diatoms

The distribution pattern of the carbon biomass of micro-sized diatoms was generally similar to that of Chl *a* biomass in the study area (Fig. 3). The carbon biomass of these diatoms at neritic Stns G9 and C1 was relatively high ($> 100\,\mu g\,C\,L^{-1}$), whereas its concentration tended to decrease offshore from the east coast of Sakhalin Island. The dominant micro-sized diatoms principally consisted of the centrics *Ditylum brightwelii*, *Chaetoceros radicans*, *Ch. debilis*, and *Coscinodiscus* spp. in the vicinity of Sakhalin Island (Table 2a). By comparison, *Ch. concavicornis* predominated the micro-sized diatoms near the Kuril Islands (Table 2b). The compositions of the micro-sized diatoms detected in the vicinity of the Kuril Islands (i.e., at Stns UrupW, UrupE, A1, and A4) were similar to each other (Fig. 4), whereas those off Sakhalin Island were rather spatially variable.

3.3 Fd index

Relative intensities of Fd and Fld were variable among stations (Fig. 5). No relationship was found between each intensity and D-Fe or carbon biomass of micro-sized diatoms (Spearman's rank correlation, $p > 0.05$). Intensities of Fd plus Fld (< 0.04) at Stns F1−G4 and G16 were lower than those at other stations (Fig. 5) and were close to the detection limit (i.e., background levels). Therefore, values of the Fd index at Stns F1–G4 and G16 were not used for further analyses. The cut off value of 0.04 corresponded to 3 % of the maximum intensity (1.33) of Fd plus Fld at Stn B1. The low intensities (< 0.04) of Fd plus Fld were probably caused by the high amount of proteins derived from detritus and micro-sized plankton other than diatoms (e.g., dinoflagellates and planktonic ciliates).

A high-Fd index (> 0.9) was measured for the micro-sized diatoms sampled at Stns G8 and G10 where D-Fe concentrations were also high (Fig. 6 and Table 1). Both the Fd index and D-Fe tended to be lower offshore from near the mouth of the Amur River. Although no significant relationship was observed between salinity and the Fd index near Sakhalin Island (Fig. 7), a high correlation was found between D-Fe and the Fd index in this area (Fig. 8a; $\rho = 0.87$, $p < 0.001$). In the vicinity of the Kuril Islands, only Fld was detected (Fig. 5), resulting in a Fd index of zero (Fig. 6a).

3.4 Pigments in micro-sized phytoplankton

The major chemotaxonomic algal pigments detected were Chl *a*, fucoxanthin (Fucox), and peridinin (Peri). Relative compositions (%) of these pigments are shown in Fig. 9. As

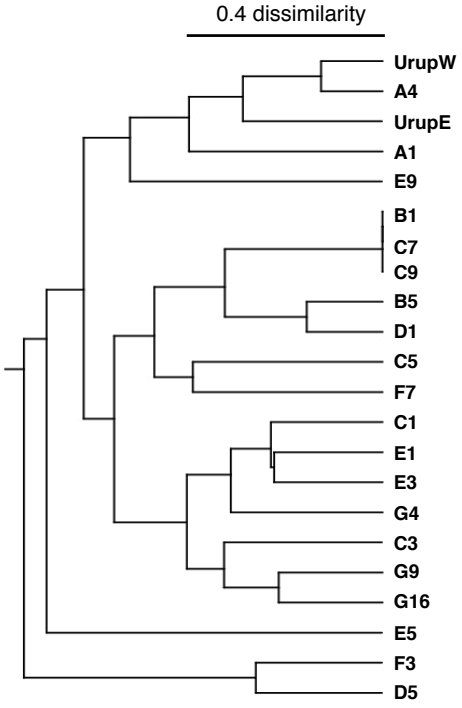

Fig. 4. Cluster dendrogram of the diatom species richness at each sampling station.

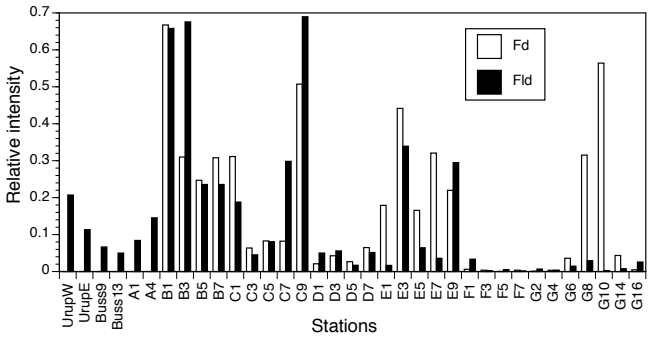

Fig. 5. Relative intensities of ferredoxin (Fd) and flavodoxin (Fld) in micro-sized diatoms in surface waters at each sampling station.

pellet size was not constant between samples, absolute pigment levels in the pellets were not estimated. Phaeopigments such as phaeophorbide *a* were rarely detected, indicating that 10 % TCA in the acetone extracts (pH ∼ 5) had minimal affect on the pigment compositions. As a haptophyte marker, 19′-hexanoyloxyfucoxanthin was occasionally detected, but the pigment was minor. A multiple regression analysis was conducted using Chl *a*, Fucox, and Peri, assuming that Fucox and Peri were derived from diatoms and dinoflagellates, respectively (Vidussi et al., 2001). Although it is known that some dinoflagellates contain Fucox (Zapata et al., 2012), these could not be identified in this study. As a result, we obtained the following regression equation:

$$[\text{Chl } a] = 1.71[\text{Fucox}] + 0.90[\text{Peri}] \quad (1)$$
$$(R^2 = 0.97, F - \text{value} = 587, p < 0.01),$$

where [Chl *a*], [Fucox], and [Peri] are the concentrations of Chl *a*, Fucox, and Peri in the acetone extracts and those correspond to the fractions of each pigment in Fig. 9. The coefficient (1.71) of Fucox was within the typical ratio between 1.4 and 1.8 for diatoms (Vesk and Jeffrey, 1977), while that of Peri (0.90) was lower than the values (1.5–2.4) reported by Jeffrey et al. (1975) and Vesk and Jeffrey (1977). The regression analysis revealed that diatoms generally became predominant in the micro-sized phytoplankton both near the Kuril Islands and near the coast of Sakhalin Island or the mainland of Russia, whereas Peri-containing dinoflagellates tended to dominate in the more open waters of the Sea of Okhotsk (Fig. 10).

3.5 PAM fluorometry

Higher F_v / F_m values for the microphytoplankton were observed in and around Sakhalin Bay (Stns F1–G16 and E1–3, respectively; Fig. 11). In particular, F_v / F_m was the highest (0.791) at Stn G8 where D-Fe levels were elevated and macronutrients had not been depleted (Table 1). The maximum theoretical value of F_v / F_m for eukaryotic algae is generally considered to be 0.6–0.8 as estimated from multiple turnover PAM fluorometry (Büchel and Wilhelm, 1993). Values of F_v / F_m tended to become lower offshore from near the mouth of the Amur River coincident with both declining D-Fe and Fd index. A significant relationship between salinity and F_v / F_m was also observed near the Sakhalin Island (Fig. 7b; $\rho = 0.56$, $p < 0.001$). Similarly, relationships between D-Fe and F_v / F_m (Fig. 8b; $\rho = 0.62$, $p < 0.001$) and between F_v / F_m and the Fd index (Fig. 12; Pearson correlation coefficient $r = 0.62$, $p < 0.01$) were also statistically significant, whereas no relationship was found between nitrate plus nitrite and F_v / F_m ($\rho = 0.30$, $p > 0.05$).

4 Discussion

In this study, nutrient limitation is defined as a reduction in the growth rate of phytoplankton due to the low concentration of a nutrient (Liebig's law of the minimum), which might be reflected in F_v / F_m (Boyd et al., 2005). On the other hand, nutrient stress is defined as a physiological adjustment of phytoplankton to lower nutrient availability, which may precede, or occur with or without a reduction in growth rate, and which may be reflected in the Fd index (Boyd et al., 2005).

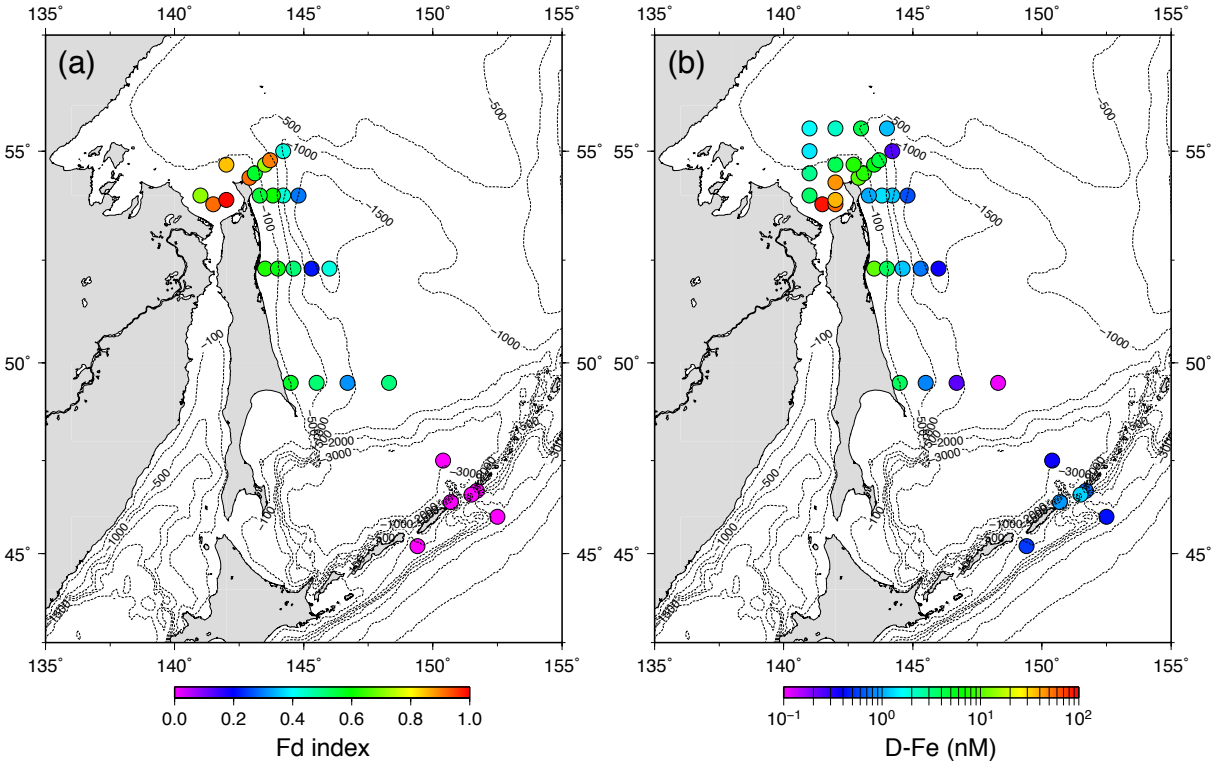

Fig. 6. Plots of **(a)** the Fd index values for micro-sized diatoms and **(b)** dissolved iron (D-Fe) concentration (nM) in surface waters at each sampling station.

4.1 Effect of riverine Fe input on the physiology of micro-sized diatoms off the Sakhalin Island

We found that there was a distinct gradient of Fe nutritional status in the micro-sized diatoms extending from near the mouth of the Amur River to open waters in the Sea of Okhotsk as estimated from the Fd index (Fig. 6a) along with D-Fe data (Fig. 6b). Values of the Fd index significantly correlated with levels of D-Fe (Fig. 8a), indicating that, in general, the Fd index could be used as a diagnostic Fe stress marker for the micro-sized diatoms in this area. However, it should be noted that, in principle, values of the Fd index would be varied with intracellular Fe levels rather than D-Fe concentrations in seawater. Additionally, the Fd index values can also be changed among species in the micro-sized diatoms. Therefore, there could be various Fd index values at a D-Fe concentration.

At Stns G8 and G10 near the Amur River, values of the Fd index were > 0.9, consistent with growth of the micro-sized diatoms under Fe-replete conditions. Nishioka et al. (2014) noted that influence of the Amur River reached to Stns C1–C9 (also see Table 1, < 32 in salinity) during the survey. The values of the Fd index at these stations became lower (0.22–0.62), indicating that the Fe nutritional status of the micro-sized diatoms declined. According to Yoshimura et al. (2010) who conducted Fe-enriched bottle incubation experiments in the Sea of Okhotsk during the study, addition of 10 nM inorganic Fe did not stimulate the growth of the large-sized phytoplankton ($> 10\,\mu m$) near Stns G9, E2, C3, and C9 (which correspond to Stns 1, 2, 3, and 4 in Yoshimura et al. (2010), respectively). Although the Fd index data were unavailable from Stns G9 and E2, the Fd index measured at nearby locations (i.e., Stn G10 and E1, respectively) was high (> 0.9); however, the Fd index values at Stns C3 and C9 were lower (0.58 and 0.42, respectively). The lower Fd index values observed at Stns C3 and C9 might reflect an early response prior to Fe limitation in the micro-sized diatoms. McKay et al. (1997) and Davey and Geider (2001) demonstrated that Fld accumulation in diatoms occurred in an early stage of Fe-deficient conditions when the growth rates of the diatoms were almost the same as those in Fe-replete conditions. At nearshore Stns C1 and E3 where both D-Fe and F_v / F_m were high (> 7 nM and > 0.6, respectively) and micro-sized diatoms were abundant ($> 4\,\mu g\,C\,L^{-1}$), the values of the Fd index were also slightly low (0.62 and 0.57, respectively). At these stations, the compositions of the micro-sized diatom species observed were similar to each other (Fig. 4). In particular, *Chaetoceros radicans* and *Dithylum brightwellii* were predominant at Stns C1 and E3, respectively. These same diatom taxa were observed at other stations where a high-Fd index was reported, suggesting that the lower index value cannot be readily attributed

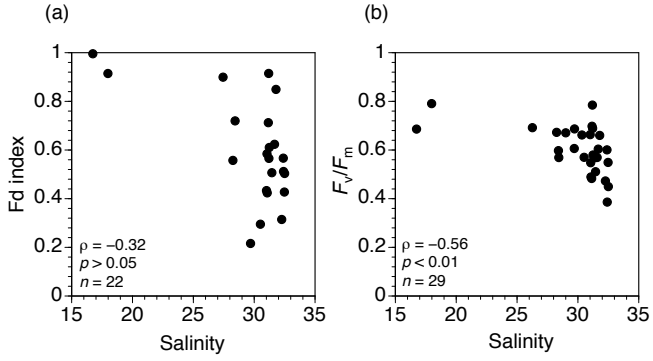

Fig. 7. Relationships between salinity and **(a)** the Fd index values for micro-sized diatoms or **(b)** F_v / F_m for total micro-sized phytoplankton in surface waters off Sakhalin Island.

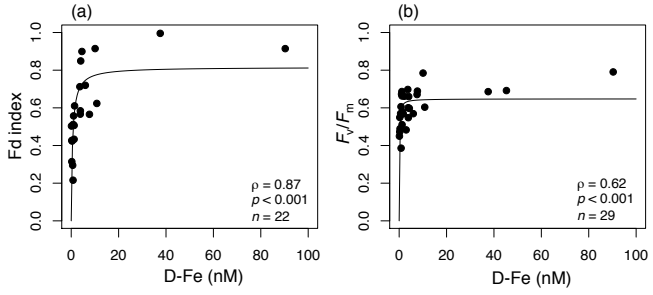

Fig. 8. Relationships between dissolved iron (D-Fe) concentrations (nM) and **(a)** the Fd index values for micro-sized diatoms or **(b)** F_v / F_m values for total micro-sized phytoplankton in surface waters off Sakhalin Island. The data obtained were fitted with the Michaelis–Menten equation (see the text).

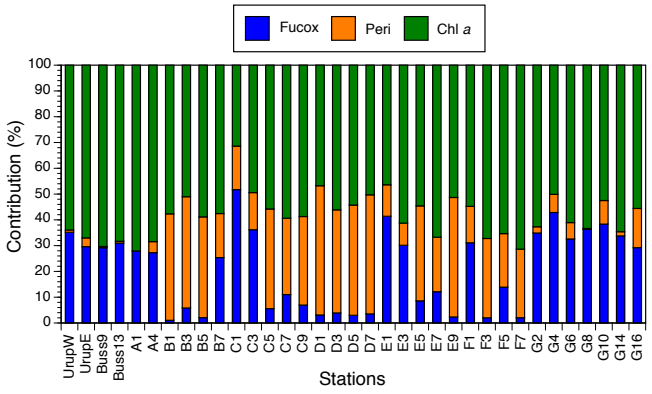

Fig. 9. Relative levels (%) of fucoxanthin (Fucox), peridinin (Peri), and Chl *a* to the sum of these pigments in the pellets collected for the Fd and Fld assays at each sampling station.

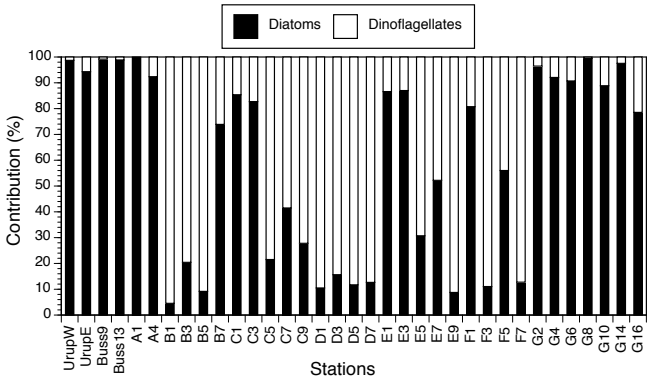

Fig. 10. Relative contributions (%) of diatoms and peridinin-containing dinoflagellates to the total micro-sized phytoplankton in terms of Chl *a* biomass.

to methodological considerations such as constitutive expression of Fld under Fe-replete conditions (McKay et al., 2000; Pankowski and McMinn, 2009a, b).

Although the Fd index was highly correlated with D-Fe, the relationship between the salinity and the Fd index was insignificant (Fig. 7a). This was probably related to the fact that the correlation coefficient between salinity and D-Fe was rather low ($\rho = -0.51$, $p < 0.01$; Fig. 2a), representing the presence of an Fe sink (i.e., utilization by microorganisms such as phytoplankton and scavenging; Boyd and Ellwood, 2010) along with Fe sources other than the Amur River discharge. Okunishi et al. (2007) and Misumi et al. (2011) pointed out that atmospheric Fe deposition and Fe flux from sediment play important roles in maintaining bioavailable Fe in surface waters of the Sea of Okhotsk. On the other hand, a correlation between the F_v / F_m values and salinity was observed (Fig. 7b). It is well known that F_v / F_m values can vary with the availability of nitrogen, phosphorous or Fe (Shelly et al., 2011). Lippemeier et al. (1999) also noted that changes in F_v / F_m could be observed in response to silica limitation and its re-supply to diatoms. Parkhill et al. (2001) proposed that F_v / F_m should be used as a diagnostic indicator for nutrient-starved unbalanced growth conditions, because F_v / F_m was not a good measure of nutrient limitation in a neritic diatom under balanced growth conditions and showed a constant high value (ca. 0.65) independent of nutrient-limited growth rate under different irradiance levels. Diurnal variations of F_v / F_m have also been reported previously (e.g., Suzuki et al., 2002; Behrenfeld and Milligan, 2013). Note that the F_v / F_m values obtained in this study were derived not only from the diatoms, but also from the dinoflagellates (Fig. 10). Nonetheless, in this study, the F_v / F_m values significantly correlated with D-Fe concentrations (Fig. 8b), but not with levels of nitrate plus nitrite, suggesting that D-Fe was a major determining factor for F_v / F_m values. A distinct gradient in F_v / F_m values was also observed from near the Amur River mouth to the neritic region at Stns C1 and C3, where diatoms were predominant in the micro-sized phytoplankton (> 80 %; Fig. 10). These results were consistent with the Fd index data. There was also a significant correlation between the F_v / F_m values and the Fd index (Fig. 11), indicating that the photosynthetic physiological conditions

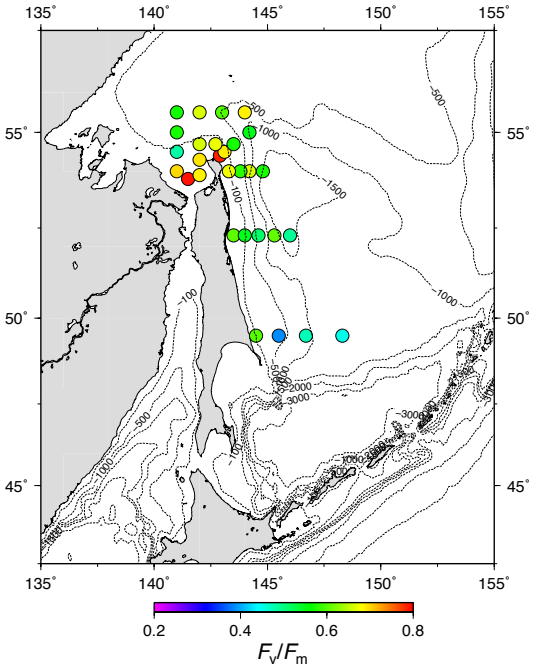

Fig. 11. Plots of F_v / F_m values for total micro-sized phytoplankton in surface waters at each sampling station.

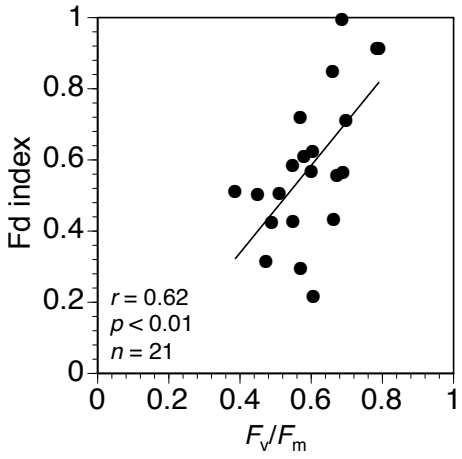

Fig. 12. A relationship between F_v / F_m values for total micro-sized phytoplankton and the Fd index values for micro-sized diatoms in surface waters off Sakhalin Island. A linear regression line is plotted with the data.

between the diatoms and other micro-sized phytoplankton (i.e., mainly dinoflagellates) were comparable.

If we fit the relationships between D-Fe and the Fd index or F_v / F_m with the Michaelis–Menten equation, the maximum values for the Fd index and F_v / F_m were estimated as 0.82 ± 0.06 and 0.65 ± 0.02 (mean $\pm$ standard error), respectively (fitting curves in Fig. 8). The lower maximum Fd index value, compared to the theoretical maximum ($= 1$), was mainly caused by the data from Stns C1 and E3 mentioned above. The maximum value (0.65 ± 0.02) of F_v / F_m was within the theoretical value between 0.6–0.8 for eukaryotic algae as estimated with multiple turnover PAM fluorometry (Büchel and Wilhelm, 1993). The half saturation constants between D-Fe and the Fd index or F_v / F_m were calculated as 0.56 ± 0.20 nM and 0.080 ± 0.028 nM (mean $\pm$ standard error), respectively. Unfortunately, no such field data for direct comparison were available from previous studies. The higher half saturation constant between D-Fe and the Fd index than that between D-Fe and F_v / F_m seems to be reasonable, because Fld accumulation could precede the declines in F_v / F_m or growth rate (McKay et al., 1997; Davey and Geider, 2001). According to Timmermans et al. (2004), the half-saturation constants between D-Fe and net growth rate in four large diatoms from the Southern Ocean were between 0.19–1.14 nM, and close to the ambient D-Fe concentrations of 0.2 nM. In this study, the half saturation constant (0.080 ± 0.028 nM) between D-Fe and F_v / F_m might be slightly lower than expected from the ambient D-Fe levels (> 0.2 nM in general; Table 1). It is known that dinoflagellates can migrate vertically to assimilate nutrients in subsurface waters (Eppley et al., 1968). Their vertical migration capability could account for the high-F_v / F_m values even under low-Fe conditions at the surface. As mentioned above, the nutrient-balanced growth conditions (Parkhill et al., 2001) might also contribute to this issue. During events of high input of D-Fe, which can sometimes be expected in coastal waters, diatoms are capable of luxury accumulation of Fe within their cells, to be used later under Fe-deficient conditions (Sunda and Huntsman, 1995; Iwade et al., 2006; Marchetti et al., 2009). Therefore, for diatoms, luxury Fe uptake and intracellular Fe storage could play an important role in maintaining the high-F_v / F_m values under low-Fe conditions.

4.2 Fe deficiency in micro-sized diatoms off the Kuril Islands

Only Fld was detected in the vicinity of the Kuril Islands (Fig. 5), resulting in a Fd index of zero (Fig. 6a), despite ambient D-Fe levels > 0.4 nM (Table 1). The results suggest that the micro-sized diatoms, which mainly consisted of *Chaetoceros concavicornis* and the species was minor off the Sakhalin Island (Table 2), were growing under Fe-deficient conditions. Otherwise, the constitutive production of Fld in the diatoms could occur even under Fe-replete conditions (McKay et al., 2000; Pankowski and McMinn, 2009a, b), whereas that has never been reported from *Chaetoceros* spp. The former is supported by the results of Fe-enriched bottle incubation experiments conducted in the Bussol' Strait by Yoshimura et al. (2010) and Sugie et al. (2013). According to Yoshimura et al. (2010), net specific growth rate of large-sized (> 10 µm) phytoplankton, which mainly consisted of the genus *Chaetoceros*, was significantly enhanced by a 10 nM inorganic Fe addition, as compared with the

unamended control. More recently, Sugie et al. (2013) also confirmed that additions of inorganic Fe (final concentration of 5 nM) or particulate Fe, which was collected from the nepheloid layer in the coastal region of the Sea of Okhotsk, stimulated the growth of diatoms in the Bussol' Strait during summer 2007, although the cell size of the diatoms was not indicated. As a plausible mechanism promoting Fe deficient conditions in the micro-sized diatoms near the Bussol' Strait, Fe and light co-limitations (Sunda and Huntsman, 1997) were considered. The strong vertical mixing in the Bussol' Strait (Yagi and Yasuda, 2012) could facilitate transport of the diatoms from the surface to deeper layer and vice versa. Indeed, vertical profiles of Chl *a* biomass in the Bussol' Strait were entirely uniform from the surface to 200 m (data not shown) as were those of temperature and salinity (see Yoshimura et al., 2010). Additionally, daily surface PAR was relatively low (21 ± 6 mol photons m^{-2} d^{-1}) in this region due to heavy cloud cover, compared to that measured at other stations during the cruise (Isada et al., 2013). These physical conditions could cause light limitation of the growth of the micro-sized diatoms. According to Sunda and Huntsman (1997) and Strzepek and Harrison (2004), higher amounts of Fe are required for diatoms growing under low-light conditions, because more Fe is required for the synthesis of the photosynthetic apparatus including pigments to capture more light energy. Therefore, the micro-sized diatoms off the Kuril Islands possibly encountered Fe and light co-limitations.

Interestingly, the values of the Fd index at Stns A1 and A4, which were slightly apart from the Bussol' Strait, showed the same results as those at other stations near the strait (i.e., Fd index = 0). These data suggest efficient water transport from the Sea of Okhotsk to the North Pacific via the Kuril Straits (Ohshima et al., 2002; Kida and Qiu, 2013). Indeed, the compositions of the micro-sized diatom species detected between Stns A1 and A4 were similar to each other (Fig. 4). Jing et al. (2009) also demonstrated similar phylotype compositions of picoplankton-sized cyanobacteria *Synechococcus* spp. between Stns A1 and A6, which was located between Stn A4 and B1 during the cruise.

5 Conclusions

The coastal area is a key environment in the global Fe cycle, where the brackish water environment changes the physicochemical speciation (i.e., mobility) of riverine Fe via aggregation, sedimentation, and redox processes (Boyd and Ellwood, 2010; Breitbarth et al., 2010). Therefore, the coastal waters become a highly dynamic transition zone, resulting in diverse spatiotemporal chemical and biological changes. In this study, we revealed that there was a distinct gradient in the Fe nutritional status of the micro-sized diatoms from near the mouth of the Amur River to the oceanic region of the Sea of Okhotsk using the Fd index. In addition, uniformly low-Fd index values suggested widespread Fe deficiency of the micro-sized diatoms near the Kuril Straits. The differential expressions of Fd and Fld in the micro-sized diatoms helped us to understand how these microorganisms responded to Fe availability in the Sea of Okhotsk without reliance on incubation approaches which are often accompanied by bottle effects (Carpenter and Lively, 1980) and trace metal contamination (Fitzwater et al., 1982). Recently, Whitney et al. (2011) succeeded in examining the expression of selected genes such as Fd and Fld genes in two diatom *Thalassiosira* strains with real-time quantitative reverse transcription PCR (qRT-PCR). Although the micro-sized diatoms play important roles in the marine ecosystems and biogeochemical processes, such molecular-based physiological knowledge has still been very scarce, especially applied to the field. We expect that recent rapid advances in metagenomics, metatranscriptomics, and metaproteomics using next-generation sequencing technologies and high-precision mass spectrometry with advanced bioinformatics (Rusch et al., 2010; Marchetti et al., 2012; Desai et al., 2012; DeLong, 2013) will lead to further understanding of how phytoplankton including the micro-sized diatom species have adapted to a wide variety of in situ Fe conditions, and the implications of their adaptations to algal productivity and biodiversity.

Acknowledgements. We wish to thank the captain, officers, crew and scientists of the R/V *Professor Khromov* for their helpful assistance during the cruise. In particular, the chief scientist Prof. Takeshi Nakatsuka must be acknowledged. We also thank Kyoko Kawanobe for light microscopy. Two anonymous referees are also acknowledged for their constructive comments and suggestions on the manuscript. This study was supported by the project "Human Activities in Northeastern Asia and Their Impact on the Biological Productivity in the North Pacific Ocean" of the Research Institute of Humanity and Nature (RIHN), JSPS Grant-in-Aid for Scientific Research (no. 19510001, no. 22221001, no. 22310002, and no. 24121004) and the CREST project of the Japan Science and Technology Agency.

Edited by:

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