A Fluorescent-Based HPLC Assay Using 4-Chloro-7-nitrobenzo-2-oxa-1, 3-diazole as Derivatization Agent for the Determination of Iron Bioavailability to Red Tide Phytoplankton

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A Fluorescent-Based HPLC Assay Using 4-chloro-7-nitrobenzo-2-oxa-1, 3-diazole as Derivatization Agent for the Determination of Iron Bioavailability to Red Tide Phytoplankton

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Abstract

A new fluorescent-based high performance liquid chromatography (HPLC) assay using 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-C1) was employed to determine iron (Fe) bioavailability to red tide phytoplankton in seawater. After growing four red tide species (*Prymnesium parvum*, *Heterosigma akashiwo*, *Eutreptiella gymnastica*, and *Oltmannsiellopsis viridis)* in f/2 artificial seawater under different Fe conditions, soluble extracts of the phytoplankton were derivatized using different fluorescent reagents (NBD-C1, 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole; NBD-F, fluorescamine, and ortho-phthalaldehyde; OPA) followed by HPLC assay. Among the four fluorescent reagents, NBD-C1 was most effective for derivatizing the phytoplankton extracts which would consist of proteins and peptides. HPLC chromatograms of the NBD-derivatized extracts showed gradual changes (decrease/increase) of six peaks for different Fe conditions. Four of the peaks decreased, while two peaks increased with the increase of Fe concentrations in the culture medium. Considering the consistency and sensitivity of chromatogram peaks E and A to different Fe, phosphate and nitrate conditions for all phytoplankton studied, the ratio of these two peaks ($I_{E/A}$) has been proposed as the indicator of Fe bioavailability to red tide phytoplankton.

Keywords: Fluorescent-based HPLC assay; Marine phytoplankton; red tide; iron bioavailability; NBD-C1.

1. Introduction

Iron (Fe) is an important nutrient for phytoplankton as it is involved in many cellular biochemical processes such as photosynthesis, respiration, nitrogen fixation and nitrate, nitrite, and sulfate reductions [1]. Therefore, Fe supply influences phytoplankton biomass, growth rate and species composition, as well as primary productivity of marine systems [1]. Studies have shown that limited Fe availability impairs phytoplankton growth by as much as 40% of the open oceans [2],

notably in the high nutrient low chlorophyll (HNLC) open seas such as the Equatorial Pacific and Southern Oceans [3, 4]. Large areas of marine systems such as the Southern Ocean, Gulf of Alaska, Subarctic Pacific, Equatorial Pacific Ocean and North Pacific Ocean have been reported to be Fe-limited [2, 5]. The hypothesis that Fe-limits phytoplankton productivity in HNLC regions of the world's oceans (Fe hypothesis) has opened numerous research fronts including analytical determination of Fe concentrations (total and dissolved) and bioavailability in marine systems.

In coastal and estuarine areas, a few species of phytoplankton have frequently grown out of control when nutrient concentrations are relatively high [6]. The phytoplankton blooms sometimes produce toxic or harmful effects on human health and marine ecosystems, which is called 'red tide' or harmful algal blooms. The growth of red tide phytoplankton is generally limited by macronutrients (primarily phosphorus and nitrogen), while temporal growth limitation by trace elements, especially iron, can occur in some coastal upwelling regions [7] and fjord systems [8]. Culture experiments indicate that marine phytoplankton have different iron requirements that are related to their growing environments; iron requirements for coastal phytoplankton species are several orders of magnitude higher than those for oceanic species [9, 10]. Wells et al. [11] suggested that pulsed inputs of iron could implicate the development of red tide blooms in coastal areas where outbreaks are initiated offshore.

With the development of analytical methods for trace metal measurement (e.g., flow injection analysis and chemiluminescence detection; FIA-CL), total dissolved Fe concentrations in seawater samples were measured at sub-nanomolar levels [12] that are unlikely to support high phytoplankton biomass. Subsequent studies also showed that Fe bioavailability to marine phytoplankton is consistent with many biotic and abiotic factors such as its influence on phytoplankton species composition [13], interactions with other nutrients [14], light intensity [15]. Therefore, a new approach was proposed for the determination of Fe status (concentration and bioavailability) in phytoplankton cells using biochemical, molecular and physiological indicators (bio-indicators) such as concentrations of flavodoxin and ferredoxin [16, 17], relative abundance and ratio of flavodoxin and ferredoxin [18], immune-probes, mRNA [13], expression of specific

proteins [19], photosynthetic pigment concentrations [20], sterol and dinosterol content [21] in phytoplankton samples using high performance liquid chromatography (HPLC) and immunocytochemical techniques.

Fluorescent-based HPLC assay is a quick, easy, efficient and powerful method for the quantitative measurement of cellular macromolecules in organisms [22-27]. This method has been used for the determination of cysteine and cysteamine adducts in *Escherichia coli*-derived proteins [26], thiol compounds in biological samples [22], human erythrocytic glutathione [25], domoic acid (DA) in mussels (*Mytilus edulis*) [24], peptides in tissue and plasma samples [28] and amines and proteins [29] using different fluorescent reagents as the derivatizer. The HPLC method has also been used for the determination of pigments in phytoplankton in order to measure marine productivity in relation of Fe status in seawater [30, 31]. However, the fluorescent-based HPLC assay of cellular macromolecules (e.g. proteins and peptides) with pre-derivatization has not been tested for the determination of Fe bioavailability to marine phytoplankton. In the present study, a fluorescent-based HPLC assay with pre-derivatization using 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-C1) was employed for the determination of Fe bioavailability to red tide species of marine phytoplankton. Three other fluorescent reagents (4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole; NBD-F, fluorescamine, and ortho-phthalaldehyde; OPA) were also used to compare the results and identify the most effective reagents for the fluorescent derivatization. The objective of the study was to test a quick and easy fluorometric method for the determination of Fe bioavailability to several red tide species using the HPLC assay.

2. Materials and Methods

2.1. Phytoplankton pre-culture and maintenance

A red tide phytoplankton, *Prymnesium parvum*, was collected from Fukuyama Bay, Hiroshima, Japan. Other red tide species, *Heterosigma akashiwo* (Osaka Bay, Osaka, Japan), *Eutreptiella gymnastica* (Hiroshima Bay, Hiroshima, Japan) and *Oltmannsiellopsis viridis* (Osaka

Bay, Osaka, Japan), were also used in this study. All red tide phytoplankton strains were provided by Dr. Ichiro Imai, Hokkaido University, Japan.

The phytoplankton strains were cultured axenically (axenicity was assessed and monitored by the 4′ 6-diamidino-2-phenylindole (DAPI) test). The strains were pre-cultured for two weeks in 30-mL polycarbonate bottles with modified f/2 culture solution in artificial seawater [32, 33] without iron (Fe; prepared from $FeCl_3$) (Table 1). Before growing *P. parvum* in nitrogen (N) and phosphorus (P) limited medium, the axenic strains were grown in f/2 culture solution in artificial seawater for two weeks without the addition of N and P.

2.2. Chemical treatments and reagents

Iron concentrations (prepared from $FeCl_3$, Kanto Chemicals, Tokyo, Japan) in the culture medium were 4, 10, 50 nM for *P. parvum* and *H. akashiwo*, and 10 and 50 nM for *E. gymnastica* and *O. viridis*, respectively. Nitrogen concentrations (prepared from $NaNO_3$, Kanto Chemicals, Tokyo, Japan) were 20 and 880 μM for N-limited and N-rich culture medium, respectively. Phosphate concentrations (prepared from $NaH_2PO_4 \cdot 2H_2O$, Wako Pure Chemicals, Osaka, Japan) were 1.0 and 38.5 μM for the P-limited and P-rich culture medium, respectively. All other chemicals and reagents used in this study were of analytical grade.

2.3. Growing phytoplankton in experimental solution

The phytoplankton strains were grown in 1-L polycarbonate bottles containing modified f/2 culture medium in artificial seawater (Table 1). The polycarbonate bottles and culture medium were sterilized (MLS-3780, SANYO, Japan) before using them. $FeCl_3$ (10^{-5} M) solution (in 1 M HCl) was sterilized by autoclaving in a separate bottle. Sterilization was done at 121 °C for 30 min. $FeCl_3$ solution was added to the modified f/2 culture medium after sterilization, and therefore, there was limited scope of Fe contamination during the sterilization process. On the other hand, all chemicals and their method/source of preparation were carefully selected to avoid any possible Fe contamination.

A 2 mL pre-culture of phytoplankton strains (2×10^3 cells mL^{-1}) of logarithmic growth phase was inoculated in 1 L of modified f/2 culture medium in a laminar air flow chamber. The phytoplankton was then incubated in growth chamber at 20±2 °C with light and dark schedule of 14:10 h and light intensity of 188 µE m^{-2} S^{-1} for 2 weeks.

2.4. Extraction of soluble extracts

Soluble extracts of the phytoplankton cells were collected following the protocol described previously [34]. Briefly, the cells in I L of the medium were collected by centrifugation at 1800 rpm (relative centrifugal force; g = 380) for 10 min at 4° C, and the cell pellets were washed for two times using 2 mL of 10 mM Tris–HCl (pH 7.2). The soluble extracts of the phytoplankton were extracted by sonication of the cells in 1.0 mL of 10 mM Tris–HCl for 20 s with an ultrasonic homogenizer (UH-50, Surface Mount Technology, Japan) at output 5 under ice-cold condition, followed by centrifugation at 15000 rpm (g = 10000) for 10 min at 4° C. The soluble proteins and peptides were concentrated using filtration on centrifugal filter units (Amicon Ultra-4, 5 kDa-cutoff, Millipore). The amounts of total proteins were estimated by the Bradford assay (Bio-Rad, Hercules, CA, USA).

2.5. Derivatization by fluorescent reagents

In the present study, four fluorescent reagents (NBD-C1, NBD-F, fluorescamine, and OPA) were used for derivatizing the phytoplankton extracts. These fluorescent reagents were reported previously to be useful for the fluorometric detection of proteins, peptides, amino- and imino-acid [24-26]. A 10 µL extract sample of each treatment was added to 100 µL of 0.266 M $NaHCO_3$ and 80 µL of 4.75 mg mL^{-1} NBD-Cl/MeOH. Then the sample was derivatized by heating at 55 °C for 60 min in a block incubator (ASTEC block incubator, Model: B1-516S, Japan). The derivatized extracts were then subjected to HPLC assay (λ_{ex} : 450 nm, λ_{em} : 540 nm) using 30% CH_3CN and 0.1% trifluroacetic acid (TFA) as mobile phase.

The derivatization of the phytoplankton extracts with NBD-F was carried out following the procedure of Miyano et al. (1985) and James et al. [35]. Briefly, 5 µL sample of each treatment was added to 100 µL of 0.1 M sodium borate and 100 µL of 0.1 mg mL^{-1} NBD-F in CH_3CN. After derivatization by heating at 60°C for 1 min in the block incubator, the reaction was stopped by adding 10 µL of 1M HCl solution, and was subjected to HPLC assay (λ_{ex} : 450 nm, λ_{em} : 540 nm). For fluorescamine derivatization, 10 µL sample of each treatment was added to 40 µL of 0.05 µM phosphate buffer (pH 9) and 20 µL of 0.28 mg mL^{-1} fluorescamine in acetone. Then the derivatized samples were subjected to HPLC assay (λ_{ex} : 390 nm, λ_{em} : 475 nm) using 70% CH_3OH and 0.1 M phosphate buffer. For derivatization with OPA, 10 µL sample of each treatment was added to 150 µL of 1.4 mg mL^{-1} OPA (pH 10) and 150 µL of 4 mL L^{-1} 2-mercaptoethanol following the protocol of Eijk et al. [36]. The derivatized samples were then subjected to HPLC assay (λ_{ex} : 340 nm, λ_{em} : 455 nm) using 50% CH_3CN as mobile phase.

2.6. HPLC chromatography

Chromatographic separation was achieved using a TOSOH 8020 series LC system, equipped with a fluorescence detector (Model: NANOSPACE SI-2, SHISEIDO, Japan). A 200 µL NBD-C1 derivatized sample of each pretreatment was transferred into a 1.5-mL micro tube and placed into an auto sampling chamber (Model: AS-8021, TOSOH, Japan), from which a 20 µL sample solution was used for the HPLC analysis. Two TSKgel ODS-100V, 5 µm particle size, 4.6 mm × 150 mm + 4.6 mm × 250 mm columns (TOSOH, Japan) was used. A mobile phase consisting CH_3CN (30%) and TFA (0.1%) in water was used with a flow rate of 0.75 mL min^{-1}. The processes described above were carried out at 4° C.

3. Results and Discussion

3.1. Effect of iron on the growth of phytoplankton

Growth curves of red tide species in modified f/2 media under iron limited conditions were reported in previous works [34]. In this study, we select 4, 10 and 50 nM of Fe(III) as Fe-limited and Fe-rich conditions. The growth of *P. parvum* was almost constant up to 6th day. The growth showed down and stopped from 7th day for 10 nM of Fe(III) and 9th day for 10 nM of Fe(III), which indicated Fe limitation in the media. On the other hand, *P. parvum* showed steady growth up to 14th day for 50 nM Fe(III) of Fe-rich condition. Both of compositions and expression levels of proteins in the cells varied significantly as iron, nitrate and phosphate concentrations increased in the culture medium [19]. Rahman et al. [34] identified several proteins highly expressed in the *P. parvum* cells under Fe-limited conditions using 2-dimensional gel electrophoresis and MALDI-TOF-MS analysis.

3.2. Selection of fluorescent reagent

In the present study, four fluorescent derivatization reagents were tested to assess their ability to detect phytoplankton extracts by HPLC (Fig. 1). The HPLC chromatogram peaks for fluorescamine were very small with poor separation of the phytoplankton extracts (Fig. 1(c)), while the chromatogram peaks for OPA were poor in quality with numerous interference peaks and long retention time for the major components (Fig. 1(d)). This is due to the limited reactivity of these reagents, which are available only for primary amines and don't react with secondary amines. Moreover, chromatogram peaks for fluorescamine and OPA in Fig. 1 were unstable. Fluorescamine and OPA were deemed to be unsuitable fluorescent reagents for derivatization of phytoplankton extracts.

NBD fluorescent reagents, NBD-Cl and NBD-F, produced better chromatograms for the soluble extracts (including proteins, peptides and amino acids) compared to those of fluorescamine and OPA with the limitation of instability of the chromatogram peaks (Fig. 1), probably because the NBD reagents react with both primary and secondary amino groups to give fluorescent adducts [37]. Recent studies on the fluorescent-based HPLC with post-column derivatization have preferred NBD-F as a NBD fluorescent reagent on the ground that NBD-F reacts faster than NBD-Cl at similar temperature during the derivatization processes (e.g. NBD-F; 1 min at 60 °C, NBD-Cl; 60

min at 55 °C). Short reaction time is favorable for HPLC post-column derivatization approaches, and hard condition during the derivatization reaction should be also avoided to prevent proteins denatured and precipitated in the extracts. However, the both NBD reagents produced stable chromatograms with clear and sharp peaks in this study (Figs. 1(a) and (b)), which suggests that the proteins, peptides and amino acids in the extracts reacted to give the fluorescent adducts and that the denaturation of the proteins is almost negligible under the both conditions during the pre-derivatization process. Maroulis *et al.* [24] reported NBD-C1 to be a better fluorescent reagent than 4-hydroxy-7-nitrobenzo-2-oxa-1,3-diazole (NBD-OH) for fluorometric detection of domoic acid (DA) in mussels (*Mytilus edulis*). In addition, NBD-C1 is commercially available at modest cost compared to other derivatization reagents such as NBD-OH and NBD-F [24]. Therefore, in the present study, NBD-C1 was selected for fluorometric detection of phytoplankton extracts to determine Fe bioavailability to marine phytoplankton.

Previous studies reported that the NBD-derivatized proteins and peptides have been separated using molecular exclusion chromatography [29] and reversed phase chromatography [26]. The phytoplankton extracts in this study would contain biological soluble molecules that have more than 5 kDa of molecular weight. [34]) have recently identified 11 proteins expressed in the cells of red tide species, *P. purvum*, under Fe-limited and Fe-rich conditions using 2D-DIGE/MALDI-TOF-MS. However, molecular exclusion chromatography did not show an expected separation for the NBD-derivatized proteins with retention times of corresponded molecular weight. The detection limits of the detector are insufficient to detect most of the minor and trace proteins in the 1 L phytoplankton cultures. Moreover, the proteins and peptides more than 5 kDa might be partially degraded during the derivatization process with the NBD reagents. Considering the wide range of molecular weight (e.g. proteins, peptides, and amino acids), separation of the NBD-derivatized extracts was achieved by reversed phase column in this study. The major NBD-derivatized extracts in Fig. 1(a) were clearly separated under isocratic conditions described in Materials and Methods.

3.3. Optimization of derivatization reaction

Optimization of derivatization reaction is vital for a stable and high quality HPLC chromatogram. Derivatization reaction of the phytoplankton extracts using NBD-C1 was found to be influenced by $NaHCO_3$ and NBD-C1 concentrations (Fig. 2). The fluorescent intensities of chromatogram peaks increased with the increase of $NaHCO_3$ concentration up to 0.14 M, while the opposite was observed for further increases of $NaHCO_3$ concentration (Fig. 2(c)). Therefore, 0.14 M was selected as the optimum concentration of $NaHCO_3$ for derivatization of phytoplankton extracts. The chromatogram peaks were also found to be influenced by NBD-C1 concentration. The chromatogram peaks increased with the increase of NBD-C1 concentrations and the peaks remained consistent for NBD-C1 concentrations between 1 – 2.25 mg mL^{-1} (Fig. 2(d)). Further increase of NBD-C1 concentration did not show substantial differences in the fluorescent intensities of the peaks. Therefore, 2 mg mL^{-1} treatment was selected as the optimum concentration of NBD-C1 for the derivatization reaction.

3.4. HPLC chromatographs of marine phytoplankton extracts under different Fe conditions

The HPLC chromatographs of NBD-derivatized extracts of the four red tide species grown under different Fe conditions are shown in Fig. 3. In general, the chromatogram peaks of six phytoplankton extracts (A-F) changed (increase/decrease) gradually for the phytoplankton grown under different Fe conditions. Three peaks (B, C and E) decreased while the remaining two (D and F) increased with increasing Fe concentrations in the culture medium. Peak A did not change with changes of Fe concentrations in the culture medium.

Peak B decreased slightly with the increase of Fe concentrations in the culture medium for *H. akashiwo*, *E. gymnastica* and *O. viridis* (Figs. 3(d)-(j)). For *P. parvum*, peak B remained unchanged when the phytoplankton was grown in 4 and 10 nM Fe (Figs. 3(a) (b)). However, a slight decrease in the intensity of peak B was observed in *P. parvum* when Fe concentration in the culture medium was 50 nM (Fig. 3(c)). These results indicate that the expression of proteins and peptides related to peak B in *P. parvum* is unlikely to be sensitive up to a specific Fe concentration.

Compared to peak B, the expression related to peak C was observed to be relatively more sensitive to Fe concentrations. However, in *H. akashiwo*, this peak did not increase consistently for cells grown in 10 and 50 nM Fe (Figs. 3(d), (e)).

For all phytoplankton species tested, peak F increased slightly with the increase of Fe concentration in the culture medium. The intensity of this peak was substantially lower compared to those of the other peaks tested. The intensity of peak F was also extremely low (sometimes, the peak cannot be distinguished) when the phytoplankton was grown in Fe concentrations below 10 nM (Fig. 3).

In general, for all phytoplankton, peak D increased substantially and consistently with increasing Fe concentrations in the culture medium (Fig. 3), indicating that the expression of the proteins and peptides related to peak D was highly sensitive to Fe conditions in the culture medium. Therefore, the expression related to peak D is likely to be useful for the determination of Fe bioavailability to marine phytoplankton. However, for *P. parvum* peak D disappeared when it was grown under N-limited condition (NO_3^- = 20 µM; Fig. 4). The role of Fe in N metabolism in phytoplankton has been well established. Timmermans *et al.* [38] reported a decrease in nitrate reductase activity in *EmiIiania husleyi*, *Isochrysis galbana*, and *Tetraselmis selmis* by 15-50% under Fe-limited conditions compared to that in Fe-rich conditions. Direct involvement of Fe in N reduction and utilization by marine phytoplankton has also been reported in numerous studies [39-41]. Therefore, we hypothesize that the proteins and peptides related to peak D are likely to be involved in N reduction, and may not be a suitable indicator of Fe bioavailability to marine phytoplankton. Rather, this expression can be used as an indicator of N reduction activity in marine phytoplankton.

3.5. Fluorescent-based HPLC peaks as an indicator of Fe bioavailability

In the present study, peak A was found to be stable under different Fe, N, and P conditions (Figs. 3 and 4). On the other hand, peak E increased consistently with the decrease in Fe concentrations in the culture medium for all red tide species studied (Fig. 3). The ratio of

fluorescence intensities of peaks E and A ($I_{E/A}$) decreased when the Fe concentration in the culture medium increased (Fig. 5). Therefore, $I_{E/A}$ can be used as an indicator of Fe bioavailability to marine phytoplankton. Specific HPLC chromatogram peaks of known proteins or the ratio of two protein peaks (e.g. flavodoxin and ferredoxin) has been used for the determination of the nutritional status of seawaters [16, 18, 27]. For example, Erdner and Anderson [18] suggested ferredoxin and flavodoxin as bio-indicators of Fe limitation in open ocean during Fe enrichment.

4. Conclusions

The present study reported that NBD-C1 can be used as an effective fluorescent derivatization reagent for the fluorometric HPLC assay in determining Fe bioavailability to red tide phytoplankton from the ratio of peaks A and E ($I_{E/A}$). Further study is required for the identification and characterization of these two compounds to fully understand their significance as bio-indicators of Fe conditions in marine systems and their roles in Fe bioavailability to marine phytoplankton.

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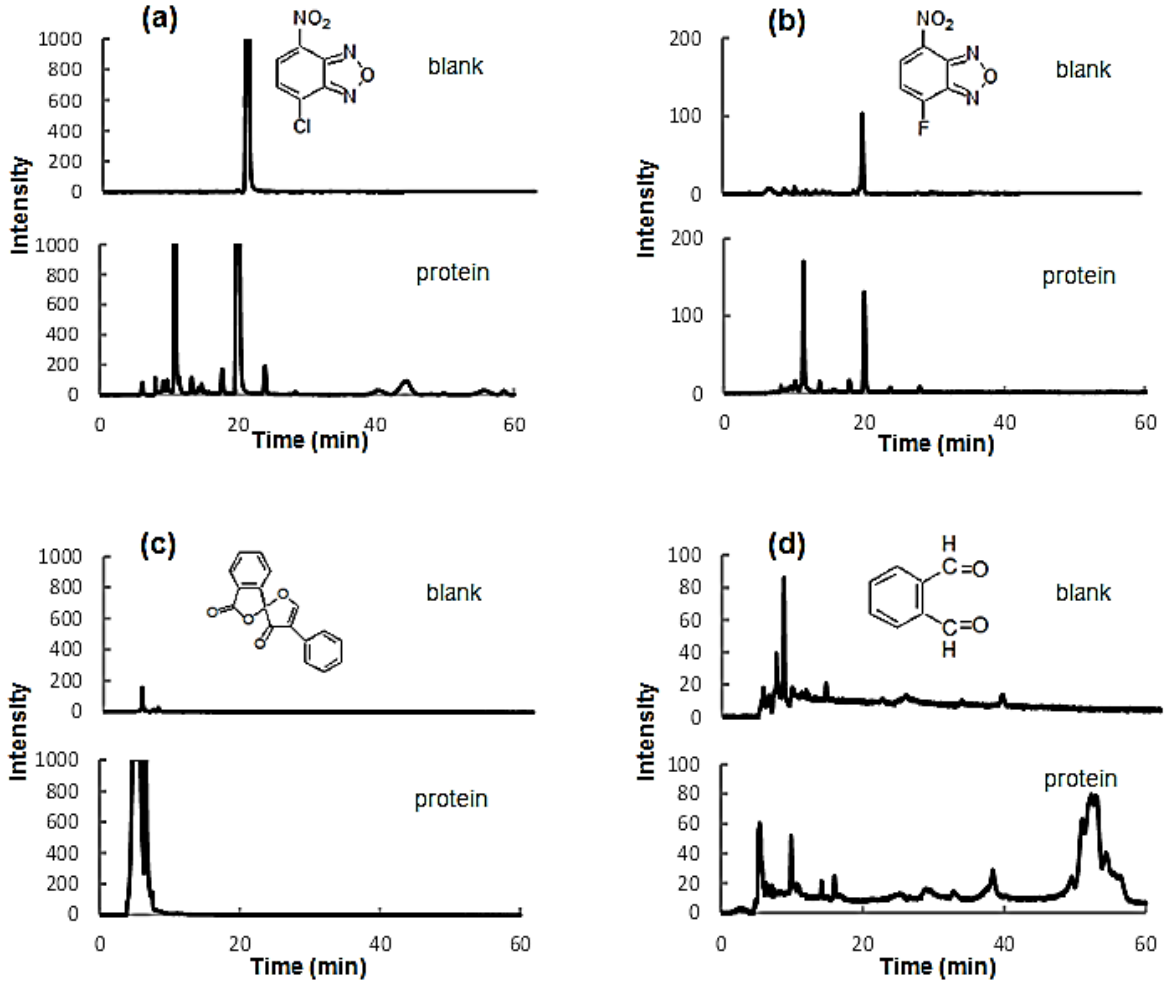

Fig. 1: HPLC chromatograms of blank and extract samples of *Prymnesium parvum* derivatized with **(a)** 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), **(b)** 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-F), **(c)** fluorescamine, and **(d)** ortho-phthalaldehyde (OPA).

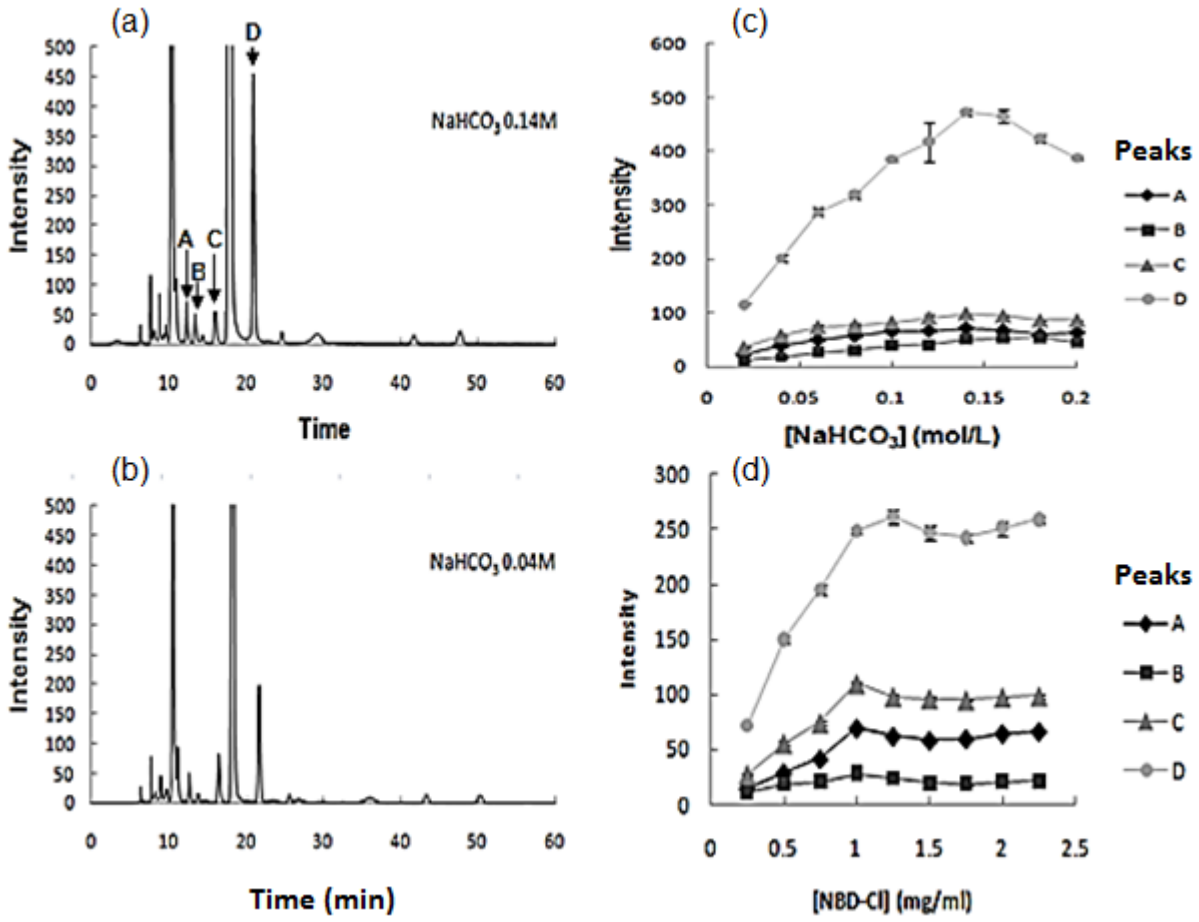

Fig. 2: HPLC chromatogram peaks as influenced by $NaHCO_3$ and NBD-Cl concentrations.

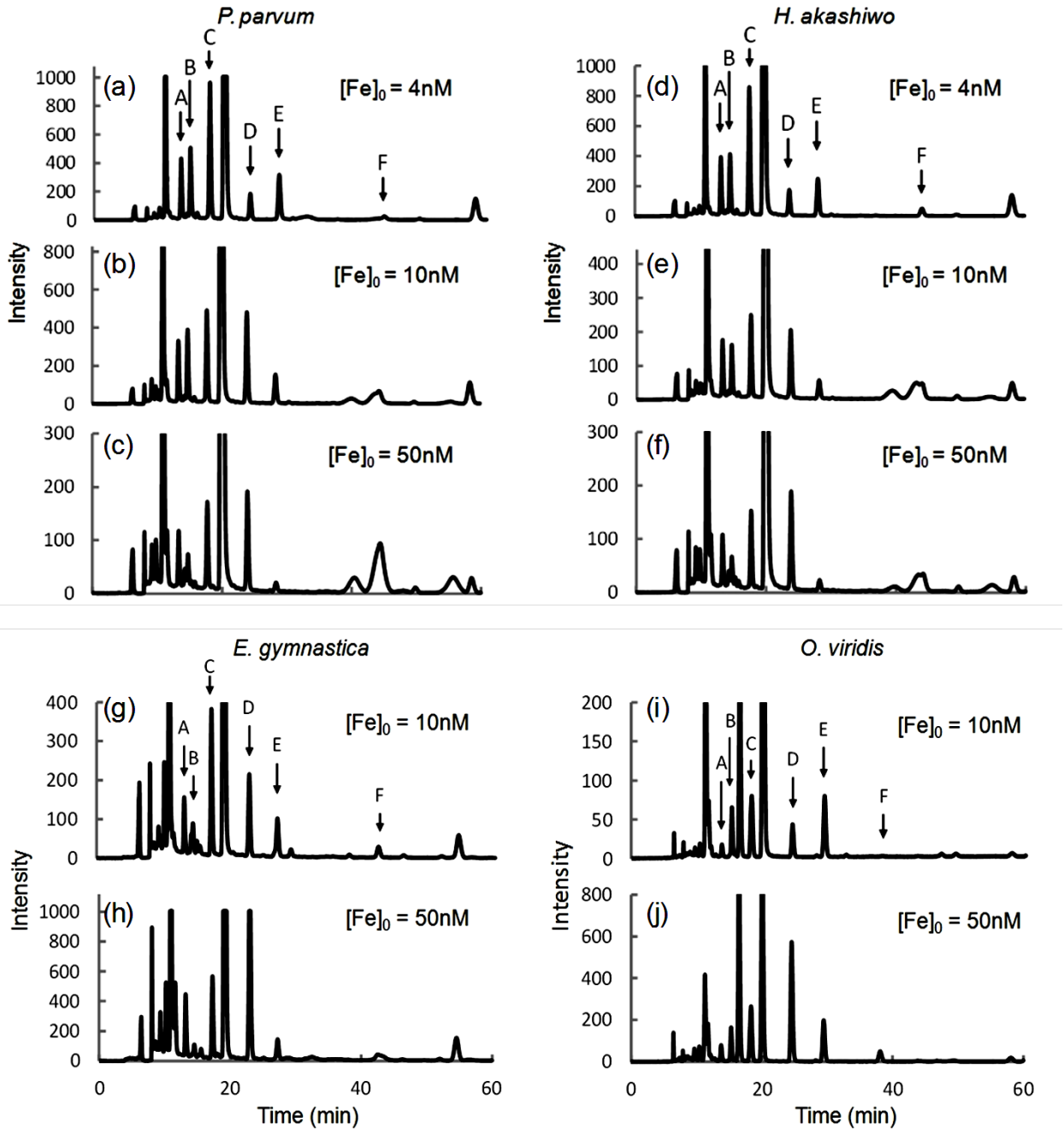

Fig. 3: HPLC chromatograms of NBD-derivatized extracts of *Prymnesium parvum, Heterosigma akashiwo, Eutreptiella gymnastica* and *Oltmannsiellopsis viridis* under different Fe conditions. The samples were collected after growing phytoplankton with Fe concentrations of 4 nM (control), 10 nM and 50 nM in f/2 artificial seawater.

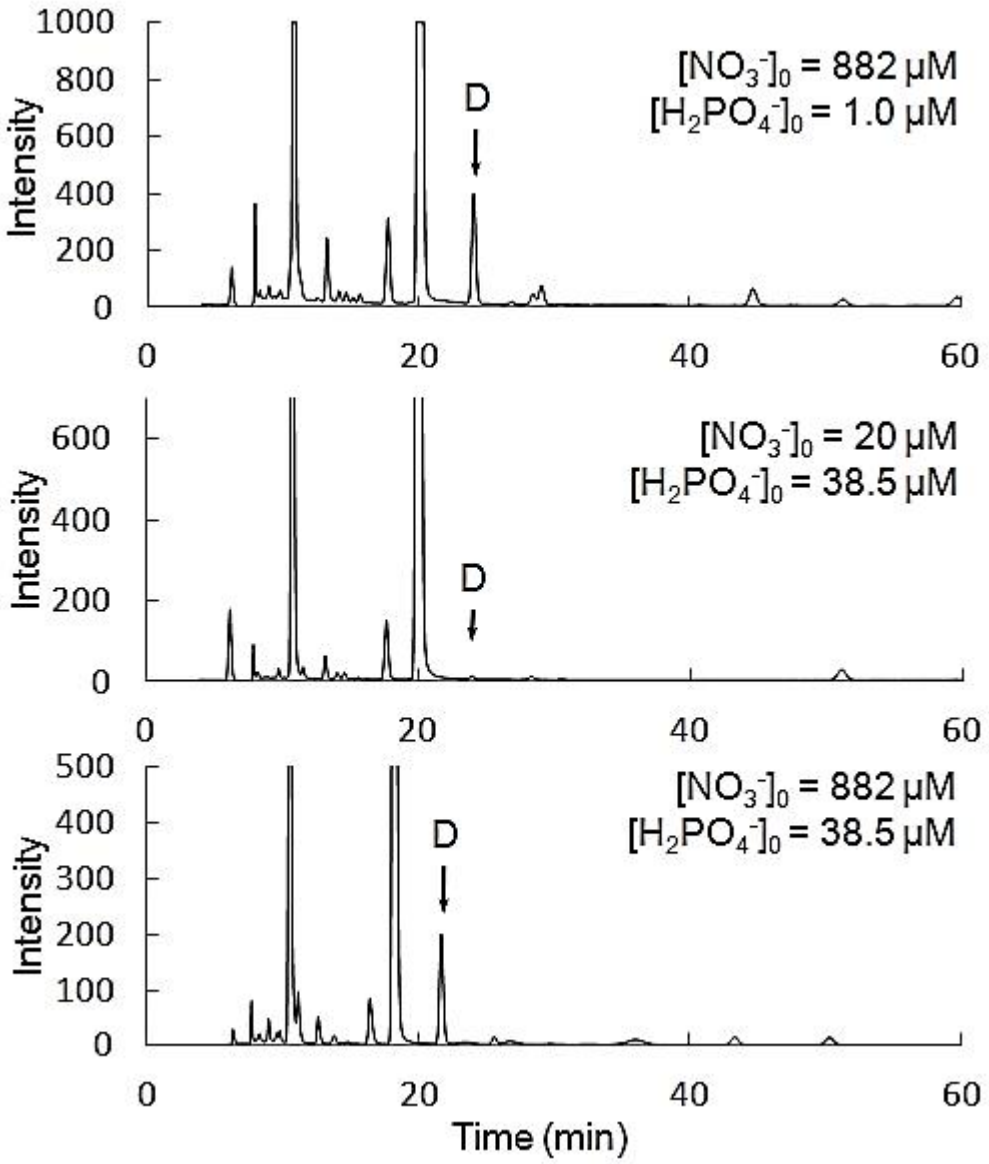

Fig. 4: The HPLC chromatograms of *P. parvum* extracts under different N and P conditions. Fluorescent derivatization was done with NBD-C1. The expression of the extracts related to peak D was highly sensitive to different N conditions, while its expression was insensitive to different P conditions.

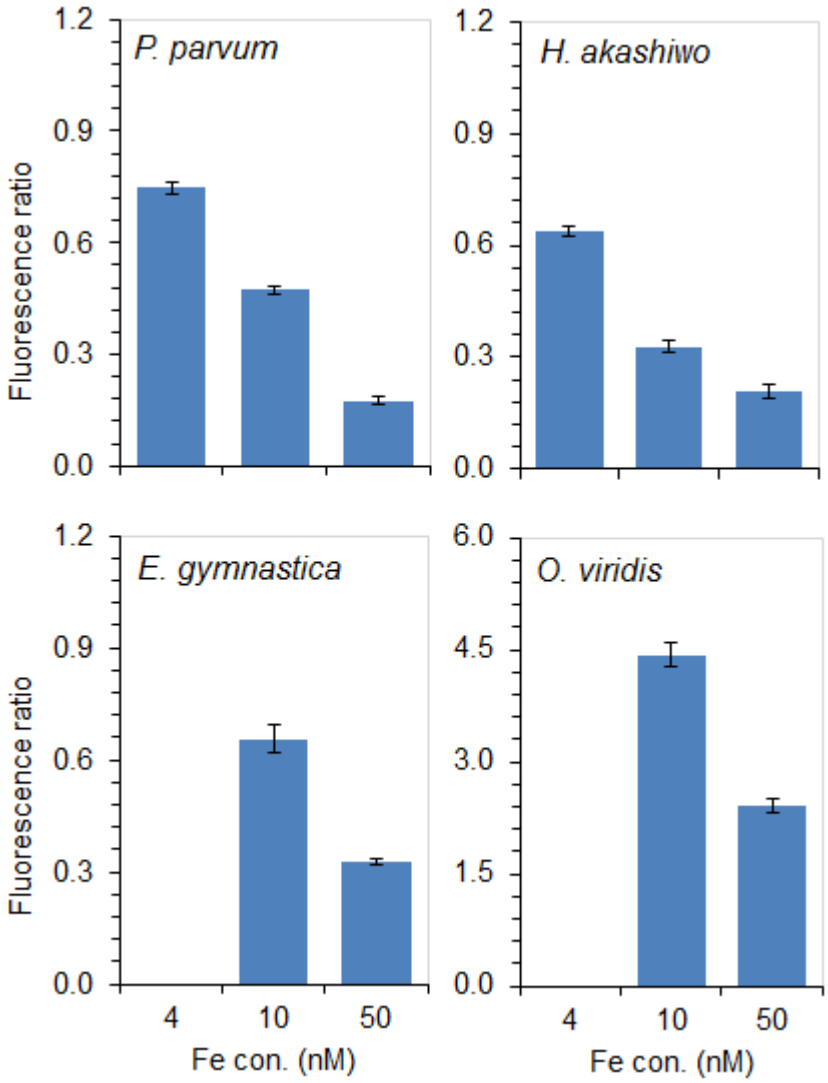

Fig. 5: Ratio of fluorescence intensities of HPLC chromatogram peaks E and A ($I_{E/A}$) according to Fe concentrations in the culture medium. Data are mean ± SD (n = 3).