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Title

Seasonal phosphorus depletion and microbial responses to the change in phosphorus availability in a subarctic coastal environment

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ABSTRACT

Temporal variations of phosphorus (P) pools (soluble reactive P (SRP), dissolved organic P (DOP), and particulate P (PP)), alkaline phosphatase activity (APA), and orthophosphate uptake were measured in Funka Bay to describe the annual P cycle in the bay and determine the phytoplankton and bacterial responses to the change in P availability. Observations over four years demonstrated that SRP concentrations in the surface waters decreased from the winter maximum of $1.0 \mu\text{mol L}^{-1}$ to $0.3 \mu\text{mol L}^{-1}$ at the end of the spring phytoplankton bloom, and then to under the detection limit by around August. The DOP concentration was relatively constant at $0.1 \mu\text{mol L}^{-1}$ throughout the year with higher concentrations up to $0.3 \mu\text{mol L}^{-1}$ just after the peak of the spring bloom. PP concentrations varied around $0.1 \mu\text{mol L}^{-1}$ with higher concentrations up to $0.6 \mu\text{mol L}^{-1}$ during the high chlorophyll *a* concentration period. SRP comprised 85% of the total P pool prior to the spring bloom, but the contribution decreased to less than 30% in the post-bloom period and DOP dominated the P pool in the surface. Most of the measured APA was less than $0.1 \text{ nmol L}^{-1} \text{ min}^{-1}$ in the whole water column, but exceptionally high values up to $1.4 \text{ nmol L}^{-1} \text{ min}^{-1}$ were observed in low SRP surface waters during summer. When SRP decreased from $0.3 \mu\text{mol L}^{-1}$ in spring to under the detection limit in summer, turnover time for orthophosphate at 10 m depth decreased from 61 days to 4.2 hours. The high APA and short turnover time for orthophosphate suggested that microbes in the surface mixed layer experienced severe P stress in summer. Under severely P stressed conditions, APA was detected mostly in the $> 10 \mu\text{m}$ fraction, suggesting that large phytoplankton would acquire P from DOP to overcome the P stress. This was supported by the result that large phytoplankton were outcompeted by the $0.2\text{--}1 \mu\text{m}$ sized microbes for orthophosphate uptake.

KEY WORDS: Phosphorus; Orthophosphate; Phosphatase; Phytoplankton; Bacteria; Coastal zone

1. Introduction

Phosphorus (P) is a key element in the regulation of the ecosystem structure and function in the ocean. Either P or nitrogen (N) controls primary production depending on the site-specific characteristics and time scale considered (Smith, 1984; Tyrrell, 1999). In particular, the P cycle is dynamic in coastal sea areas due to high nutrient input and high biological productivity. P is introduced to the coastal surface layer with other nutrients through many pathways, but when N input surpasses P input relative to the N:P consumption ratio within a system, orthophosphate (PO_4), the most accessible P form for microbes (phytoplankton and bacteria) typically becomes depleted. In fact, external nutrient input through rivers (Horne and Goldman, 1994), groundwater (Greenaway and Gordon-Smith, 2006; Slomp and Van Cappellen, 2004), atmospheric precipitation (Herut et al., 1999; Zhang et al., 2007) and dust particles (Chen et al., 2007; Zhang et al., 2007) show a much higher N:P ratio than the average N:P consumption ratio of 16 (molar) by planktonic microbes (Redfield et al., 1963). As a result, seasonal depletion of the P standing stock is observed commonly in estuarine and coastal oceans of the world (Howarth and Marino, 2006). In some environments, nitrogen fixation by cyanobacteria and subsequent supply of their reduced N into the water also lowers in situ PO_4 availability (Nausch et al., 2004). Under low PO_4 availability conditions, microbes will acquire P from dissolved organic P (DOP) via ectoenzyme activity (Björkman and Karl, 2003; Nausch and Nausch, 2007). Increase in the excess N input worldwide is likely to continue, leading to the potential P limitation in these ecosystems, and thus understanding the P cycle will become more important (Dyhrman et al., 2007).

Under certain P depleted environments, the degree of P deficiency for ambient microbes is a matter of concern. P nutritional status in planktonic microbes has been examined using several indicators. Occurrence of alkaline phosphatase (APase) is commonly used as a sensitive indicator of inorganic P stress of ambient microbes (Jansson et al., 1988). APase is the enzyme which catalyzes the hydrolysis of phosphomonoesters. Planktonic microbes synthesize the enzyme on the outer membrane of the cell under P depleted conditions for utilizing ambient DOP (Jansson et al., 1988). Although it is difficult to determine whether the low concentration of P limits the growth, the induction of APase activity (APA) indicates that their growth is stressed by low PO_4 availability (Duhamel et al., 2010). The early work by Perry (1972) on APA in marine environments examined P stressed phytoplankton growth in the subtropical North Pacific. PO_4 turnover time also is a sensitive

indicator of P stress with a shorter turnover time under more severely P stressed conditions. In a highly P stressed marine system, PO₄ turnover time is reported as several hours or less (Sylvan et al., 2006; Thingstad et al., 1998). Tanaka et al. (2006) proposed that biomass specific affinity for PO₄ uptake and biomass specific APA are useful for examining P availability for different microbial components.

However, differences in sensitivity to low P availability among planktonic microbes are largely unknown. Bulk measurements of APA and PO₄ turnover time provide information on the overall microbial assemblages, not on individual members. A qualitative technique for measuring APase in individual cells under microscopy has been developed (González-Gil et al., 1998). Differences in the mechanism of APase synthesis among phytoplankton groups and species have been shown using this technique (Dyhrman and Ruttenberg, 2006; González-Gil et al., 1998; Nicholson et al., 2006). Another technique would be size fractionation using several different pore size filters. Filtration treatment may cause serious artifacts such as cell lysis (Duhamel et al., 2010) and flocculation (Logan et al., 1994), but such artifacts could be minimized by care being taken during the analytical procedure. In a strict sense, particle size may not necessarily correspond exactly to the organism's size, for example, particle-associated bacteria. It is expected that different size fractions would show different responses to the change in P availability because various physiological and ecological processes and functions in planktonic ecosystems are size dependent (Friebele et al., 1978; Timmermans et al., 2004; Wen et al., 1997). Size fractionation studies have been actively carried out for freshwater ecosystems (e.g. Bentzen et al., 1992). Such fractionation studies have also been carried out for marine environments, but are still limited. The pioneering work of Faust and Correll (1976) demonstrated the characteristic dynamics of PO₄ uptake among microbes with different cell size using a size fractionation technique. Regardless of the interest in P uptake behavior, concurrent measurements of size fractionated PO₄ uptake and APA are surprisingly scarce in marine systems.

Funka Bay, a subarctic coastal area of Japan is characterized by a massive spring phytoplankton bloom with a chlorophyll *a* (Chl *a*) concentration of more than 20 µg L⁻¹, which occurs between March and April (Kudo and Matsunaga, 1999). The bloom is mainly composed of chain-forming diatoms *Thalassiosira* spp. and *Chaetoceros* spp. (Shinada et al., 1999). The bloom is terminated by nitrate (NO₃) depletion, and then silicic acid (Si(OH)₄) also is depleted during the bloom decline (Kudo et al., 2000). The post-bloom season (late spring to summer) is characterized by dissolved inorganic N (DIN) and Si(OH)₄ depleted

conditions in the surface waters, while active nutrient regeneration occurs in the bottom water (Kudo et al., 2007). Nutrients accumulated in the bottom layer during summer are returned to the surface by vertical water mixing in winter. Seawater temperature, salinity, and nutrients are vertically homogeneous throughout the water column around February (Lee et al., 2002). Then, the spring diatom bloom occurs in the high nutrient waters under the condition of increasing solar radiation and water column stability (Kudo and Matsunaga, 1999). Since the spring diatom bloom has a significant impact on the biogeochemistry in Funka Bay, previous studies have mainly focused on this event. In contrast, the post-bloom period is represented by low nutrient and low phytoplankton biomass conditions. The planktonic microbial community is dominated by pico-sized ($< 2 \mu\text{m}$) cyanobacteria and heterotrophic bacteria (Ban, 2000; Lee et al., 2001b) and large-sized phytoplankton are scarce (Ban, 2000). During this period, surface waters inside the bay are considered to be a semi-enclosed system as a result of the stable clockwise circulation, restricting the exchange of the surface water in the bay with that outside of the bay (Isoda et al., 2000). The freshwater supply from rivers and precipitation tends to accumulate in the bay, resulting in a lowering of the surface salinity (Hasegawa and Isoda, 1997). Nutrient input with high inorganic N:P from these sources can affect the P cycle in the bay especially during summer (Yoshimura and Kudo, 2003). However, less attention has been paid to the dynamics of P in Funka Bay.

To illustrate the P dynamics in Funka Bay, we observed the seasonal variation of the various P pools with other nutrients and Chl *a* during 1997 to 2000. Responses of planktonic microbes to the change in P availability were examined by assessing APA and PO_4 uptake as well as primary production, focusing on the post spring bloom period with temporarily decreasing P availability under near steady state chemical and biological conditions. Size fractionation techniques were used to determine P acquisition strategy of microbes with different sizes.

2. Materials and methods

2.1. Study site

Funka Bay is located in southwestern Hokkaido, northern Japan (Fig. 1). The bay has an area of $2.08 \times 10^9 \text{ m}^2$, a volume of $1.23 \times 10^{11} \text{ m}^3$, and mean and maximum depth of 59 m and 100 m, respectively. The bay has a caldera-like morphology and is open to the Pacific

Ocean.

2.2. Field samplings

Sampling was conducted at station 30 with a 92 m depth (42.27°N, 140.6°E) during 36 cruises onboard the R/V *Ushio-Maru* and T/S *Oshoro-Maru* from April 1997 to October 2000 (Fig. 1). Seawater samples for P pools, nutrients, and Chl *a* analyses were collected vertically (typically from 0, 5, 10, 20, 30, 40, 50, 60, 70, and 80 m depth) during the whole study period with 5 L Niskin bottles suspended on a stainless wire. Particulate P (PP) was measured for the samples obtained during June 1997 through November 1999. Seawater samples for APA were collected at 10 m depth in August 1998 and April 1999, and vertically during May 1999 through October 2000. Samples for PO₄ uptake, and primary production measurements were collected at 10 m depth on four occasions in August 1998 and April through June 1999 with an acid-washed lever action Niskin bottle suspended on a Kevlar wire. Subsamples were transferred to acid-washed polyethylene bottles. The samples were kept in a cooler box for no more than 12 h until being processed in the onshore laboratory. Salinity and temperature were recorded by a CTD profiler SBE 19 (SeaBird) aboard the *Ushio-Maru* or Mark IIIB (Neil Brown) aboard the *Oshoro-Maru*. Photosynthetically active radiation (PAR) was measured vertically with an integrating quantum photometer (LI-188B, LI-COR).

2.3. Chemical analyses and bioassays

Samples for dissolved nutrients were filtered through a Millipore HA filter (0.45 µm pore size). Care, such as gentle vacuum for filtration (< 0.01 MPa) and pre-washing of the filter with sample water, were carried out to avoid any alterations of the nature of dissolved nutrients. Soluble reactive P (SRP) concentration was measured manually by the molybdenum blue method (Parsons et al., 1984) using a spectrophotometer (U-2001, Hitachi) with a 5 cm optical path length glass cell. Note that, in this paper, we differentiated the term SRP and PO₄ since SRP is not necessarily identical to biologically accessible PO₄ (Hudson et al., 2000). Total dissolved P (TDP) concentration was measured as SRP after persulfate oxidation (Menzel and Corwin, 1965). Unfiltered seawater was used for total P (TP) analysis with the same procedure of TDP. Although the persulfate oxidation method may not degrade some organic P compounds completely, it yields comparable results for natural water samples compared to other methods with a stronger degradation ability (Monaghan and Ruttenberg, 1999). Thus our TDP and TP analyses provide suitable results to show the changes in organic

P pools. Particulate P (PP) and soluble non-reactive P (SNP) concentrations were obtained by subtracting TDP from TP and SRP from TDP, respectively. Here, SNP is assumed to correspond to DOP. The detection limit, given as three times the standard deviation from ten blank measurements using Milli-Q water, for P analyses was $0.01 \mu\text{mol L}^{-1}$. The precision for TP, TDP, and SRP analysis was $\pm 0.01 \mu\text{mol L}^{-1}$ ($n = 5$), and thus, taking into account error propagation, the precisions of the PP and DOP concentrations were $\pm 0.02 \mu\text{mol L}^{-1}$. Nitrate plus nitrite (N+N) and Si(OH)_4 concentrations were measured with an air-segmented continuous flow system (Autoanalyzer II, Technicon). The coefficient of variation (CV) for N+N and Si(OH)_4 analyses were 0.4% (at a level of $4 \mu\text{mol L}^{-1}$) and 0.2% ($25 \mu\text{mol L}^{-1}$), respectively ($n = 5$).

For size fractionations of planktonic microbes, polycarbonate filters (Nuclepore) with 0.2, 1, and $10 \mu\text{m}$ pore-size were used. However $2 \mu\text{m}$ pore-size filters were used in place of $1 \mu\text{m}$ pore-size filters in August 1998. Chl *a* concentration, PO_4 uptake, and primary production were measured on the filter, while APA was measured in the filtrate. APA in $0.2 \mu\text{m}$ filtrate was defined as dissolved APA. Size fractionation bioassays were conducted only in the conditions of relatively low SRP concentration compared to the half saturation constant for PO_4 uptake of $0.02\text{--}0.5 \mu\text{mol L}^{-1}$ (Lalli and Parsons, 1997). For this purpose, size fractionations were conducted on four occasions during the post bloom period in August 1998 and April through June 1999 only for the 10 m depth samples as the representative of the surface mixed layer waters because the surface mixed layer was $30\text{--}40 \text{ m}$ depth during late spring to summer (Kudo et al., 2007; Lee et al., 2002). Additionally, APA size fractionation was conducted in August 1999.

For Chl *a* measurements, aliquots of $100\text{--}300 \text{ mL}$ of subsamples were filtered onto a Millipore HA filter or Nuclepore filters. The filters were soaked in *N,N*-dimethylformamide over 24 h in the dark at 5°C (Suzuki and Ishimaru, 1990) and the extracted Chl *a* was measured fluorometrically (Parsons et al., 1984) with a spectrofluorometer (F-2000, Hitachi). The precision for Chl *a* analysis was $\pm 2.6\%$ at $10 \mu\text{g L}^{-1}$ ($n = 5$).

APA was measured fluorometrically based on the hydrolysis of 4-methylumbelliferyl phosphate (MUF-P) (Pettersson, 1980). Ten mL of 0.1 mmol L^{-1} MUF-P (Sigma) in 0.1 mol L^{-1} tris-HCl buffer of pH 8.3 was added to each 80 mL sample and incubated at 20°C in the dark. Final concentration of MUF-P in samples was $11 \mu\text{mol L}^{-1}$. Milli-Q water was used for the control sample to subtract the background activity. Fluorescence was read initially and at

least another three data points at 10–20 min intervals within 1 hour with 360 nm excitation and 450 nm emission using a spectrofluorometer (F-2000, Hitachi). APA in $\text{nmol L}^{-1} \text{ min}^{-1}$ was estimated from the slope of fluorescence vs. time. The CV was 3.0% at a level of $0.44 \text{ nmol L}^{-1} \text{ min}^{-1}$ ($n = 5$) and detection limit was $0.01 \text{ nmol L}^{-1} \text{ min}^{-1}$. The substrate concentration is assumed to be at the saturation level for APA in a broad range of marine environments (Hoppe, 1983; Ivančić et al., 2009). Therefore the APA in the present study represents maximal potential hydrolysis rate.

Kinetics of PO_4 uptake was determined using carrier-free $\text{Na}_2\text{H}^{32}\text{PO}_4$. An aliquot of $^{32}\text{PO}_4$ was added to 500 mL seawater, incubated at the in situ water temperature and PAR, to give a total activity of $20,000 \text{ cpm mL}^{-1}$. At several minute intervals, aliquots (3–5 mL) were taken and filtered through Nuclepore filters. To minimize background counts, the filters were presoaked with 0.1 mmol L^{-1} non-radioactive KH_2PO_4 solution. After filtration, filters were rinsed immediately with freshly filtered seawater and then counted in a liquid scintillation counter (LS6000TA, Beckman). Abiotic controls killed with formaldehyde were run in the same manner. Rate constant for PO_4 uptake (k) was estimated from the equation (Lean and White, 1983):

$$k = \ln(100 - \text{P-}^{32}\text{P}_t)/t \quad (1)$$

where t is the time and $\text{P-}^{32}\text{P}_t$ is the percent ^{32}P incorporated into the particulate fraction at time t . The reciprocal of k was used to estimate the turnover time for PO_4 , which is the time required for an amount of PO_4 equivalent to the ambient pool to be taken up by the planktonic microbes and the same amount to be released into solution (Bentzen and Taylor, 1991).

Primary production was measured with a ^{14}C technique (Parsons et al., 1984). In the laboratory, samples in two light bottles and one dark bottle were spiked with 92.5 KBq ($2.5 \mu\text{Ci}$) of $\text{NaH}^{14}\text{CO}_3$ and incubated for 3 h at the in situ water temperature and PAR. After the incubation period, samples were filtered onto Nuclepore filters. Incorporated ^{14}C was determined using a liquid scintillation counter (LS6000TA, Beckman).

3. Results

3.1. Seasonal variations of chlorophyll a, nutrients, and phosphorus pools

Spring phytoplankton blooms occurred during February to April every year (Figs. 2c and 3a). The maximum values of Chl *a* concentration observed in the present study varied interannually from ~ 3 to $> 20 \mu\text{g L}^{-1}$. Due to the monthly intervals between our observations, we could not necessarily observe the peak of the spring phytoplankton blooms. In October to November, a smaller intensity phytoplankton bloom than the spring bloom was observed.

The spring blooms had large impacts on the nutrient dynamics. Nutrient concentrations were high and uniform throughout the water column prior to the spring blooms (Figs. 2d–f). This is due to vertical water mixing induced by surface water cooling which develops from autumn through winter (Figs. 2a and b) and supplies nutrient rich water accumulated in bottom layer during the previous summer to the whole water column (Figs. 2d–f). The concentration of N+N increased to $> 10 \mu\text{mol L}^{-1}$ in winter and dropped to $< 0.1 \mu\text{mol L}^{-1}$ during the spring blooms (Figs. 2d and 3c). Si(OH)_4 concentration was $> 15 \mu\text{mol L}^{-1}$ in winter with an interannual variability, and decreased to $\sim 3 \mu\text{mol L}^{-1}$ during the spring bloom and continued to decrease even after the termination of the bloom due to the depletion of N+N (Figs. 2e and 3d), as reported in Kudo et al. (2000). SRP concentration was around $1.0 \mu\text{mol L}^{-1}$ prior to the spring blooms (Figs. 2f and 3e). By the end of the spring bloom, SRP concentration decreased to typically $0.3 \mu\text{mol L}^{-1}$. DOP concentration was relatively constant at $0.10\text{--}0.13 \mu\text{mol L}^{-1}$ throughout the year, but a higher concentration up to $0.3 \mu\text{mol L}^{-1}$ was observed just after the peak of the spring bloom (Figs. 2g and 3f). Distribution of PP (Figs. 2h and 3b) is well correlated with Chl *a* (e.g. at 10 m depth: $r = 0.84$, $n = 24$, $p < 0.0001$). The PP concentrations varied around $0.1 \mu\text{mol L}^{-1}$ but higher concentrations up to $0.63 \mu\text{mol L}^{-1}$ were observed during the high Chl *a* concentration period.

In the post-bloom seasons (April to September), relatively low Chl *a* (typically $< 0.5 \mu\text{g L}^{-1}$) and Si(OH)_4 concentrations (typically $< 5 \mu\text{mol L}^{-1}$), and a depleted N+N condition ($< 0.1 \mu\text{mol L}^{-1}$) were maintained at the surface in each year (Figs. 2c, and 3a, c, and d). On the other hand, SRP concentration continued to decrease from $0.3 \mu\text{mol L}^{-1}$ to under the detection limit (UDL; $< 0.01 \mu\text{mol L}^{-1}$) toward late summer (Figs. 2f and 3e). The depletion of SRP was observed from the surface to 20 m depth layer during late summer (Fig. 2f).

Our data set on the three P pools showed characteristic patterns of seasonal variation in each P pool at the surface (Fig. 4). In winter, seasonal vertical water mixing increased the surface SRP concentration to ca. $1 \mu\text{mol L}^{-1}$ and the SRP comprised 85% of the total P pool. The rest was distributed equally into DOP and PP. During the spring bloom, rapid utilization

of SRP by phytoplankton depressed the proportion of SRP to around 30% and increased that of PP and then DOP. In summer, the proportion of SRP was 0–20%, while DOP dominated the P pool (30–60%) in the surface layer, typically shallower than 30 m.

3.2. Dynamics of alkaline phosphatase activity

The annual cycle of vertical profiles of APA in Funka Bay (Fig. 2i) showed that in most of the samples APA was lower than $0.1 \text{ nmol L}^{-1} \text{ min}^{-1}$. High APA was observed only in the surface waters during summer. The highest value of $1.36 \text{ nmol L}^{-1} \text{ min}^{-1}$ was observed at 10 m depth in August 1999, where SRP concentration was at $0.04 \text{ } \mu\text{mol L}^{-1}$. Temporal and spatial distributions of Chl *a*-specific APA (SAPA; UDL– $3.91 \text{ nmol } \mu\text{g Chl } a^{-1} \text{ min}^{-1}$) corresponded to those of APA (Figs. 2i and j). Some data points of SAPA over $0.5 \text{ nmol } \mu\text{g Chl } a^{-1} \text{ min}^{-1}$ were obtained in the deeper layers ($> 70 \text{ m}$) where measured APA was low due to the very low Chl *a* concentrations. In the upper 50 m, high APA and SAPA were observed only in the SRP depleted water, but APA and SAPA were not necessarily high in water with a low SRP concentration (Fig. 5).

3.3. Size fractionations

Size fractionated Chl *a*, primary production, PO_4 uptake, and APA at 10 m were comparable at four occasions covering SRP depleted (August) and SRP available (April–June) conditions (Fig. 6 and Table 1). In all the size fractionated samples, total Chl *a* concentrations were relatively constant at $0.37\text{--}0.56 \text{ } \mu\text{g L}^{-1}$ and the smallest size fraction ($0.2\text{--}1 \text{ } \mu\text{m}$) dominated more than half of the total Chl *a*, while the $> 10 \text{ } \mu\text{m}$ fraction was less than 14% (Figs. 6a and b). Primary production was higher at $5.6 \text{ } \mu\text{g C L}^{-1} \text{ h}^{-1}$ in August than April–June which ranged $0.9\text{--}1.3 \text{ } \mu\text{g C L}^{-1} \text{ h}^{-1}$. When the size distributions of primary production and Chl *a* were compared, the $1\text{--}10 \text{ } \mu\text{m}$ fraction in April showed a higher proportion for primary production (61%) than for Chl *a* (24%), and the $> 10 \text{ } \mu\text{m}$ fraction in June showed a higher contribution for primary production (43%) than for Chl *a* (6%) (Figs. 6c and d).

PO_4 turnover time was 61 days in conditions of relatively high SRP concentrations, while it was 4.2 h under SRP depleted condition (Table 1). PO_4 uptake rate constant was high at 5.7 day^{-1} in August 1998, while in 1999 increased from 0.017 day^{-1} in April to 0.11 day^{-1} in June. PO_4 uptake was dominated by the $0.2\text{--}1 \text{ } \mu\text{m}$ fraction with more than 90% in August 1998 (Figs. 6e and f). From April to June 1999, PO_4 uptake was distributed equally for the $0.2\text{--}1 \text{ } \mu\text{m}$ and $1\text{--}10 \text{ } \mu\text{m}$ fractions and the $> 10 \text{ } \mu\text{m}$ fraction was still minor. On the other hand,

APA was highly dominated by the $> 10 \mu\text{m}$ fraction with 75% of the total activity of $0.14 \text{ nmol L}^{-1} \text{ min}^{-1}$ in August 1998 (Figs. 6g and h). The values of APA were near or UDL from April to June, and therefore size fractionations of APA were not conducted. Similar to August 1998, 76% of particle-associated APA was also detected in $> 10 \mu\text{m}$ fraction in August 1999 (Fig. 6h). Size distributions in PO_4 uptake showed agreement with that in primary production. In August 1998, contribution of the $> 10 \mu\text{m}$ fraction was negligible in primary production and contributed only 2% in PO_4 uptake, but the $> 10 \mu\text{m}$ fraction dominated APA. Size fractionated SAPA (Table 1) showed two orders of magnitude difference between fractions in August 1998; SAPA was $0.07 \text{ nmol } \mu\text{g Chl } a^{-1} \text{ min}^{-1}$ for the $0.2\text{--}1 \mu\text{m}$ and $1\text{--}10 \mu\text{m}$ fractions, whereas $2.5 \text{ nmol } \mu\text{g Chl } a^{-1} \text{ min}^{-1}$ for the $> 10 \mu\text{m}$ fraction. In August 1999, SAPA in the $> 10 \mu\text{m}$ fraction was more than two orders of magnitude higher than in the $0.2\text{--}1 \mu\text{m}$ fraction, while 2.5 times higher than in the $1\text{--}10 \mu\text{m}$ fraction (Table 1).

4. Discussion

Multiyear observations confirmed that N seemed to control microbial community growth in Funka Bay at least during the spring phytoplankton bloom period as also shown in Kudo et al. (2000). Si(OH)_4 also was depleted in the bloom but thereafter elevated sporadically due to riverine input (Yoshimura and Kudo, 2003), so Si(OH)_4 would control the diatom abundance in the post-bloom period. Our finding of SRP depletion under very low DIN and Si(OH)_4 availability is noteworthy to understand the coastal nutrient cycle. Clarification of which element limits the biomass or physiological activity of microbes is complex as discussed in Tanaka et al. (2011). We focused on P dynamics in the post-bloom period, and discuss the differences in response among size fractions of the microbial community to the changing P environment.

4.1. Factors leading to SRP depletion in the summer surface water

The present study demonstrated that the SRP concentration in surface waters gradually decreased to UDL during summer in Funka Bay. During the SRP decreasing post-bloom period, stratification of the upper ca. 30 m depth (Fig. 2) restricted the nutrient input to the surface from the deep nutrient rich layer. Since the decrease in SRP concentration in the surface waters occurred under N+N depleted conditions, external nutrient input and consumption of other forms of N in the surface water were necessary for this SRP

consumption.

Freshwater nutrient input was speculated because the decrease in SRP concentration coincided with the decrease in salinity (Fig. 7). The rivers flowing into Funka Bay have a high concentration of nutrients (N+N of $24 \mu\text{mol L}^{-1}$; NH_4 of $2.9 \mu\text{mol L}^{-1}$; SRP of $0.30 \mu\text{mol L}^{-1}$; Si(OH)_4 of $270 \mu\text{mol L}^{-1}$; all in volume-weighted mean values) with an inorganic N:P ratio of 90 (Yoshimura and Kudo, 2003). Precipitation collected in Hakodate (30 km south of Funka Bay) (Fig. 1) contained high amounts of nutrients (N+N of $17 \mu\text{mol L}^{-1}$; NH_4 of $35 \mu\text{mol L}^{-1}$; SRP of $0.28 \mu\text{mol L}^{-1}$; Si(OH)_4 of $0.2 \mu\text{mol L}^{-1}$; all in volume-weighted mean values) with an extremely high inorganic N:P ratio of 190, and these excess N inputs from the two sources accounted for $> 40\%$ of the decrease in SRP standing stock if N and P were consumed at a ratio of 16 (Kudo, unpubl. data). Dry deposition and groundwater also are potential nutrients sources with high N:P, although the data are not available for the bay. Earlier studies in other coastal systems indicate that P depletion is induced in the period with high river discharge with high N:P nutrient input (Pennock and Sharp, 1994; Miao et al., 2006; Sylvan et al., 2006). Unlike other studies, depletion of SRP occurred in summer, in conditions of minimal river discharge into Funka Bay (Yoshimura and Kudo, 2003). River discharge shows its maximum in April, originating from the thaw in the mountain areas around Funka Bay. Funka Bay is a semi-enclosed system which is induced by a stable clockwise circulation (Isoda et al., 2000) and this physical forcing by maintaining the water mass within the bay enhances the influence of the external nutrient supply with high N:P on the SRP depletion in the summer surface waters.

Bioavailable N other than N+N in ambient water for reducing SRP concentration could be ammonium (NH_4) and dissolved organic N (DON). Decreases in the surface standing stock of NH_4 and urea were observed in the post-bloom period during the observations in 2000, and corresponding PO_4 consumption accounted for $> 40\%$ of the observed decrease in SRP standing stock if N and P were consumed at a ratio of 16 (Kudo, unpubl. data). Furthermore, DON other than urea also can be the N source to consume SRP, although the change in DON standing stock is unknown in the bay. These discussions revealed that the external nutrient input and in situ utilization of NH_4 and DON explain $> 80\%$ of the decrease in SRP standing stock.

The depletion of SRP in summer surface waters may have appeared in recent decades. Previous studies in the 1970's did not mention about the SRP depletion in summer

surface waters. Yanada et al. (1976) reported the minimum surface SRP concentration at Stn 30 to be $0.20 \mu\text{mol L}^{-1}$ in 1974. Yoshimura et al. (2000) demonstrated that riverine nutrient input doubled DIN while SRP remained constant from the 1970's to 1990's, close to the watershed of rivers flowing into Funka Bay. If this is also the case in Funka Bay, the increase of N:P in nutrient input could have led to the SRP depletion in recent years, although other factors should also be considered to identify the cause of the recent SRP depletion. Published data on surface SRP concentration and riverine nutrient input in the last few decades are limited, so further data are required to evaluate human impacts on nutrient dynamics in Funka Bay. Since the increase in DIN input is a general feature (Galloway et al., 2004), P availability could have decreased historically in worldwide coastal environments. Further, since atmospheric DIN depositions also have increased through the last century (Bowen and Valiela, 2001), qualitatively the same phenomena, as experienced in Funka Bay, could occur in summer stratified surface waters even in the open ocean. In fact, a shift of the system from N to P limitation was reported in the subtropical North Pacific and this is caused by increased N_2 fixation which is a biologically mediated external N input to the system (Karl et al., 1997). Temporal and spatial expansion of P depletion in coastal and open ocean areas requires a better understanding of the P biogeochemical cycle.

4.2. Change in the degree of P stress ~~in Funka Bay~~

APA and PO_4 turnover rates measured in this study provide insights into P nutritional status of the in situ plankton community during decreasing SRP availability in the surface mixed layer. One of the issues for comparing the degree of P stress is how to standardize the APA values for biomass of microbes. APA normalized to Chl *a* (i.e. SAPA) is now used extensively. However, APase can be synthesized not only by phytoplankton but also by bacteria. Healey and Hendzel (1980) standardized APA by ATP because ATP is a useful index for all living biomass. Tanaka et al. (2006) used microscopically estimated PP to show biomass specific APA. Duhamel et al. (2010) showed cell abundance specific APA in picoplankton dominated systems. Note, however, that APA should be normalized by Chl *a* in the $> 1 \mu\text{m}$ size fraction, since free living bacteria fall in the size range of $< 1 \mu\text{m}$ (Robinson, 2008). Another issue is that a large fraction of the total APA is often observed in the dissolved fraction. For example, around 80% of APA was found in the dissolved fraction in the Gulf of Aqaba and Red Sea (Li et al., 1998). Multiple size fractionations can resolve this problem and furthermore, we can estimate SAPA for each of the fractions using size fractionated Chl *a* and APA. Size fractionated SAPA showed contrasting responses to reduced P availability among

different size fractions (Table 1). Bulk SAPA showed a difference of an order of magnitude between August 1998 and August 1999, and we can comprehend this difference to have originated from the large difference in the activity of the 1–10 μm fraction, although the underlying mechanisms are unknown. Thus, size fractionation can be a powerful tool for understanding the P nutrition among the microbial community.

From the results of SAPA in the present study, the microbial assemblage would not necessarily experience P stressed growth even under SRP depleted conditions (Fig. 5). Although our detection limit of SRP measurement was much higher than that of a high sensitivity method such as the one of Karl and Tien (1992), 0.01 $\mu\text{mol L}^{-1}$ would be near the range in which planktonic microbes are considered to be stressed (Lalli and Parsons, 1997). Low SAPA regardless of a depleted SRP condition could be explained by acquiring fast recycling of bioavailable P to satisfy the microbial demand. Zooplankton mediated P release into the water should have a central role in rapid nutrient regeneration. Phytoplankton biomass was dominated by the 0.2–1 μm fraction in the summer seasons (Fig. 6A and B), so the energy and materials flowed via the microbial loop. Rapid regeneration of materials in the microbial loop is well known in aquatic environments (e.g. Fogg, 1995). Another explanation also is possible if a certain amount of P is stored intracellularly possibly just after a sporadic supply of P. Litchman and Nguyen (2008) revealed that APA strongly correlated with cellular P rather than external P concentrations. Therefore, the variation of SAPA could be an indicator of the status of intracellular P conditions, and thus the phytoplankton P nutritional status regardless of ambient SRP concentration.

4.3. Microbial responses to the decrease in SRP concentration

The present study demonstrated that large phytoplankton were more sensitive to the decrease in SRP availability than smaller plankton in Funka Bay, judging from size fractionated SAPA. If we consider the ecological significance of the APase synthesis, large phytoplankton would acquire P from DOP to overcome the P stress under low SRP availability conditions. This was supported by the result that the $> 10 \mu\text{m}$ fraction was outcompeted for PO_4 uptake by smaller plankton. Large phytoplankton were unsuccessful in PO_4 uptake and deficient in their P demands and subsequently forced to synthesize APase under low SRP availability. Similar conclusions have been drawn in previous studies on freshwater ecosystems (e.g. Bentzen et al., 1992). This conclusion in the present study although very clear is based on only two APA and one PO_4 uptake data sets. However,

consistent results of large phytoplankton-dominated APA were obtained in two summers running (1998 and 1999). DOP has been suggested to be an important P resource for phytoplankton and bacteria under low SRP concentration in the oligotrophic open ocean (Björkman et al., 2000; Björkman and Karl, 2003; Suzumura and Ingall, 2004). Suzumura et al. (1998) reported that bulk DOP in coastal water contained easily hydrolyzable mono- and diesters, and utilization of this P resource is also likely to be the case in summer surface waters of Funka Bay. Studies in the Baltic Sea also indicate the importance of DOP in primary and bacterial production (Nausch and Nausch, 2006, 2007). The concentration, composition, and dynamics of DOP are key factors to support the production particularly of large phytoplankton in Funka Bay during summer.

For the marine environment, there are a few reports on concurrent measurements of size fractionated APA and PO_4 uptake. In the brackish central Baltic Sea, when P depletion occurs in summer, the $> 10 \mu\text{m}$ fraction, representing N_2 fixing cyanobacteria *Nodularia* and *Aphanizomenon*, accounted for only a few percent of the PO_4 uptake, while they comprised 42% of the total APA (Nausch et al., 2004). Under such conditions, 91% of the PO_4 uptake occurred in the $0.2\text{--}3 \mu\text{m}$ fraction, and the fraction contributed only 14.7% of the total APA (Nausch et al., 2004). Sohm and Capone (2006) drew a similar conclusion in the western North Atlantic that *Trichodesmium* spp. colonies can satisfy their P needs by utilizing DOP via APase, although other plankton assemblages outcompeted *Trichodesmium* spp. for PO_4 uptake. Unlike these studies, large phytoplankton assemblages of Funka Bay are composed of eukaryotic phytoplankton such as diatoms and flagellates (Ban, 2000) when high APA was observed. It has been already confirmed that a broad range of phytoplankton species, including diatoms, dinoflagellates, and prymnesiophytes, produce APase under P stressed conditions (González-Gil et al., 1998; Møller et al., 1975), our data demonstrated that larger eukaryotic phytoplankton have a central role in producing APase to overcome P stress.

In the present study, we cannot rule out the contribution of heterotrophic bacteria in PO_4 uptake in the $0.2\text{--}1 \mu\text{m}$ fraction, although 85% of the total Chl *a* was found in this fraction (Fig. 6B). Bacterial abundance and production during our size fractionation study were reported in Lee et al. (2001a, b). Furthermore, during the post-bloom period, the phytoplankton community was dominated by the $< 2 \mu\text{m}$ fraction and composed of an overwhelming proportion of cyanobacteria and a small proportion of eukaryotes (Lee et al., 2002). If bacterial C:P (molar) of 50 (Kirchman, 2000) and phytoplankton C:P of 106 (Redfield et al., 1963) are assumed, P requirements for bacteria and $0.2\text{--}1 \mu\text{m}$ sized

phytoplankton can be estimated from each C production as a similar range of 0.63–1.47 and 0.23–3.73 nmol P L⁻¹ hr⁻¹, respectively (Table 2). We cannot determine whether the 23% of bulk APA for the 0.2–1 µm fraction on 28 August 1998 (Figs. 6G and H) was derived from bacteria or phytoplankton or both of them, 0.2–1 µm sized microbes fulfilled a considerable part of the P requirement through the PO₄ uptake relative to large size fraction, because significantly low P stress was estimated from the size fractionated SAPA.

In conclusion, the strategy for P acquisition under P depleted conditions may be controlled primarily by the cell size (i.e., the surface area to the volume ratio), regardless of algal species or nutritional group (autotrophs or heterotrophs). Among phytoplankton, the cell size influences many aspects of nutrient acquisition (Litchman et al., 2007). However the role of bacteria in nutrient uptake behavior varies with DOC availability, as reported in the mesocosm experiment of Thingstad et al. (2008). In the case of Funka Bay, DOC availability from autochthonous and allochthonous sources would not limit bacterial production (Lee et al., 2001b), and hence bacteria could play a significant role in PO₄ uptake.

Contributors

All authors have approved the article for publication. Yoshimura and Kudo equally contributed on research design and management, field operations, data analysis and interpretation, manuscript drafting.

Disclosure Statement

The authors declare no actual or potential conflict of interest.

Role of the funding source

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Figure Legends

Fig. 1. Location of sampling station 30, Stn 30, in Funka Bay, Japan.

Fig. 2. Temporal variations in vertical distributions of (a) salinity, (b) water temperature, (c) chlorophyll *a*, (d) nitrate+nitrite (NO_3+NO_2), (e) silicic acid (f) soluble reactive P (SRP), (g) dissolved organic P (DOP), (h) particulate P (PP), (i) alkaline phosphatase activity (APA), and (j) Chl *a*-specific APA (SAPA) at station 30 in Funka Bay.

Fig. 3. Temporal variations at 10 m depth of (a) chlorophyll *a*, (b) particulate P (PP), (c) nitrate+nitrite (N+N), (d) silicic acid, (e) soluble reactive P (SRP), (f) dissolved organic P (DOP), (g) alkaline phosphatase activity (APA), and (h) Chl *a*-specific APA (SAPA) at station 30 in Funka Bay.

Fig. 4. Temporal variations at 10 m depth in proportions of particulate P (PP), dissolved organic P (DOP), and soluble reactive P (SRP) in the total P pool at station 30 in Funka Bay.

Fig. 5. Alkaline phosphatase activity (APA) and chlorophyll *a*-specific APA (SAPA) as a function of soluble reactive P (SRP) concentrations for samples taken from 0 to 50 m depth at station 30 in Funka Bay.

Fig. 6. Temporal variations and relative contribution in size fractionated (a) (b) chlorophyll *a*, (c) (d) primary production, (e) (f) orthophosphate uptake rate constant, and (g) (h) alkaline phosphatase activity (APA) at 10 m depth of station 30 in Funka Bay.

Fig. 7. Relationship between soluble reactive P (SRP) concentration and salinity in 0 to 50 m depth at station 30 in Funka Bay.

Tables

Table 1. Soluble reactive phosphorus (SRP) concentrations, chlorophyll *a* (Chl *a*) concentrations, orthophosphate turnover time (TT), alkaline phosphatase activity (APA), and Chl *a*-specific APA (SAPA) measured in 10 m depth waters at station 30 (Funka Bay) surface from August 1998 to August 2000. UDL, under detection limit: 0.01 $\mu\text{mol L}^{-1}$ for SRP; 0.01 $\text{nmol L}^{-1} \text{min}^{-1}$ for APA. NM, not measured.

Date	SRP ($\mu\text{mol L}^{-1}$)	Chl <i>a</i> ($\mu\text{g L}^{-1}$)	TT (h)	APA ($\text{nmol L}^{-1} \text{min}^{-1}$)	SAPA ($\text{nmol } \mu\text{g Chl } a^{-1} \text{min}^{-1}$)	SAPA ($\text{nmol } \mu\text{g Chl } a^{-1} \text{min}^{-1}$)		
						0.2–1 μm	1–10 μm	>10 μm
28 Aug 1998	UDL	0.56	4.2	0.14	0.24	0.066	0.068	2.5
20 Apr 1999	0.26	0.41	1454	0.02	0.05	UDL	UDL	UDL
18 May 1999	0.10	0.37	639	UDL	UDL	UDL	UDL	UDL
17 Jun 1999	0.05	0.45	212	0.02	0.03	UDL	UDL	UDL
26 Aug 1999	0.04	0.38	NM	1.16	3.07	UDL	1.5	3.8
27 Jul 2000	UDL	0.31	NM	0.45	1.45	NM	NM	NM
28 Aug 2000	UDL	0.46	NM	0.21	0.46	NM	NM	NM

Table 2. Comparison of abundance, carbon biomass, and production for bacteria and phytoplankton in 0.2–1 μm fraction with estimated phosphorus requirement. Phosphorus requirements were estimated from each C production, assuming bacterial C:P (molar) of 50 (Kirchman, 2000) and phytoplankton C:P of 106 (Redfield et al., 1963).

	Bacterial abundance* $10^6 \text{ cells mL}^{-1}$	Bacterial production* $\mu\text{g C L}^{-1} \text{ hr}^{-1}$	Primary production $\mu\text{g C L}^{-1} \text{ hr}^{-1}$	Estimated P requirement in bacteria $\text{nmol P L}^{-1} \text{ hr}^{-1}$	Estimated P requirement in phytoplankton $\text{nmol P L}^{-1} \text{ hr}^{-1}$
28 Aug 1998	1.10	0.63	4.75	1.05	3.73
20 Apr 1999	0.35	0.58	0.29	0.96	0.23
18 May 1999	0.66	0.38	0.79	0.63	0.62
17 Jun 1999	0.44	0.88	0.37	1.47	0.29

*Data reported in Lee et al. (2001a, b)

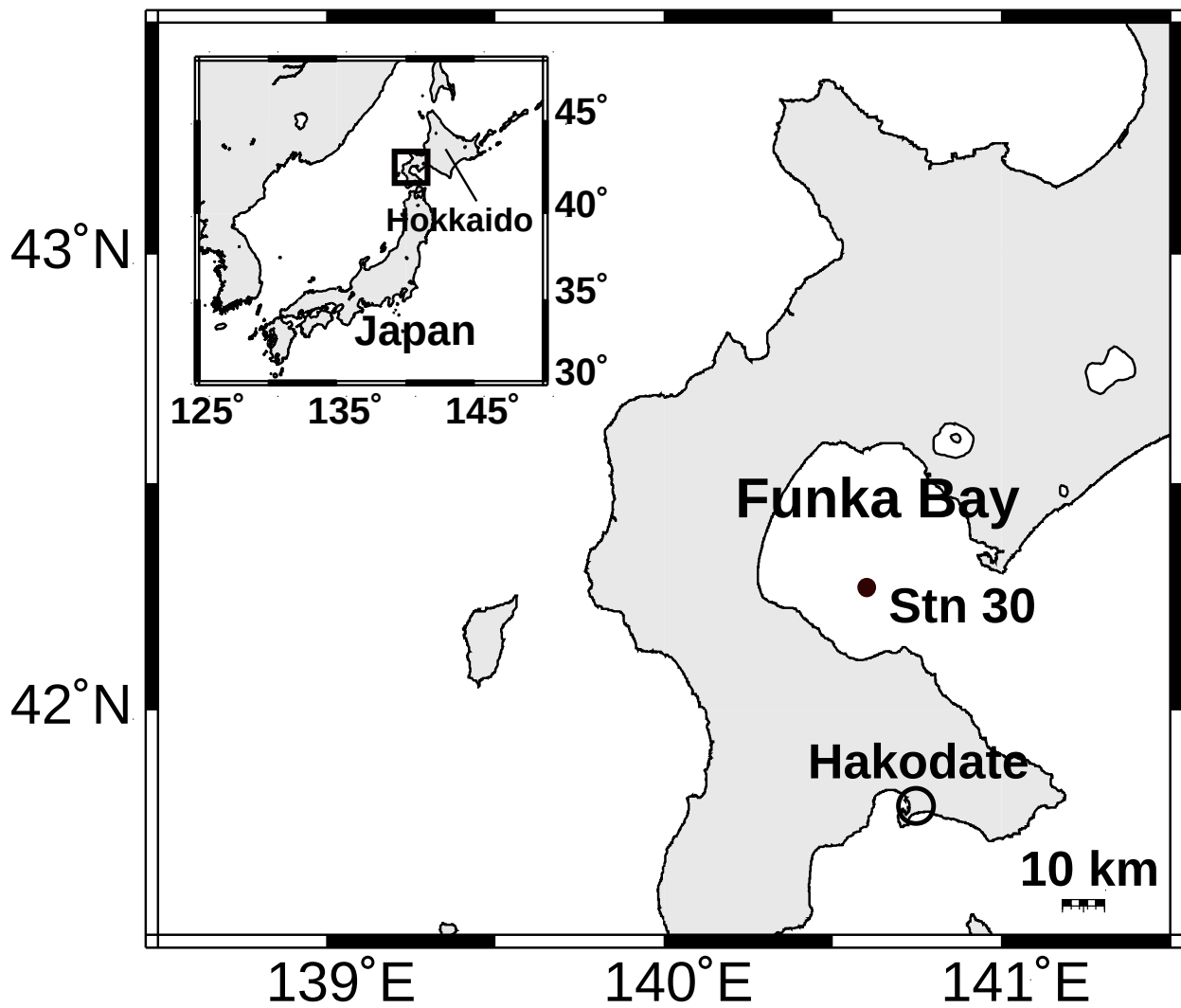


Fig. 1. Location of sampling station 30, Stn 30, in Funka Bay, Japan.

Yoshimura and Kudo Figure 2

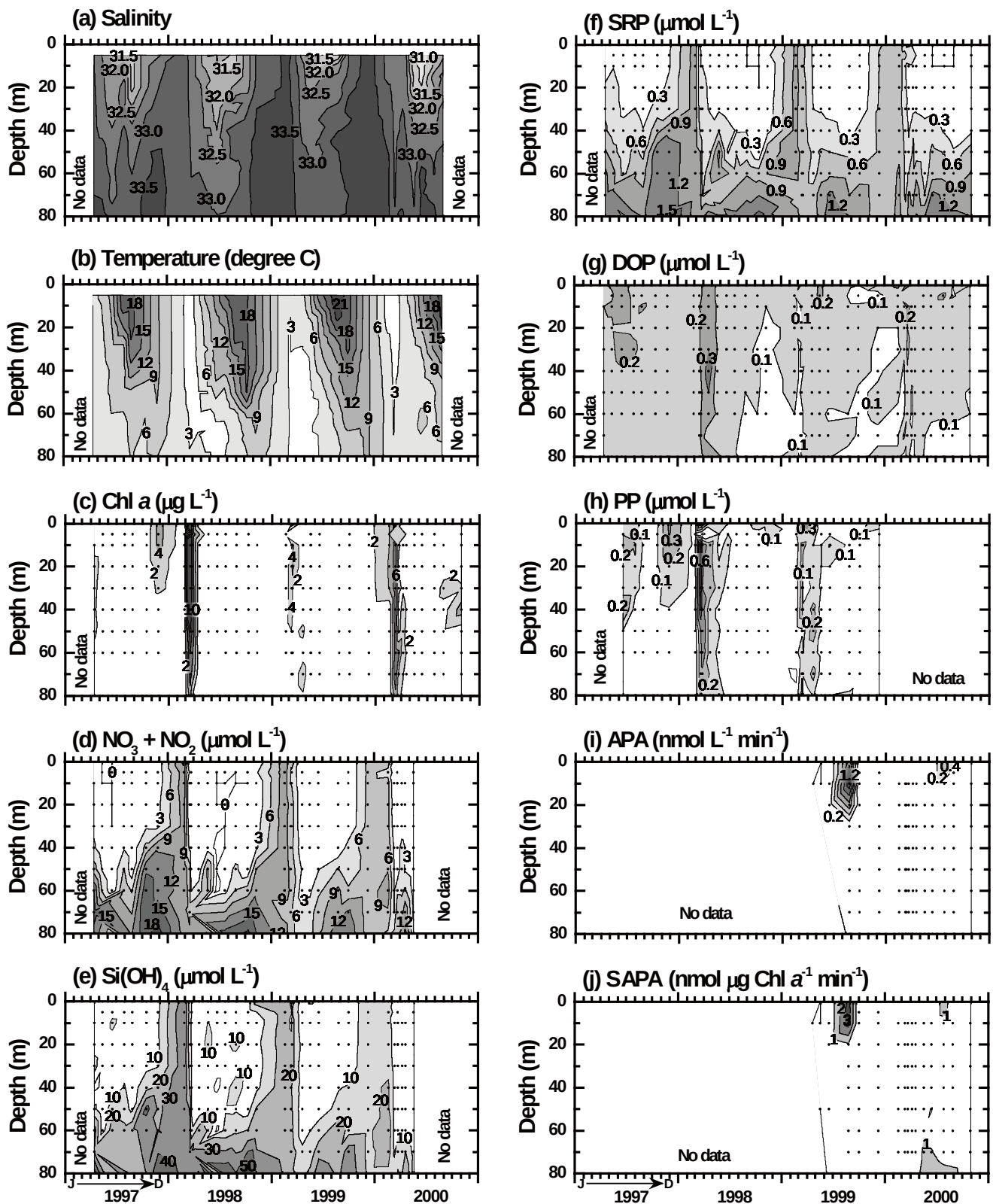


Fig. 2. Temporal variations in vertical distributions of (a) salinity, (b) water temperature, (c) chlorophyll *a*, (d) nitrate+nitrite ($\text{NO}_3 + \text{NO}_2$), (e) silicic acid (f) soluble reactive P (SRP), (g) dissolved organic P (DOP), (h) particulate P (PP), (i) alkaline phosphatase activity (APA), and (j) Chl *a*-specific APA (SAPA) at station 30 in Funka Bay.

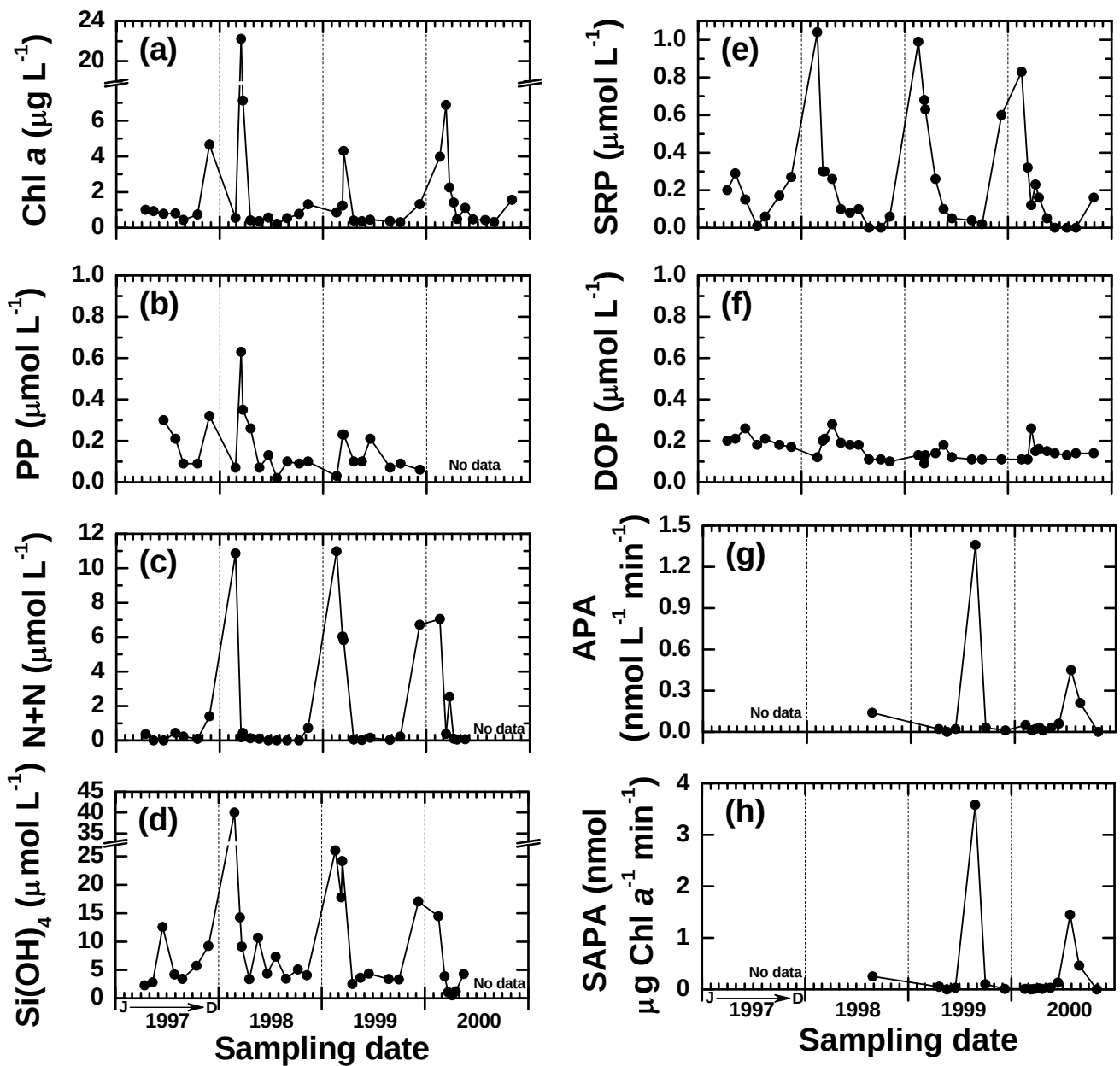


Fig. 3. Temporal variations at 10 m depth of (a) chlorophyll *a*, (b) particulate P (PP), (c) nitrate+nitrite (N+N), (d) silicic acid, (e) soluble reactive P (SRP), (f) dissolved organic P (DOP), (g) alkaline phosphatase activity (APA), and (h) Chl *a*-specific APA (SAPA) at station 30 in Funka Bay.

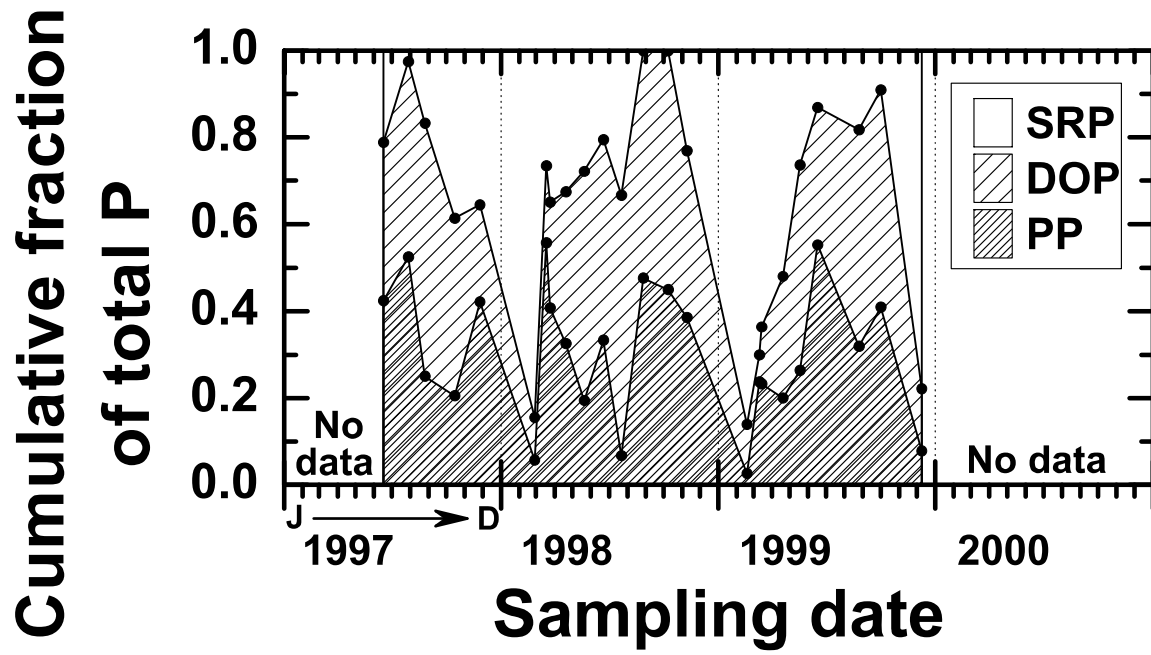
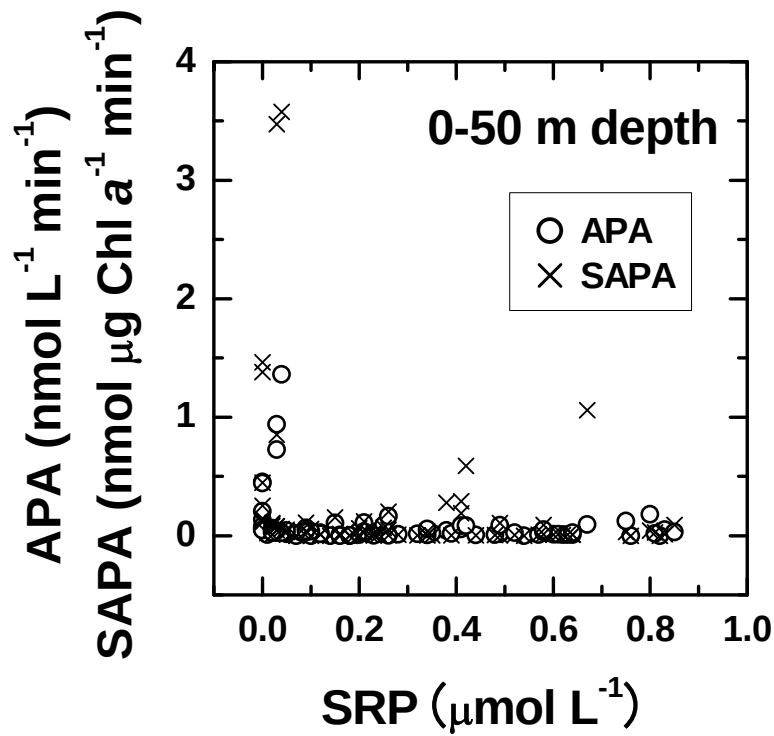


Fig. 4. Temporal variations at 10 m depth in proportions of particulate P (PP), dissolved organic P (DOP), and soluble reactive P (SRP) in the total P pool at station 30 in Funka Bay.



Yoshimura and Kudo Figure 6

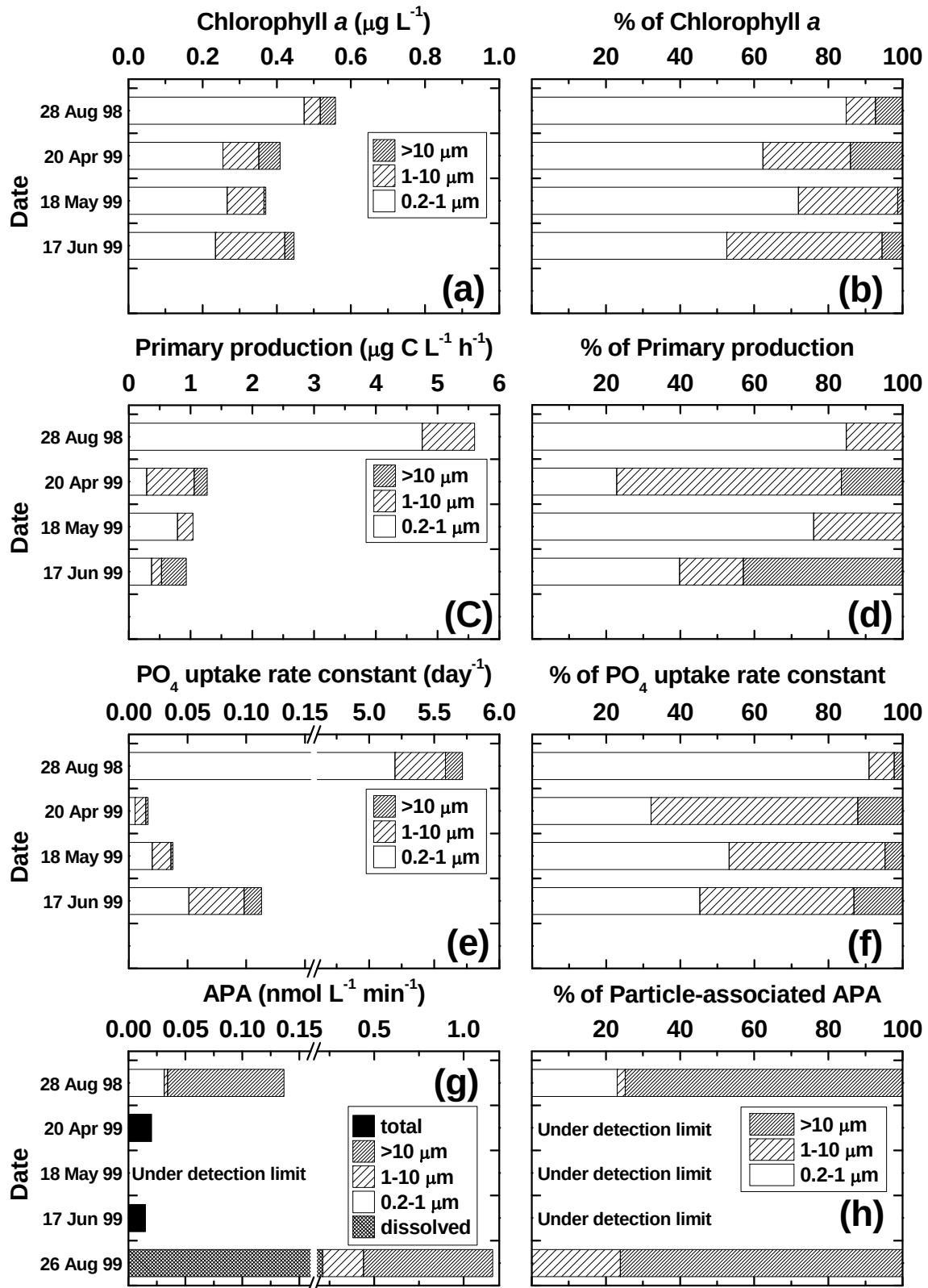


Fig. 6. Temporal variations and relative contribution in size fractionated (A) (B) chlorophyll *a*, (C) (D) primary production, (E) (F) orthophosphate uptake rate constant, and (G) (H) alkaline phosphatase activity (APA) at 10 m depth of station 30 in Funka Bay.

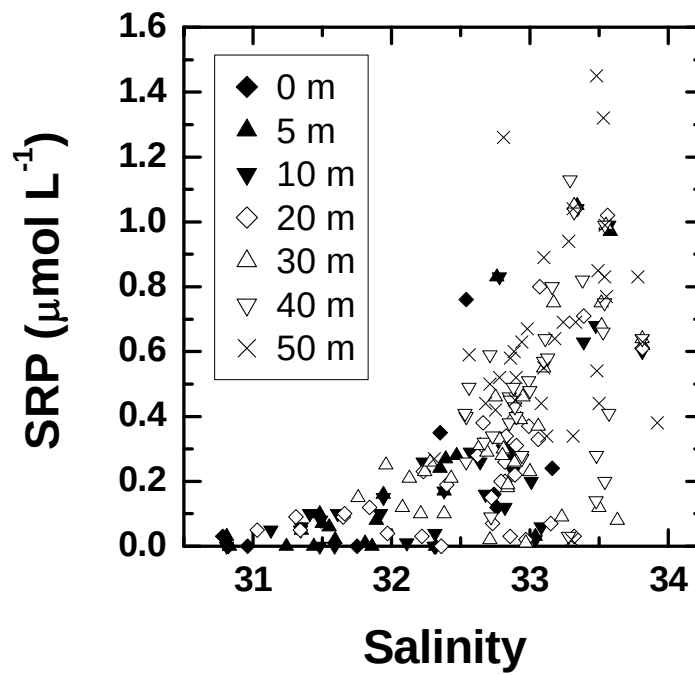


Fig. 7. Relationship between soluble reactive P (SRP) concentration and salinity in 0 to 50 m depth at station 30 in Funka Bay.