

# Orthophosphate uptake by heterotrophic bacteria, cyanobacteria, and autotrophic nanoflagellates in Villefranche Bay, northwestern Mediterranean: Vertical, seasonal, and short-term variations of the competitive relationship for phosphorus

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## Abstract

Previous studies have suggested that Mediterranean surface water becomes phosphorus limited for both bacteria and phytoplankton during stratified periods and that orthophosphate uptake in these situations was close to diffusion limitation for both cyanobacteria and autotrophic nanoflagellates. In order to better understand vertical and seasonal variations of this system, we measured orthophosphate uptake by bacteria and phytoplankton in Villefranche Bay (northwestern Mediterranean) monthly for the 0–75-m layer and weekly or biweekly at 10 m from June to December 2002. Turnover time of orthophosphate was relatively short (<2 h) in the surface mixed layer (0–30 m) during the stratified period, and long (>30 h) in the deeper layer during the stratified period and in the whole water column during the mixing period. Short-term fluctuations in turnover time were repeatedly observed from the stratified through the mixing periods. The dominance of PO<sub>4</sub> uptake drastically shifted from both bacteria and cyanobacteria to cyanobacteria when there were slight increases in turnover time (1–10 h). Compared to the theoretical maximum calculated for diffusion limitation, mean affinity constants at 10 m were similar for autotrophic nanoflagellates and greater for cyanobacteria in situations with turnover time <2 h, but observed values were much smaller than theoretical for heterotrophic bacteria even in samples with turnover time <1 h.

Phosphorus is one of the essential elements for all living organisms. In a pelagic plankton food web, dissolved phosphorus is taken up by osmotrophs (e.g., heterotrophic bacteria and phytoplankton) and then transferred through the food web via trophic interactions. The concept that nitrogen is the key limiting nutrient in euphotic layers has prevailed for most marine pelagic systems (e.g., Dugdale and Goering 1967). In contrast, phosphorus has been known to be the dominant limiting nutrient in many freshwater systems (Berman 1988; Hecky and Kilham 1988) and some systems subject to freshwater input such as the Chesapeake Bay (Fisher et al. 1992), Baltic Sea (Lignell et al. 1992), Norwegian fjords (Thingstad et al. 1993), and the Bay of Biscay (Labry

et al. 2002). However, recent studies have suggested phosphorus as the most limiting nutrient also in some open oceanic systems (Karl et al. 1995; Cotner et al. 1997; Ammerman et al. 2003) and in the Mediterranean Sea (e.g., Krom et al. 1991; Zweifel et al. 1993; Thingstad et al. 1998; Zohary and Robarts 1998).

In Villefranche Bay (northwestern Mediterranean), previous studies have found that (1) estimates of bioavailable orthophosphate concentrations can be very low (0.8 nmol L<sup>-1</sup>; Thingstad et al. 1996), (2) bioassays based on response in bacterial abundance and production indicate P-limited bacterial growth (Zweifel et al. 1993; Thingstad et al. 1998), (3) a response in the cell cycle of *Synechococcus* cells was induced by P but not by N addition (Vaulot et al. 1996), and (4) turnover time of orthophosphate became relatively short (<2 h) during the stratified period (Dolan et al. 1995; Thingstad et al. 1998). These results suggest that the surface mixed layer becomes P limited for both bacteria and phytoplankton, with the expected consequence that the competition between bacteria and phytoplankton for P becomes important during the stratified period.

The specific affinity constant for a substrate uptake is the parameter that determines the competitive ability among osmotrophs in an environment with a permanently low substrate concentration (Thingstad and Rassoulzadegan 1999; Vadstein 2000). We recently reported that the mean specific affinity constant for orthophosphate uptake by cyanobacteria was highest among osmotrophs in autumn in Villefranche Bay (Tanaka et al. 2003). For cyanobacteria and autotrophic

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nanoflagellates (ANFs), the estimated values were almost as high as the theoretical maximum based on the assumption that the physical process of molecular diffusion toward small autotrophs is the rate-limiting process (diffusion limitation). This suggests that cyanobacteria and ANFs were P limited with cyanobacteria as the superior competitors at permanently low  $\text{PO}_4$  concentrations. Despite the accumulated evidence of P limitation in surface mixed layer during the stratified period in Villefranche Bay, information on the spatial and temporal variations of orthophosphate uptake has been limited.

We here report vertical and seasonal variations of orthophosphate uptake rates and affinity constants for orthophosphate by bacteria, cyanobacteria, and ANFs together with concentrations of soluble reactive phosphorus (SRP), dissolved organic phosphorus (DOP), and particulate P in Villefranche Bay. Two sampling strategies were used to cover both spatial and temporal variations at a coastal site from June to December 2002: vertical profiles were taken monthly and seasonal and short-term variations were monitored at one depth weekly or biweekly. Our objective was to examine how orthophosphate uptake by bacteria and phytoplankton (i.e., the competition for P) varies vertically and seasonally with seasonal cycle of hydrographic conditions.

## Materials and methods

**Sampling**—Sampling was conducted at Point B in Villefranche Bay in the northwestern Mediterranean ( $43^\circ 41' 10''\text{N}$ ,  $7^\circ 19' 00''\text{E}$ ; 80 m maximum depth) from June to December 2002. Water samples were collected at 0, 10, 20, 30, 50, and 75 m for monthly sampling and at 10 m for short-term sampling using 5-liter Niskin bottles. In order to remove large zooplankton, water samples were prescreened through 200- $\mu\text{m}$  nylon mesh.

**Phosphorus measurements**—SRP, total dissolved phosphorus (TDP), and particulate P were measured spectrophotometrically using the molybdenum blue reaction according to Koroleff (1976). SRP was measured in triplicate without filtration. TDP was measured in triplicate after acid persulfate oxidation at  $121^\circ\text{C}$  of 0.2- $\mu\text{m}$  filtrates. The detection limit was  $\sim 0.03 \mu\text{mol L}^{-1}$  for both SRP and TDP. DOP was calculated by subtracting SRP from TDP. As for particulate P, 500-ml samples were filtered sequentially through 47-mm polycarbonate filters with 2-, 0.6-, and 0.2- $\mu\text{m}$  pore sizes. Filters were transferred to polypropylene test tubes with 15 ml of Milli-Q water and then treated as TDP samples. No replicates were done for particulate P samples. Data of particulate P are presented as total (i.e., 0.2–200- $\mu\text{m}$  fraction) in the present paper.

**Uptake of  $^{32}\text{PO}_4$** —Uptake kinetics of orthophosphate was measured according to Thingstad et al. (1993). Carrier-free  $^{32}\text{P}$  orthophosphate (ICN Biomedicals) was added to 20-ml samples in polyethylene vials to give a final radioactive concentration of  $\sim 10^5$ – $10^6$  counts per minute (CPM)  $\text{ml}^{-1}$ . Samples were incubated at in situ temperature ( $\pm 2^\circ\text{C}$ ) and subdued (laboratory) illumination for an incubation time varying between 30 min and 4 h, according to expected turn-

over time. Incubation was stopped by a cold chase of 100  $\text{mmol L}^{-1}$   $\text{KH}_2\text{PO}_4$  (final concentration, 1  $\text{mmol L}^{-1}$ ). Sub-samples were filtered in parallel on 25-mm polycarbonate filters with 10-, 5-, 2-, 1-, 0.8-, 0.6-, 0.4-, and 0.2- $\mu\text{m}$  pore sizes. All filters were placed on a Millipore 12-place manifold and supported on Whatman glass fiber filters (grade GF/C) saturated with 100  $\text{mmol L}^{-1}$   $\text{KH}_2\text{PO}_4$ . After filtration, filters were placed in polyethylene scintillation vials with Milli-Q water and radioassayed with a Beckman liquid scintillation counter (model LS 1800).

Kinetics of orthophosphate uptake was determined for samples from 10 m by the Rigler bioassay (1966). Unlabelled  $\text{KH}_2\text{PO}_4$  was added to 10-ml samples (0, 25, 50, 75, and 100  $\text{nmol L}^{-1}$ ) prior to isotope addition. The incubation was stopped after 15 min by adding cold  $\text{KH}_2\text{PO}_4$ , and uptake of  $^{32}\text{P}$  was measured as mentioned above. All counting results were transformed into the fraction of particulate  $^{32}\text{P}$  to total  $^{32}\text{P}$  per hour. Turnover time, maximum uptake rates ( $V_{\text{max}}$ ), and the sum  $K_{\text{PO}_4} + S_n$  (the half-saturation constant and the natural concentration of bioavailable orthophosphate, respectively) were calculated according to the method of Wright and Hobbie (1966) as modified by Thingstad et al. (1993).

**Size distribution of  $^{32}\text{PO}_4$  uptake**—In order to characterize the size distribution of  $\text{PO}_4$  uptake, a cumulative gamma distribution was fitted to the observed cumulative distribution of  $^{32}\text{PO}_4$  uptake. The calculations were performed using the Solver option in Microsoft Excel, minimizing the squared residual differences between the observations and the fitted cumulative gamma distribution as implemented in the Excel function library. The gamma distribution with 0  $\mu\text{m}$  shifted to  $X = 0.2 \mu\text{m}$

$$y(X - 0.2, \alpha, \beta) = \frac{1}{\beta^\alpha \Gamma(\alpha)} (X - 0.2)^{\alpha-1} e^{-(X-0.2)/\beta}$$

for  $X > 0.2 \mu\text{m}$

was chosen because it is 0 for an organism size of 0.2  $\mu\text{m}$  (assuming all organisms to be  $> 0.2 \mu\text{m}$ ) and it has a skewed form that appeared to fit the data and has two parameters,  $\alpha$  and  $\beta$ , allowing adjustment both of the mean ( $\alpha\beta$ ) and variance ( $\alpha\beta^2$ ). The gamma distribution was thus used for reasons of convenience, not because we have any theoretical argument for this particular distribution. The median of the fitted gamma distribution was calculated using Excel's inverse gamma function with probability 0.5 and the fitted  $\alpha$  and  $\beta$  values.

**Calculation of affinity constants**—Specific affinity constants for orthophosphate uptake were estimated according to the procedure proposed by Thingstad and Rassoulzadegan (1999) and modified by Tanaka et al. (2003) as follows. Under the assumption that the uptake in heterotrophic bacteria is proportional to the substrate concentration ( $S_n$ :  $\text{nmol P L}^{-1}$ ) and biomass ( $B_B$ :  $\text{nmol P L}^{-1}$ ), one has  $\alpha_B S_n B_B = f_B V$ , where  $\alpha_B$  is the specific affinity of bacteria ( $\text{L nmol P}^{-1} \text{h}^{-1}$ ) and  $f_B$  is the fraction (no dimension) of total uptake ( $V$ :  $\text{nmol P L}^{-1} \text{h}^{-1}$ ) that goes into heterotrophic bacteria. Because turnover time (h)  $T = S_n/V$ , solving for  $\alpha_B$  gives  $\alpha_B = (f_B/$

Table 1. Surface temperature (0 m), temperature difference between 0 and 75 m, integrated Chl *a* in the upper 75 m, turnover time of orthophosphate, and median size for orthophosphate uptake at 10 m at Point B (Villefranche Bay, NW Mediterranean) in 2002. The former two parameters are presented as the monthly mean, and the latter three are in the monthly mean with SD. ND, no data.

Month	Surface temperature (°C)	Temperature difference (°C)	<i>n</i> *	Chl <i>a</i> (mg m <sup>-2</sup> )	<i>n</i>	Turnover time (h)	Median size (μm)	<i>n</i>
Jan	14.0	0.0	5	16±3	5	ND	ND	—
Feb	13.2	-0.1	3	36±11	3	ND	ND	—
Mar	13.4	0.1	2	23±8	5	ND	ND	—
Apr	14.7	0.9	5	22±4	4	ND	ND	—
May	18.1	2.5	4	24±6	4	ND	ND	—
June	21.8	7.4	4	24±11	4	1.0±0.2	0.71±0.08	4
July	24.1	9.9	5	21±6	5	1.4±0.8	0.83±0.30	3
Aug	23.8	9.5	4	16±2	4	3.3±3.3	0.91±0.31	3
Sep	23.0	8.6	4	15±1	4	2.3±1.6	0.85±0.10	5
Oct	20.5	5.5	5	21±5	4	2.5±1.2	0.94±0.14	7
Nov	17.9	1.2	4	19±3	4	26.8±49.1	1.37±0.48	7
Dec	16.0	0.1	4	18±3	5	26.7±24.2	1.29±0.05	2

\* *n*, number of data. Data on temperature and Chl *a* from SOMLIT (2002).

$T)/B_B$ . Correspondingly, for  $\alpha_P$  and  $\alpha_N$  (the specific affinity of picoplankton and ANFs, respectively),  $\alpha_P = (f_P/T)/B_P$  and  $\alpha_N = (f_N/T)/B_N$ , where  $B_P$  and  $B_N$  are the biomass of autotrophic picoflagellates (APFs) plus cyanobacteria and ANFs, respectively.

Under the assumption that the 0.2–0.6-μm fraction contains a fraction ( $b$ : 0.75 in the present paper, see Tanaka et al. 2003) of total heterotrophic bacteria and that all bacteria are equally active,  $f_B = f_{0.2-0.6\mu\text{m}}/b$ , so that

$$\alpha_B = [(f_{0.2-0.6\mu\text{m}}/b)/T]/B_B$$

The 0.6–2-μm fraction contains both the uptake by another fraction of bacteria  $(1 - b)f_B V$  and by APFs and cyanobacteria  $f_P V$ , so that  $f_{0.6-2\mu\text{m}} = (1 - b)f_B + f_P$ . Thus,

$$\alpha_P = \{(f_{0.6-2\mu\text{m}} - [(1 - b)/b] f_{0.2-0.6\mu\text{m}})/T\}/B_P$$

Under the assumption that the uptake in the 2–10-μm fraction is only by ANFs ( $f_N = f_{2-10\mu\text{m}}$ ), similar reasoning gives

$$\alpha_N = (f_N/T)/B_N$$

**Abundance and biomass of microbial components**—Samples of picoplankton and nanoplankton for microscopic counts were fixed with glutaraldehyde (final concentration, 1%). Heterotrophic bacteria, cyanobacteria, APFs (<2 μm), and ANFs (2–10 μm) were counted by epifluorescence microscopy after staining with 4,6-diamidinophenyleindole (DAPI) (Porter and Feig 1980). In order to estimate biomass P of microbial components, we chose the same conversion factors as in Tanaka et al. (2003): 20 fg C cell<sup>-1</sup> for bacteria (Lee and Fuhrman 1987), 200 fg C cell<sup>-1</sup> for cyanobacteria, and 183 fg C μm<sup>-3</sup> for flagellates (Caron et al. 1995). With regard to flagellates, a constant volume was assumed for each size class: 4.2 μm<sup>3</sup> cell<sup>-1</sup> for <2 μm, 35 μm<sup>3</sup> cell<sup>-1</sup> for 2–5 μm, and 294 μm<sup>3</sup> cell<sup>-1</sup> for 5–10 μm. We used a C:P ratio of 50 for bacteria (Fagerbakke et al. 1996) and 106 for the other groups (Redfield et al. 1963).

**Statistical analysis**—The effects of season (months) and depth on estimated affinity constants for bacteria, picophy-

toplankton, and ANFs were tested by analyses of variance (ANOVA). To describe the relationship between the fraction of particulate <sup>32</sup>P to total <sup>32</sup>P (%) for the 0.2–0.6- and 0.6–2-μm fractions and turnover time of orthophosphate (h), the semilog linear regression was used after the latter was logarithmically transformed. The type of linear regression was simply chosen in order to give the highest value of the coefficient of determination. The statistical significance was tested by *F*-test for ANOVA and by Student's *t*-test for regression slope (Sokal and Rohlf 1995).

## Results

**Environmental variables and microbial abundance**—Based on the data from Service d'Observation en Milieu Littoral (SOMLIT 2002), monthly means of surface temperature, vertical difference of temperature in the water column, and integrated chlorophyll *a* are summarized in Table 1. With the simple hydrographic characterization of the temperature difference in 0 and 75 m ( $\Delta\text{Temp}$ ; Bustillos-Guzmán et al. 1995), three phases of the water column structure were identified: the mixing period from January to April and November–December ( $\Delta\text{Temp}$  almost 0°C), the semistratified period in May and October ( $\Delta\text{Temp}$  2–7°C), and the stratified period from June to September ( $\Delta\text{Temp}$  >7°C). This characterization corresponded well to the density profile showing the existence of the strong pycnocline from mid-June to the beginning of September (Fig. 1, SOMLIT 2002). Phytoplankton biomass was highest in February, indicating a spring phytoplankton bloom, and thereafter it gradually decreased until September (Table 1).

Concentrations of SRP, DOP, and particulate P varied from less than detection limit to 0.2 μmol L<sup>-1</sup>, from 0.15 to 0.93 μmol L<sup>-1</sup>, and from 0.003 to 0.055 μmol L<sup>-1</sup>, respectively, from June to December 2002 (Fig. 2). SRP was low in the surface mixed layer and high in the deeper layer during the stratified period except in September, while DOP and particulate P concentrations were slightly higher in the surface mixed layer. Average dissolved P (TDP = SRP + DOP)

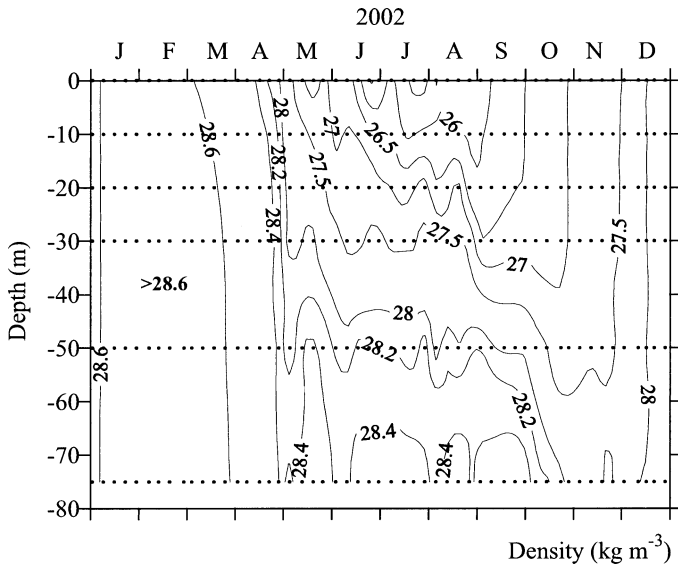


Fig. 1. Seasonal variations of water density ( $\text{kg m}^{-3}$ ) at Point B, Villefranche Bay in 2002. Dots denote the positions of sampling depths and dates. Data from SOMLIT (2002).

was 95% (range, 90–99%,  $n = 39$ ) of total P (TDP + particulate P) from 0 to 75 m during the study period. Note that SRP was on average 11% of total P, and concentrations of DOP were 1 order of magnitude higher than those of SRP during the period and the depth with relatively short  $\text{PO}_4$  turnover time (see below). From November to December, vertical distributions of all three P forms were uniform, which suggests a well-mixed water column.

Integrated P stocks (SRP + DOP + particulate P < 200  $\mu\text{m}$ ) varied little from June to September at 0–30 m and from July to October at 0–75 m (Fig. 3). However DOP in the upper 30 m increased (6  $\text{mmol P m}^{-2}$ ) from September to October and decreased (–12  $\text{mmol P m}^{-2}$ ) from October to November. DOP concentrations at 10 m were as high on 1 and 8 October as on 24 October (data on the former dates not shown), which suggests that this DOP increase was not sporadic. In November and December, P stocks decreased in both the upper 30 and 75 m.

Bacterial abundance ranged from  $2.8 \times 10^8$  to  $8.3 \times 10^8$  cells  $\text{L}^{-1}$  (Fig. 4A). Although vertical and seasonal changes were small (a factor of 3), small peaks in bacterial abundance were observed at the subsurface depths during the stratified period. Cyanobacteria abundances were mostly between  $1 \times 10^7$  and  $4 \times 10^7$  cells  $\text{L}^{-1}$  in the upper 50 m, while the abundance drastically decreased to the order of  $10^6$  cells  $\text{L}^{-1}$  at 75 m (Fig. 4B). The vertical distribution of APFs was different from that of the other osmotrophs (Fig. 4C). APFs were less abundant in the upper layers and increased in the deeper layer. Peaks ( $>1.5 \times 10^6$  cells  $\text{L}^{-1}$ ) were observed at 50/75 m in July and December. ANFs ranged from  $7.4 \times 10^4$  to  $4.4 \times 10^6$  cells  $\text{L}^{-1}$  (Fig. 4D). They showed marked peaks at 0 m from June to July and in November, and around 30/50 m in October, while their distribution was relatively mixed during the rest periods.

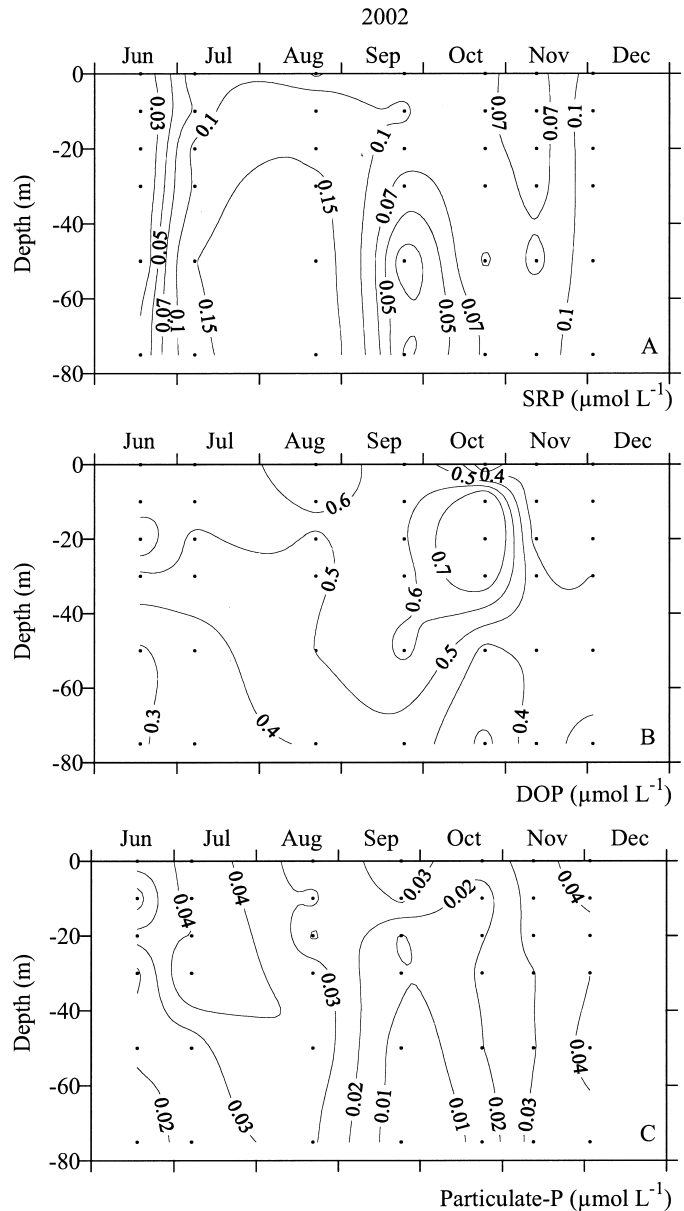


Fig. 2. Seasonal variations in concentration ( $\mu\text{mol L}^{-1}$ ) of (A) SRP, (B) DOP, and (C) particulate P from June to December 2002. Dots denote the positions of sampling depths and dates.

**P uptake kinetics**—Turnover time of orthophosphate ranged from 0.7 to 273.6 h (Fig. 5A). In the upper 30 m, it was shorter than 1 h from June to August and then gradually increased toward December. Below 30 or 50 m, turnover time steeply increased. Because planktonic osmotrophs (i.e., heterotrophic bacteria, picophytoplankton, ANFs, and large phytoplankton) in Villefranche Bay can largely be separated into the 0.2–0.6-, 0.6–2-, 2–10-, and  $>10$ - $\mu\text{m}$  fractions, respectively (see Tanaka et al. 2003), the fraction of  $^{32}\text{PO}_4$  uptake was presented using these four size fractions (Fig. 5B–E). From June to October except September,  $^{32}\text{PO}_4$  uptake in the upper 30 m was dominated by the 0.2–0.6- and 0.6–2- $\mu\text{m}$  fractions (mean, 93%) (Fig. 5D,E). The uptake by the 0.2–0.6- $\mu\text{m}$  fraction gradually decreased in the upper 30

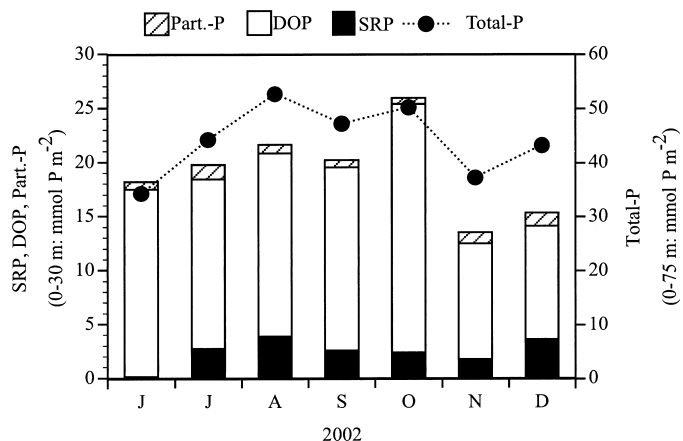


Fig. 3. Phosphorus stocks integrated for the 0–30 m (SRP, DOP, and particulate P <200  $\mu\text{m}$ ) and for the 0–75 m (total P) from June to December 2002. Note that different scales are used.

m from June to October, while uptake by the 0.6–2- $\mu\text{m}$  fraction showed an opposite increasing trend. The 2–10- $\mu\text{m}$  fraction contributed almost always less than 5% of total uptake from June to October and thereafter increased to  $\sim 10\%$  throughout the water column (Fig. 5C). During the whole study period, any considerable ( $>10\%$ ) contribution by the  $>10\text{-}\mu\text{m}$  fraction was observed only at 50 and 75 m, where turnover time was always longer than 5 h (Fig. 5B).

A short-term sampling at 10 m clearly showed short-term fluctuations in seasonal variation of orthophosphate turnover time (Fig. 6A). While the turnover time was mostly  $\sim 1$  h from June to August, sporadic increases were observed in July and August. This resulted in two to threefold longer monthly means and relatively larger SD of turnover time in August, September, and October compared to those in June and July (Table 1). The isotope dilution experiments gave  $K_{PO_4} + S_n$  estimates (0.6–41  $\text{nmol P L}^{-1}$ ) for the bacterial fraction that are lower than the SRP concentrations measured (Table 2). This indicates that bioavailable phosphate concentrations were lower than the measured SRP. The relationship between turnover time of orthophosphate and the percent of total  $^{32}PO_4$  uptake was different for the four size fractions (Fig. 6B). When turnover time was shorter than 2 h, almost all  $^{32}PO_4$  was used by the two smaller fractions (0.2–0.6- and 0.6–2- $\mu\text{m}$  fractions). However when turnover time increased from 2 to 10 h,  $^{32}PO_4$  uptake by the former fraction decreased to 10% and that by the latter fraction increased up to 80%. For the data with turnover  $<10$  h (Fig. 6B), the semilog linear regression analysis gave slopes ( $\pm\text{SE}$ ) and intercepts ( $\pm\text{SE}$ ) that were significantly different from zero ( $p < 0.0001$ , Student's  $t$ -test):

$$\begin{aligned} \text{\% of total } ^{32}\text{P uptake} \\ &= 40.0(\pm 2.4) - 41.6(\pm 4.9) \times \log_{10}[\text{turnover time}] \\ r^2 &= 0.740, \quad n = 27 \quad \text{for the 0.2–0.6-}\mu\text{m fraction} \end{aligned}$$

$$\begin{aligned} \text{\% of total } ^{32}\text{P uptake} \\ &= 55.3(\pm 2.5) + 31.8(\pm 5.2) \times \log_{10}[\text{turnover time}] \\ r^2 &= 0.600, \quad n = 31 \quad \text{for the 0.6–2-}\mu\text{m fraction} \end{aligned}$$

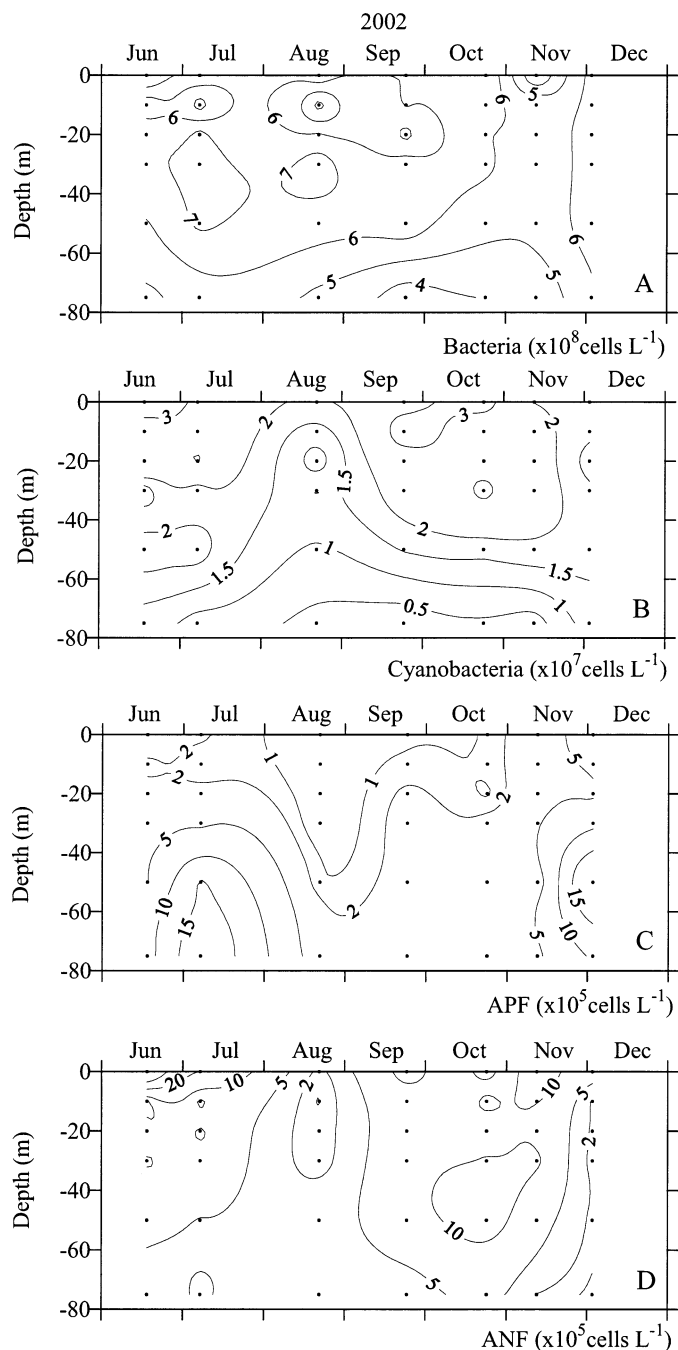


Fig. 4. Same as in Fig. 2 but in abundance ( $\text{cells L}^{-1}$ ) of (A) bacteria, (B) cyanobacteria, (C) autotrophic picoflagellates (APFs), and (D) autotrophic nanoflagellates (ANFs).

When turnover time was longer than 10 h,  $^{32}PO_4$  uptake by the 0.6–2- $\mu\text{m}$  fraction decreased. Uptake by the two larger fractions (2–10- and  $>10\text{-}\mu\text{m}$  fractions) slightly increased with the increase of turnover time.

Figure 7 shows two examples of the observed cumulative distribution of  $^{32}PO_4$  uptake with the fitted gamma function lines at 10 and 75 m, respectively, on 8 July 2002. At 10 m, 97% of total  $^{32}PO_4$  uptake occurred in the  $<10\text{-}\mu\text{m}$  fraction, in which most of uptake was by the 0.4–2- $\mu\text{m}$  fraction.

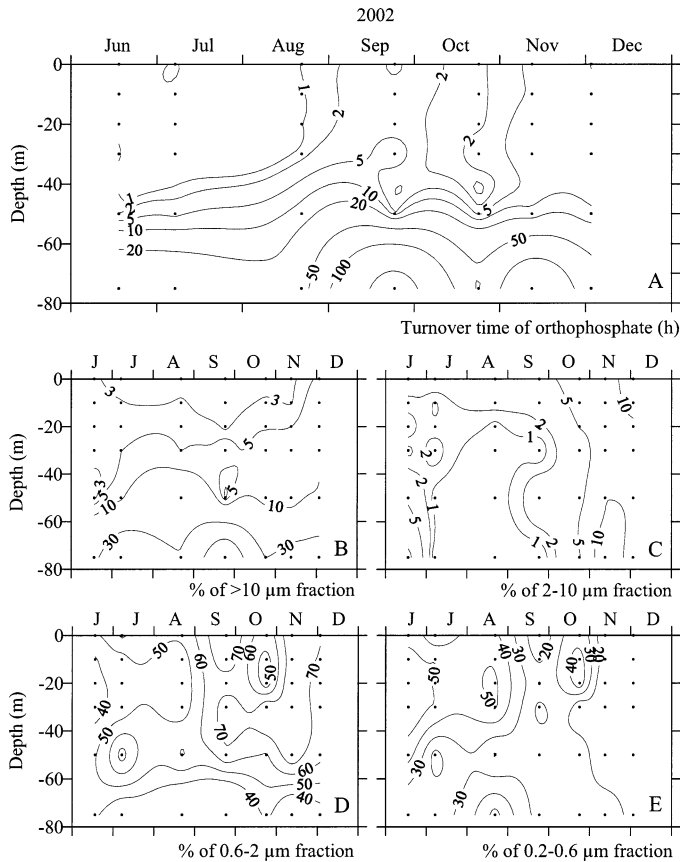


Fig. 5. Same as in Fig. 2 but (A) turnover time of orthophosphate (h), and fraction of  $^{32}\text{PO}_4$  uptake (%) by (B)  $>10\ \mu\text{m}$ , (C)  $2\text{--}10\ \mu\text{m}$ , (D)  $0.6\text{--}2\ \mu\text{m}$ , and (E)  $0.2\text{--}0.6\ \mu\text{m}$ .

Turnover time of orthophosphate at 75 m was 40 h, and half of total  $^{32}\text{PO}_4$  uptake occurred in the  $<10\text{-}\mu\text{m}$  fraction. The median size was estimated to be  $0.6\ \mu\text{m}$  at 10 m and  $3.3\ \mu\text{m}$  at 75 m. Fitness of the cumulative gamma function (sum of the squared residual differences) tended to be better with shorter turnover time of orthophosphate ( $<10\ \text{h}$ ). Monthly means of the median size for cumulative distribution of  $^{32}\text{PO}_4$  uptake showed twofold increase ( $0.7$  to  $1.4\ \mu\text{m}$ ) at 10 m, as the mean turnover time of orthophosphate increased from 1 h in June to 27 h in November–December (Table 1).

Our procedure for estimating the affinity constants as-

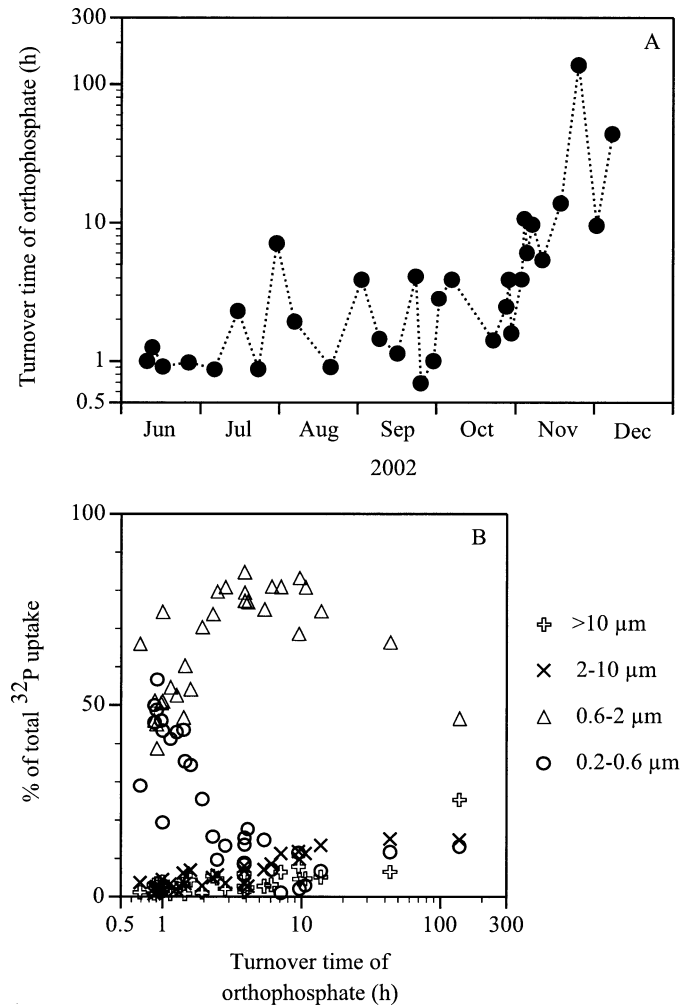


Fig. 6. (A) Short-term variations in turnover time of orthophosphate (h) at 10 m and (B) relationships between percent of isotope uptake by the four different size fractions ( $0.2\text{--}0.6\ \mu\text{m}$ ,  $0.6\text{--}2\ \mu\text{m}$ ,  $2\text{--}10\ \mu\text{m}$ , and  $>10\ \mu\text{m}$ ) and turnover time of orthophosphate.

sumes a linear relationship between substrate concentration and uptake. We calculated affinity constants only for the period and the depth with turnover time shorter than 5 h (i.e., 0–30 m from June to October except at 0 and 30 m in September, *see* Fig. 5A). The affinity constants for picophyto-

Table 2. Turnover time for orthophosphate, estimates of maximum uptake rates for the  $>0.2\ \mu\text{m}$  fraction ( $V_{\text{max}}$ ), the half-saturation constant plus the natural concentration of bioavailable orthophosphate for the  $0.2\text{--}0.6\ \mu\text{m}$  fraction ( $K_{\text{PO}_4} + S_n$ ), and SRP (mean  $\pm$  SD,  $n = 3$ ). ND, no data.

Date	Turnover time (h)	$V_{\text{max}}$ , $>0.2\ \mu\text{m}$ (nmol P L <sup>-1</sup> h <sup>-1</sup> )	$K_{\text{PO}_4} + S_n$ , $0.2\text{--}0.6\ \mu\text{m}$ (nmol P L <sup>-1</sup> )	SRP ( $\pm$ SD, nmol P L <sup>-1</sup> )
14 Jun 02	1.3	8	2	48 $\pm$ 3
28 Jun 02	1.3	16	12	170 $\pm$ 3
25 Jul 02	3.3	27	38	73 $\pm$ 12
8 Aug 02	1.9	17	41	69 $\pm$ 24
3 Sep 02	5.7	26	ND	<30
17 Sep 02	1.3	22	0.6	<30
8 Oct 02	5.0	26	ND	106 $\pm$ 5
5 Nov 02	3.9	5	11	88 $\pm$ 27

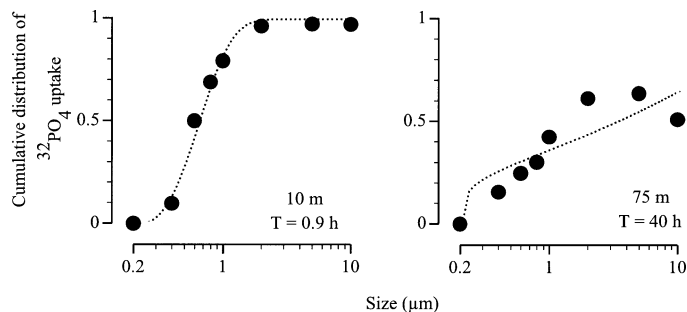


Fig. 7. Example of cumulative distribution of  $^{32}PO_4$  uptake (filled circles) measured at 10 m and 75 m on 8 July 2002. The fitted cumulative distribution (dotted lines) was estimated using the function of cumulative gamma distribution (see text for details). Turnover times of orthophosphate (h) are also shown.

plankton ( $\alpha_p$ ) were greater than those for ANF ( $\alpha_N$ ) and bacteria ( $\alpha_b$ ), ranging from 0.001 to 0.060  $L\ nmol\ P^{-1}\ h^{-1}$  for bacteria, from 0.029 to 0.244  $L\ nmol\ P^{-1}\ h^{-1}$  for picophytoplankton, and from 0.001 to 0.127  $L\ nmol\ P^{-1}\ h^{-1}$  for ANF (data not shown). Two-way ANOVA showed that affinity constants from June to October except September were significantly different between months for picophytoplankton ( $p < 0.05$ ,  $F$ -test) and between depths and months for bacteria ( $p < 0.01$  for depth,  $p < 0.05$  for months,  $F$ -test) but insignificant for ANF ( $p > 0.05$ ,  $F$ -test).

Similarly, affinity constants at 10 m (from June to October except on 1 August, see Fig. 6A) ranged from 0.002 to 0.060  $L\ nmol\ P^{-1}\ h^{-1}$  for bacteria, from 0.029 to 0.171  $L\ nmol\ P^{-1}\ h^{-1}$  for picophytoplankton, and from 0.002 to 0.127  $L\ nmol\ P^{-1}\ h^{-1}$  for ANFs, whose ranges were quite similar to those at 0–30 m (Fig. 8). One-way ANOVA did not detect significant differences between months for each group ( $p > 0.05$ ,  $F$ -test).

## Discussion

Our results present full-depth profiles of turnover time of orthophosphate and affinity constants of heterotrophic bacteria, picophytoplankton, and ANFs for orthophosphate in Villefranche Bay, northwestern Mediterranean, from June to December 2002 covering stratified, semistratified, and mixing periods. An overwhelming dominance of dissolved P (average, 95% of total P) found from 0 to 75 m during the study period corresponded well to our previous study at 10 m of the same site in autumn 2001 (average 93%; Tanaka et al. 2003), which suggests a common feature throughout the water column at the study site.

Small variations of the integrated P stocks in the upper 30 m during the stratified period suggest an internal P cycling in the surface mixed layer (Fig. 3). On the other hand, the DOP increase in the upper 30 m in October may suggest either less DOP use or increased DOP production or both in the food web. The decline of the water column stratification presumably allows the nutrient-rich deeper water to mix with the surface water in October (Table 1, Fig. 1). This was supported, not by the vertical profile of SRP, but by the increase in turnover time of orthophosphate (Fig. 5). However, the integrated P stocks decreased both in the upper 30 and

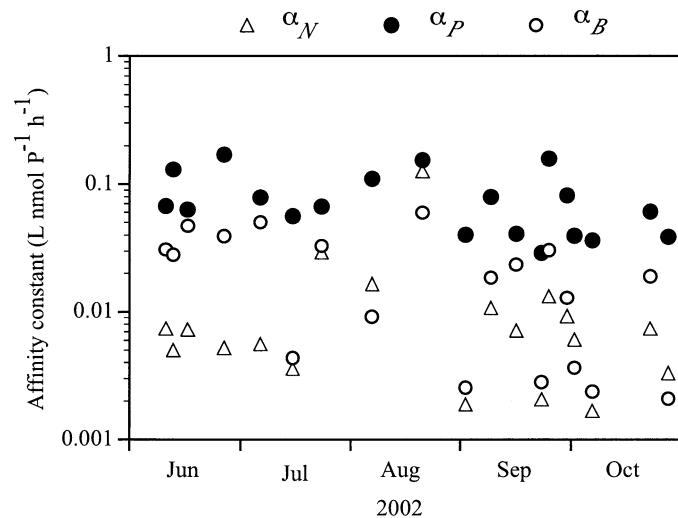


Fig. 8. The estimated affinity constants ( $L\ nmol\ P^{-1}\ h^{-1}$ ) for bacteria ( $\alpha_b$ ), picophytoplankton ( $\alpha_p$ ), and ANFs ( $\alpha_N$ ) at 10 m from June to October 2002, when turnover time of orthophosphate was relatively short ( $< 5\ h$ ).

75 m during the mixing period. Because Villefranche Bay is not a closed system and we did not measure particulate P  $> 200\ \mu m$ , a thorough budget analysis remains to be done.

Vertical and seasonal variations in turnover time of orthophosphate coincided well with the changes in water column structure (Figs. 1, 5, and 6), i.e., shorter in the surface mixed layer during the stratified period and longer in the deeper layer during the stratified period and in the whole water column during the mixing period. It should be noted that when turnover time gradually increased in the upper 30 m from June to October, the dominance of  $^{32}PO_4$  uptake shifted from the bacteria (0.2–0.6  $\mu m$ ) plus picophytoplankton (0.6–2  $\mu m$ ) fractions to the picophytoplankton fraction. This seasonal succession was also shown as the increase in median size of  $^{32}PO_4$  uptake (Table 1). High-performance liquid chromatography analysis has shown that cyanobacteria are abundant in the upper 30 or 40 m, while *Prochlorococcus* are abundant only below 30 m during the stratified period at the study site (Bustillos-Guzmán et al. 1995). APFs were two orders of magnitude less abundant than cyanobacteria (Fig. 4). Thus, it is well considered that  $^{32}PO_4$  uptake by the 0.6–2- $\mu m$  fraction was mostly by cyanobacteria in the upper 30 m during the stratified period.

Since turnover time of orthophosphate is a function of concentration and total uptake rate of  $PO_4$ , the increase in turnover time is a result of an increase in the pool of bioavailable phosphate or decreased  $PO_4$  uptake (decreased biomass of osmotrophs or  $PO_4$  uptake rate per cell) or both. Dissolved inorganic phosphate is considered to be the most readily available form of P to osmotrophs. Unless direct uptake of orthophosphate released by the membrane-bound enzymes is a significant process of DOP use by osmotrophs (e.g., Ammerman and Azam 1985), DOP remineralization can be mixed into the orthophosphate pool in the water. The standard method for measuring SRP usually overestimates concentrations of the bioavailable phosphate due to the background such as acid labile P compounds and arsenate (re-

Table 3. Comparison estimated affinity constants (mean  $\pm$  SD, L nmol P<sup>-1</sup> h<sup>-1</sup>) for heterotrophic bacteria, picophytoplankton, and autotrophic nanoflagellates (ANFs) at 10 m with the theoretical maximum predicted by a diffusion model.

Turnover time of orthophosphate (h)	Heterotrophic bacteria	Picophytoplankton	ANFs	n*
<1	0.044 $\pm$ 0.011	0.116 $\pm$ 0.051	0.012 $\pm$ 0.010†	6
1–2	0.020 $\pm$ 0.008	0.082 $\pm$ 0.030	0.009 $\pm$ 0.004	7
2–5	0.003 $\pm$ 0.001	0.040 $\pm$ 0.009	0.003 $\pm$ 0.002	6
Theoretical maximum‡	0.346	0.046	0.011	

\* n, number of data.

† n = 5.

‡ the theoretical maximum of affinity constants ( $\alpha$ ) based on the diffusion model assumes  $\alpha = (3D/\sigma)/r^2$ , where  $D$  is the diffusion constant for the substrate molecules (assumed  $\approx 10^{-5}$  cm<sup>2</sup> s<sup>-1</sup>),  $\sigma$  is the internal concentration of P, and  $r$  is the cell radius of osmotroph. The parameter  $\sigma$  is derived from the assumption that bacteria and phytoplankton cells have a density of 1.2 g cm<sup>-3</sup>, 50% dry weight of wet weight, 50% carbon of dry weight, and that a C:P ratio is 50 for bacteria (Fagerbakke et al. 1996) and 106 for phytoplankton (Redfield et al. 1963). The parameter  $r$  is assumed to be 0.25, 1, and 2  $\mu$ m for heterotrophic bacteria, cyanobacteria, and ANFs, respectively. See also Thingstad and Rassoulzadegan (1999).

viewed by Karl and Björkman 2002). The kinetics measurement of orthophosphate uptake cannot separate PO<sub>4</sub> concentration from the half-saturation constant (Thingstad et al. 1993). However, under the assumption of an approximate steady-state food web, the bioavailable phosphate can be estimated by using the data on particulate P concentration and <sup>32</sup>P release rate from the particulate fraction (Dolan et al. 1995; Thingstad et al. 1996; Tanaka et al. 2003). The estimates, which were obtained in different months and years but at the same site with relatively short turnover times of orthophosphate, ranged from 0.8 to 2 nmol P L<sup>-1</sup>, far below measurable levels with the standard technique. This suggests that the bioavailable phosphate concentration is kept very low compared to SRP in the surface mixed layer during the stratified period, although no information is available for its

seasonal variation. Ratios of SRP to the maximum potential uptake of orthophosphate (based on turnover time of orthophosphate and  $V_{\max}$ ) ranged from 0.8 to 8. This difference remains to be explained but again suggests that our SRP values sometimes included significant background. It is obviously a straight way to measure the size of the bioavailable phosphate pool, although this can only be assessed indirectly. Biomass P of osmotrophs at 10 m was dominated by heterotrophic bacteria (mean, 71%, n = 25), and the abundance of heterotrophic bacteria showed small seasonal variations (Fig. 4). Affinity constants for picophytoplankton and ANFs were almost at diffusion limitation during the stratified period (see below). We thus suggest that a slight increase in turnover time during the stratified period was a result of decrease in PO<sub>4</sub> uptake rate by bacteria.

As shown in Fig. 6B, <sup>32</sup>PO<sub>4</sub> uptake by the 0.2–0.6- and 0.6–2- $\mu$ m fractions was very sensitive to the increase of orthophosphate turnover time up to 10 h. This trend is similar to that found in the otherwise very different environment of Sandsfjord, western Norway (Thingstad et al. 1993), which suggests a generic pattern, robust to environmental differences in, e.g., salinity, temperature, and light conditions. Extrapolation of the regression for 0.2–0.6  $\mu$ m implies that the complete dominance of orthophosphate uptake (i.e., 100%) by bacteria is expected when turnover time is as short as 2 min, although such a short turnover time has not been observed in the Mediterranean (Dolan et al. 1995; Thingstad et al. 1998; Zohary and Robarts 1998; Moutin et al. 2002; Tanaka et al. 2003).

As long as there is diffusion limitation, the equation  $f/T = \alpha B$  (see Materials and methods) predicts that pairs of measured  $B$  and  $f/T$  fall along a straight line through the origin with slope  $\alpha$  (Fig. 9). When a substrate concentration increases beyond the level of diffusion limitation, measured points should fall below this line. The expected pattern in a plot of  $y = f/T$  versus  $x = B$  should thus be a cloud of points with the line  $y = \alpha x$  forming the upper envelope. Since, in our data, longer turnover time ( $T$ ) corresponded with smaller percent of total <sup>32</sup>P uptake by the bacterial fraction ( $f_B$ ; Fig. 6B), there was a large variation in the ratio  $f_B/T$ , with particularly low estimates when turnover time exceeded 2 h. All estimates fell well below the line corresponding to the theoretical maximum estimate for  $\alpha_B$  (Fig. 9A). For cyano-

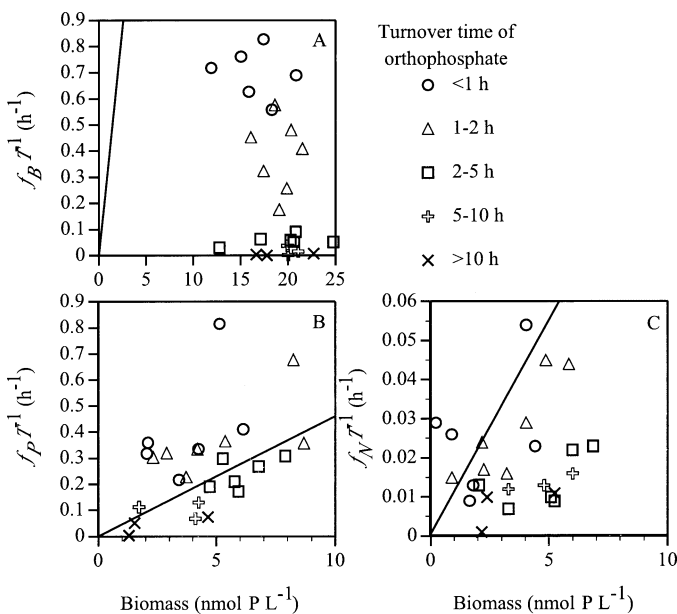


Fig. 9. Relationships between  $f/T$  (fraction of isotope uptake divided by orthophosphate turnover time) and biomass P of osmotroph for (A) bacteria, (B) picophytoplankton, and (C) ANFs, at 10 m from June to December 2002. Lines denote the theoretical maximum of affinity constant that is predicted by a diffusion model (see Table 3).

bacteria and ANFs, the cloud of points resembled the expected pattern with an upper envelope suggested by points for which the turnover time was less than 2 h (Fig. 9B,C). For cyanobacteria, this upper envelope indicated, however, an even higher affinity constant than our theoretical maximum estimate. We calculated the mean affinity constants ( $\pm$ SD) for different ranges of turnover time (Table 3). The affinity constant of ANFs on 22 August appeared to be outlier and not included for this calculation. Compared to the theoretical maximum, mean affinity constants for ANFs were fairly close and those of cyanobacteria were twofold to threefold greater in the range of turnover time <2 h. On the other hand, the mean affinity constant for bacteria was 1 order of magnitude smaller even when turnover time was <1 h. This appears to be contrary to the result of Thingstad et al. (1998) that showed the clear-cut evidence of P limitation on bacterial growth rate at the same site during the stratified period. We speculate that P was the most limiting factor for bacteria, but bioavailable phosphate concentrations were still not so extremely low that heterotrophic bacteria were diffusion limited. We can, however, not rule out the possibility that only a fraction of the microscopically observed bacteria were active and that our estimates of P in active bacteria were, thus, too high.

The mean ( $\pm$ SD) ratio of plankton counting-based P (picoplankton, nanoplankton, and ciliates) to chemically measured particulate P (0.2–200  $\mu$ m) was  $1.3 \pm 0.7$  ( $n = 20$ ) at 10 m during the study period (data on heterotrophic nanoplankton and ciliates not shown). Given that nonliving particles were not measured, our conversion factors for estimating biomass P could be uncertain for estimating affinity constants. The conversion factors for cyanobacteria (0.16 fmol P cell<sup>-1</sup>) and bacteria (0.03 fmol P cell<sup>-1</sup>) used in our study (Tanaka et al. 2003; the present study) were within the reported range in which cellular contents of phosphorus were directly measured (0.02 to 0.25 fmol P cell<sup>-1</sup> for *Synechococcus* spp. in P-limited medium, Bertilsson et al. 2003; Heldal et al. 2003; 0.01 to 0.07 fmol P cell<sup>-1</sup> for heterotrophic bacteria in P-limited medium and in different aquatic systems, Fagerbakke et al. 1996; Vrede et al. 2002). These studies have shown that cellular contents of phosphorus tend to decrease with increase in magnitude of P limitation but are variable among species and conditions. It will be necessary to measure both isotope uptake and biomass P of active osmotrophs in terms of P uptake for each time in order to improve the measurement of affinity constants in the field.

In summary, we presented that, in Villefranche Bay, (1) a significant portion of phosphorus was in the dissolved form in the whole water column (0–75 m), (2) turnover time of orthophosphate was relatively short (<2 h) in the surface mixed layer during the stratified period, (3) the dominance of PO<sub>4</sub> uptake gradually shifted from bacteria plus cyanobacteria to cyanobacteria with a slight increase of orthophosphate turnover time, (4) short-term fluctuations were observed in seasonal change of orthophosphate turnover time, and (5) mean affinity constants were highest for cyanobacteria. We believe that this information contributes to our understanding of dynamic features of the competitive relationship between bacteria and phytoplankton for P in a coastal system.

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