

Microbial stimulation of the aggregation process between submicron-sized particles and suspended particles in coastal waters

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ABSTRACT: Transfer rates of submicron-sized particles (SMP) to micro-suspended particles (5 to 20 μm in diameter) in coastal waters around Japan were measured using fluorescent beads (0.5 μm in diameter) and flow cytometry, with and without metabolic inhibitors (thiuram or NaN_3). This flow cytometric technique has the advantage that it allows the number of beads stuck to suspended particles to be counted without any pre-treatment, such as filtration, that can introduce artifacts. The transfer rates to particles in the 5–20 μm fraction without inhibitors were on average 4.3 to 5.8 times higher than with added NaN_3 . On the other hand, ingestion rates of fluorescent beads by protozoa in the 5–20 μm fraction were less than 10% of the transfer rates to particles in the 5–20 μm fraction. These results indicate that biological activities were more efficient than physical mechanisms for the transfer process of SMP into the 5–20 μm fraction separately from the grazing process. Also, the inhibitory effect of thiuram on the transfer rates suggests an involvement of eukaryotic cells in this stimulation. The intensity of this stimulation was positively correlated with the density of attached nanoflagellates per particle (Da) ($R = 0.630$, $n = 22$), bacterial abundance ($R = 0.489$, $n = 22$), concentration of chlorophyll *a* ($R = 0.518$, $n = 18$) and water temperature ($R = 0.569$, $n = 22$). These results indicate that biological processes are key mechanisms for the coagulation process of submicro- and micro-scale particles in coastal environments.

KEY WORDS: Particle aggregation · Colloidal particles · Sinking flux · Microbial food web · Collision efficiency · Sickness · Extracellular release

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INTRODUCTION

Submicron particles (SMP, 0.4 to 1 μm) belong to the larger group within the size range of marine colloids (0.001 to 1 μm) and are one of the most abundant particles classes in marine environments, consisting mainly of organic materials produced by plankton (Koike et al. 1990). Through the adsorption process of inorganic and organic compounds, SMP play a significant role in material transfer in planktonic food webs (Nagata & Koike 1995, Quigley et al. 2002, Kerner et al. 2003).

Much attention has been paid to physical aggregation processes as the major factor controlling the fate of various particles in marine environments. Aggregation processes were examined by comparing field data or laboratory experiments with aggregation models describing the physico-chemical behavior of particles

(McCave 1984, Jackson & Burd 1998, Mari & Burd 1998). Classical coagulation theory focuses on 3 particle collision mechanisms: Brownian motion, laminar and turbulent shear, and differential sedimentation (reviewed in Jackson & Burd 1998). However, biological activities are also, occasionally indispensable, factors in the aggregation process (Biddanda 1985). Planktonic organisms produce particulate organic matter, increase particle collision frequency (Johnson & Kepkay 1992, Fukuda & Koike 2000) and increase particle stickiness (Kjørboe & Hansen 1993, Passow 2000).

In previous studies, relative importance of biological processes and physico-chemical processes in particle aggregation was examined by observing the amount of aggregates in cultures with or without biological activities (reviewed in Biddanda 1985). Therefore, it was dif-

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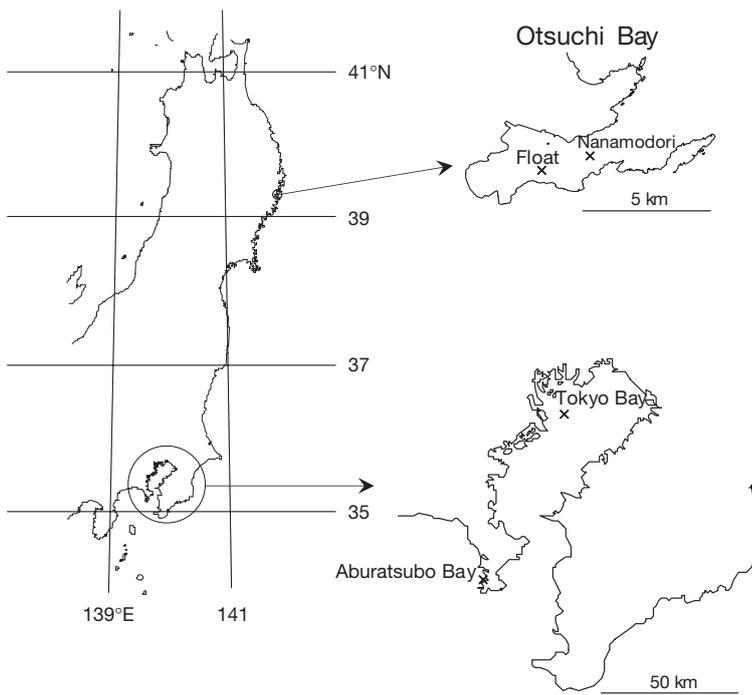


Fig. 1. Study sites (X) in Tokyo Bay, Otsuchi Bay and Aburatsubo Bay

difficult to quantify the relative contributions of biological and physico-chemical processes to aggregation. To understand the role of biological activities in marine particle dynamics, however, it is crucial to obtain the coagulation rate with and without biological processes using ambient seawater samples.

This study quantitatively examined the contribution of biological processes to the transfer process of SMP onto micro-suspended particles (5 to 20 μm in diameter) in coastal waters by using fluorescent beads as model SMP (0.5 μm in diameter) and flow cytometric technique. Transfer rates of fluorescent beads to particles in the 5–20 μm fraction were compared with biological and other parameters obtained in coastal waters, to examine the factors controlling biological stimulation of SMP aggregation transferred into particles in the 5–20 μm fraction.

MATERIALS AND METHODS

Study site and sample collection. The incubation experiments were conducted by using seawater samples collected from the coastal area of Japan. Fig. 1 summarizes the location of each station and the date of the survey. In Otsuchi Bay, the samples were collected by a 5 l Van Dorn sampler at the center of the bay (39° 20' N, 141° 56' E), or from the pier (39° 20' N, 141° 57' E) with a plastic bucket. The bucket was washed with

HCl and Milli-Q water. Tokyo Bay samples (35° 34' N, 139° 49' E) were collected by a 24 l Van Dorn sampler. Aburatsubo Bay samples (35° 54' N, 139° 20' E) were collected from a pier, using a metal jug, which was washed with detergent and then rinsed with Milli-Q water.

To examine the role of the microbial community associated with settling particles on the SMP collision process, settling organic aggregates and associated microorganisms were collected by using a sediment trap at the station (Float or Nanamodori) in Otsuchi Bay. Two sediment traps, which had square meshed mouths (23 cm in diameter), were moored 15 m from the bottom of Float or Nanamodori stations for ca. 48 h. The trap was washed with detergent and Milli-Q water, but the acrylic cups were washed with HCl and Milli-Q water. To fill the trap before the mooring, we used seawater filtered through a polycarbonate filter (pore size: 5 μm ; Whatman) or a GF/C filter (Whatman). This procedure was to remove large particles, but to keep

small particles such as bacteria as food for possible suspension feeding protozoa on settling particles during a ca. 48 h mooring time. Collected aggregates were re-suspended and diluted with filtered seawater using a similar procedure for use in the experiments. During all of the sampling procedures, plastic gloves were worn to minimize the contamination by organic and inorganic compounds.

Transfer rate of fluorescent beads to 5–20 μm particles by natural microbial assemblages. Experiments were designed to observe the stimulation of fluorescent bead (0.5 μm) attachment to particles in the 5–20 μm fraction by microbial assemblages in coastal seawater. Since solid surfaces are negatively charged in solution in most cases, carboxylate-modified beads were chosen as tracer particles (Molecular Probes). The water samples or the re-suspended organic aggregates were filtered through a 94 μm mesh net to remove net-zooplankton and then split into four 250 ml polycarbonate bottles. Two controls were also prepared, one with the eukaryotic inhibitor, thiuram (final concentration 20 μM ; Taylor & Pace 1987) and the other with an inhibitor of the respiratory chain (sodium azide; final concentration 0.02%, w/v). One subsample and 2 controls were amended with fluorescent beads (final concentration 1.5×10^6 particles ml^{-1}) and incubated for 24 or 36 h at *in situ* temperature ($\pm 1.5^\circ\text{C}$) in the dark. One of the subsamples was used to determine the ingestion rate, biological parameters and the

number of particles in the 5–20 μm fraction. The other subsample was incubated under the same conditions without the addition of fluorescent beads. For Tokyo Bay samples, however, the incubation was conducted at 20°C, which was close to the *in situ* temperatures of 24, 22 and 22°C at 0, 10 and 16 m, respectively.

The abundance of beads associated with particles in the 5–20 μm fraction was monitored every 6 or 8 h during the incubation using a flow cytometer (BRYTE, Bio-Rad; Fukuda & Koike 2000). The flow cytometers were equipped with a 100 μm orifice and 2 filters: 470 to 490 nm and 515 to 565 nm for excitation and emission, respectively. Fluorescent beads sticking onto particles in the 5–20 μm fraction were distinguished from mono-dispersed beads in the solution by increases in the signals of forward angle light scattering, which was calibrated by comparison with the standard latex beads (1.18, 5.06, 10.0 and 17.6 μm in diameter; Beckman-Coulter). The number of fluorescent beads on a particle in the 5–20 μm fraction was estimated from the intensity of yellow-green fluorescence assuming that fluorescence increases linearly with increasing number of beads included in the aggregate. Autofluorescence of suspended particles in the 5–20 μm fraction under the combination of excitation and emission filters was less than 25% of single fluorescent beads (data not shown). Quenching of fluorescence from the opposite side of the suspended particle, however, may occur, which would cause an underestimation of the number of transferred beads. The number of beads on particles in the 5–20 μm fraction in 75 μl of subsamples was counted 3 times for each sample.

During the middle of the incubation period, an experiment to determine ingestion rates of beads by protozoa was conducted. A subsample was amended with fluorescent beads (final concentration 1.5×10^6 particles ml^{-1}) and incubated in the dark for 15 min at the same temperature as the 24 or 36 h incubation experiments. To observe the initial accumulation process into the protozoan food vacuole, ingestion rate was obtained from a 15 min incubation period, since the number of fluorescent beads in the food vacuole saturates at about 20 min (Sherr et al. 1987). After incubation, the sample was fixed with ice-cold glutaraldehyde and the number of beads in food vacuoles was counted to estimate the ingestion rates of flagellates and ciliates (Sherr et al. 1987). Organisms were stained by FITC and DAPI (Sherr & Sherr 1983) to discriminate between beads attached to cell surfaces and those ingested. Counting was conducted 3 times for each sample and at least 400 or 500 attached eukaryotes were counted in all. The detection limit of ingestion rate is 1.0 or 0.80×10^{-2} beads $\text{cell}^{-1} \text{h}^{-1}$, respectively. During the middle of the incubation period, samples for the determination of biological and other parameters (abundance of bacteria, free-living nano-

flagellates and attached nanoflagellates, chlorophyll *a* (chl *a*), total number of particles in the 5–20 μm fraction and density of attached nanoflagellates per 5–20 μm particle) were also collected from untreated incubations without the addition of fluorescent beads.

Subsamples for the determination of bacterial abundance and the number of particles in the 5–20 μm fraction were fixed with formaldehyde (final concentration 2%, v/v), whereas those for the determination of abundance of nanoflagellates were fixed with glutaraldehyde (final concentration 1%, v/v). These fixed samples were stored at 4°C in the dark until analysis. Subsamples for determination of chl *a* were immediately filtered on GF/F filters and then stored at –20°C in the dark.

Determination of biological parameters and abundance of particles in the 5–20 μm fraction. Bacterial abundance was counted directly by epifluorescence microscopy after DAPI staining (Porter & Feig 1980), whereas those of free-living and attached nanoflagellates were counted after staining with DAPI and FITC without discriminating pigmented or non-pigmented cells (Sherr & Sherr 1983). Attached nanoflagellates were differentiated from nanoflagellates by checking the contact of nanoflagellates with particles under epifluorescent microscopy. After counting, the number of attached nanoflagellates was doubled to account for them on the invisible side of particles (modification of Caron 1987). Chl *a* concentrations were determined using fluorometry (Fluorometer 10-AU, Turner Designs; Holm-Hansen et al. 1965) after extraction with *N,N*-dimethylformamide (Suzuki & Ishimaru 1990) at –20°C for more than 2 d. The number of particles in the 5–20 μm fraction was determined by using a coulter particle counter (Multisizer II, Beckman Coulter) equipped with a 50 μm orifice. The density of attached nanoflagellates was calculated by dividing the abundance of attached nanoflagellates by the number of particles in the 5–20 μm fraction.

Statistical analysis. All of the statistical analyses were conducted using Sigma Stat (SPSS). The transfer rate of fluorescent beads was calculated from the time course, applying linear regression. The difference among transfer rates and ingestion rates were examined with a *t*-test. To examine the relationship between the transfer rate and other variables, Model II regression (Ricker 1973) was used. For regression analysis, log transformed variables were used to test whether the Kolmogorov-Smirnov distance was made smaller by the transformation. A backward stepwise multiple linear regression analysis was used to examine whether the variable was useful for explaining the variation of the dependent variable. At each step of the analysis, the independent variable was removed if its *F*-value was the minimum among independent variables and was lower than 2.

RESULTS

Vertical profiles of transfer rates of fluorescent beads to particles in the 5–20 μm fraction at Tokyo Bay and Otsuchi Bay

Fig. 2 shows vertical profiles of water temperature, salinity, total number of particles in the 5–20 μm fraction, microbial cell abundance, concentration of chlorophyll *a* (chl *a*) and density of attached nanoflagellates at the Tokyo Bay station on 29 September

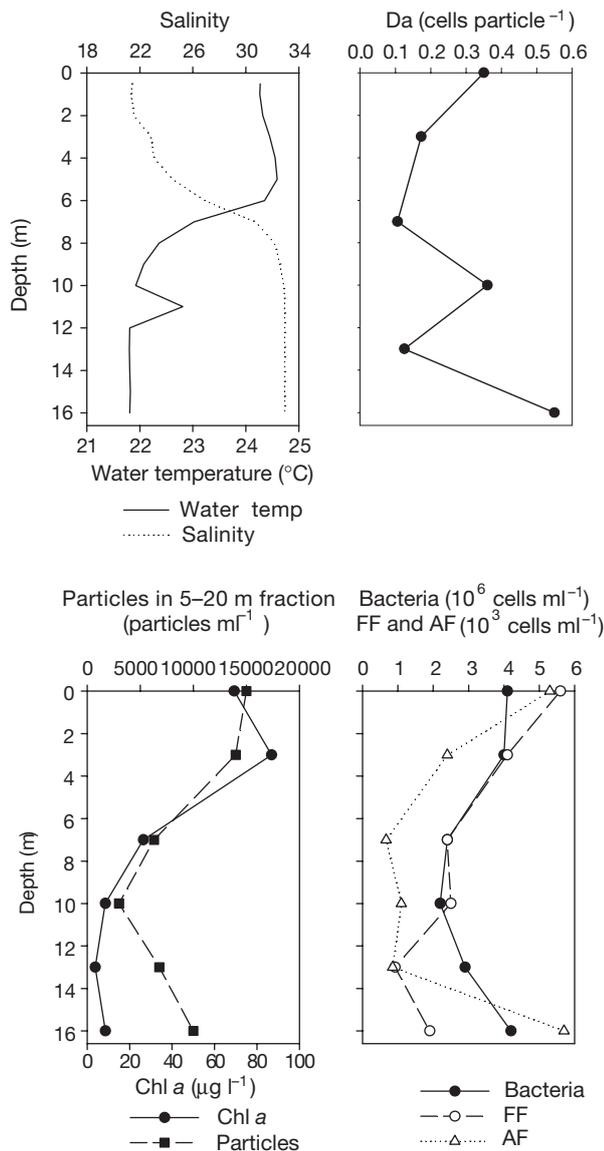


Fig. 2. Vertical profiles of water temperature, salinity, density of attached nanoflagellates (Da), total particle number in the 5–20 μm fraction, concentration of chlorophyll *a* (chl *a*), bacterial abundance (Bacteria) and abundance of free-living or attached nanoflagellates (FF or AF, respectively) at Tokyo Bay on 29 September 1999

1999. The surface mixed layer was about 4 to 5 m deep. The thermocline and halocline were located at a depth of ca. 5 to 7 m. Chl *a* in the 0–3 m layer was very high (65 to 85 $\mu\text{g l}^{-1}$) and decreased sharply below that depth. On the other hand, the number of particles in the 5–20 μm fraction had a minimum value at 10 m, suggesting accumulation and/or resuspension of settled organic detritus near the bottom (16.6 m) (Fig. 2). Incubation experiments were conducted using the water samples collected from the surface, from 10 m, where the number of particles in the 5–20 μm fraction was at a minimum, and from the bottom, where an increased number of particles in the 5–20 μm fraction was observed.

Fig. 3 shows the time course of the number of fluorescent beads on particles in the 5–20 μm fraction using the samples collected from 0, 10 and 16 m. The measured transfer rates in untreated cultures were 3.5 to 8.5 times higher than those in NaN_3 -treatment (V_N/V_A) (Table 1). The V_N/V_A ratios showed some decrease with depth, although all of the ratios were significantly higher than 1 ($p < 0.001$). The differences between those treated with thiuram and NaN_3 were not significant or smaller than those with no treatment. The number of fluorescent beads in the food vacuoles of microorganisms in the 5–20 μm size range did not increase significantly ($p > 0.05$) in all incubations (Table 1). From the abundance of attached nanoflagellates and the detection limit of ingestion rate (1.0 or 0.80×10^{-2} beads cell⁻¹ h⁻¹), the contributions of ingestion rate by the population of attached nanoflagellates to the transfer rate in the untreated incubations were less than 5%.

Similar depth profiles of the transfer rates and environmental and biological parameters were also obtained using Otsuchi Bay samples collected on 24 May 2000 (Fig. 4, Table 1). Low salinity between depths of 1 to 2 m was probably caused by river inflow. Chl *a* in the bay ranged from 1 to 2 $\mu\text{g l}^{-1}$ within the top 13 m and decreased sharply towards the bottom at a depth of 44 m (Fig. 4). The number of particles in the 5–20 μm fraction at Otsuchi Bay (2.1 to 4.1×10^2 particles ml⁻¹) was more than 1 order of magnitude lower compared to those samples from Tokyo Bay in the summer (3.0 to 15×10^3 particles ml⁻¹). The number of particles in the 5–20 μm fraction had a minimum at a depth of 13 m and then increased remarkably with depth towards the bottom. The transfer rates in untreated incubations were significantly higher than those in NaN_3 -treatment incubations except at a depth of 42 m ($p < 0.001$), and the V_N/V_A ratio varied drastically from 1.4 at a depth of 42 m to 10 at the surface. Similarity in vertical trends between the V_N/V_A ratio and other parameters was not observed (Fig. 4, Table 1). The contribution of ingestion by protozoa to the transfer rate was less than 10% in these incubations.

Changes in transfer rates of fluorescent beads to particles in the 5–20 μm fraction at Otsuchi Bay and Aburatsubo Bay

Using surface waters of Otsuchi Bay, periodic changes in biological parameters and in the transfer rates of fluorescent beads to particles in the 5–20 μm fraction were examined from February to May 2000 (Fig. 5, Table 2). Water temperature decreased slightly from February to April and then increased rapidly in May (Fig. 5). The number of particles in the 5–20 μm

fraction, abundance of free-living or attached nanoflagellates and chl *a* showed some variability with time, but did not increase significantly compared to the increase in water temperature. One exception was the abundance of bacteria, which showed a significant increase in the May sample.

The transfer rates in untreated incubations were significantly higher ($p < 0.01$) than those in the thiuram-treatment incubation, except on 16 February and 12 April (0 and 43 m depth), and also significantly higher ($p < 0.001$) than the NaN_3 -treatment incubation

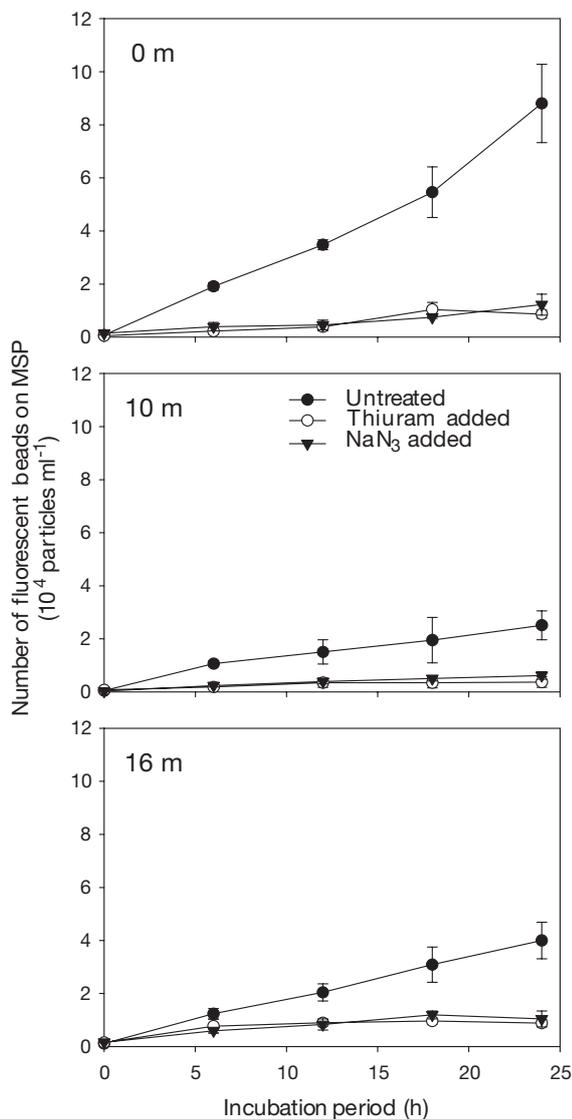


Fig. 3. Time course of the number of fluorescent beads on 5–20 μm particles in 3 different cultures: untreated, thiuram added, and NaN_3 added; using water samples collected from Tokyo Bay on 29 September 1999 at different depths (0, 10 and 16 m). Error bars indicate 1 SD

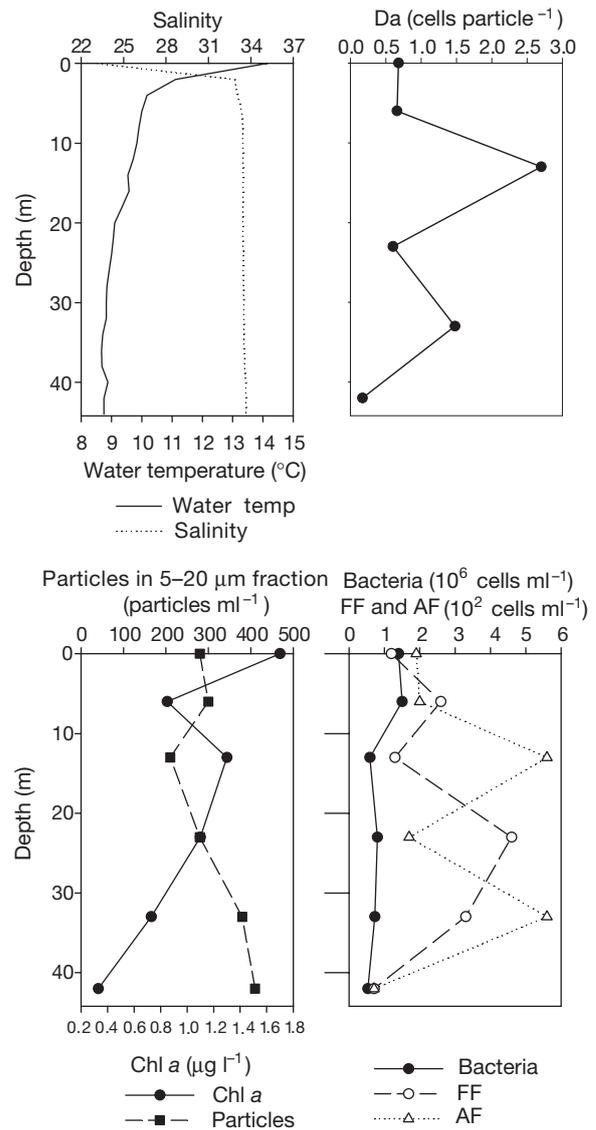


Fig. 4. Vertical profiles of water temperature, salinity, density of attached nanoflagellates (Da), total particle number in the 5–20 μm fraction, concentration of chlorophyll *a* (chl *a*), bacterial abundance (Bacteria) and abundance of free-living or attached nanoflagellates (FF or AF, respectively) at Otsuchi Bay on 24 May 2000

except for 12 April (0 and 43 m depth) (Table 2). The V_N/V_A ratio ranged from 1.1 to 10 during the observation period, but significant correlations between the change of the V_N/V_A ratio and other parameters were not observed ($p > 0.05$, $n = 8$ or 9), except for water temperature ($R = 0.693$, $p < 0.05$, $n = 9$). The ingestion rate of fluorescent beads by protozoa was under the

detection limit and the contribution of ingestion rate by protozoa to the transfer rate was less than 10% (Table 2).

Periodic changes in biological parameters and in the transfer rates of fluorescent beads to particles in the 5–20 μm fraction from August to September 2000 were also examined using the water samples collected from

Aburatsubo Bay (Fig. 6, Table 2). Water temperature at Aburatsubo Bay remained almost constant, while chl *a* changed from 0.76 to 5.3 $\mu\text{g l}^{-1}$ during the observation period (Fig. 6). In addition, the densities of attached nanoflagellates, the number of particles in the 5–20 μm fraction and the abundance of attached nanoflagellates changed significantly, and had their maximum on 29 August 2000, which was similar to chl *a*. The transfer rates in untreated incubations were significantly higher ($p < 0.001$) than those in the thiuram- or NaN_3 -treatment incubations (Table 2). Although biological parameters changed as indicated above, the V_N/V_A ratio did not change much during the observation period and the trend of the V_N/V_A ratio was similar to that in incubation temperature. The contribution of ingestion rate by protozoa to the transfer rate was also less than 5% (Table 2).

Table 1. Depth profile of transfer rate (mean, $n = 5$) of fluorescent beads to micro-suspended particles (5 to 20 μm in diameter) with or without metabolic inhibitors, ingestion rate (mean, $n = 3$) of beads in cultures without metabolic inhibitors and the V_N/V_A ratio during the incubation of water samples collected from Tokyo Bay on 29 September 1999 and Otsuchi Bay 24 May 2000. V_N : no treatment; V_T : thiuram treatment; V_A : NaN_3 treatment; ns: statistically significant increase was not observed ($p > 0.05$). $\pm\text{SE}$ is shown

Depth (m)	Transfer rate (10^2 particles $\text{ml}^{-1} \text{h}^{-1}$)			Ingestion rate (10^2 particles $\text{ml}^{-1} \text{h}^{-1}$)	V_N/V_A ($\pm\text{SE}$)
	V_N	V_T	V_A		
Tokyo Bay					
0	33 \pm 2	3.3 \pm 0.5	3.9 \pm 0.4	ns	8.5 \pm 0.1
10	11 \pm 0.1	1.5 \pm 0.2	2.6 \pm 0.1	ns	4.2 \pm 0.1
16	16 \pm 0.2	4.1 \pm 0.8	4.7 \pm 0.1	ns	3.5 \pm 0.1
Otsuchi Bay					
0	7.7 \pm 0.5	ns	0.50 \pm 0.10	0.051 \pm 0.019	10 \pm 0.2
13	2.5 \pm 0.6	ns	0.25 \pm 0.06	ns	9.9 \pm 0.3
42	0.38 \pm 0.01	0.30 \pm 0.07	0.27 \pm 0.02	0.017 \pm 0.002	1.4 \pm 0.1

Table 2. Daily changes of transfer rate (mean, $n = 5$) of fluorescent beads to micro-suspended particles (5 to 20 μm in diameter) with or without metabolic inhibitors, ingestion rate (mean, $n = 3$) of beads in cultures without metabolic inhibitors and the V_N/V_A ratio during the incubation of water samples collected from the surface layer (0 to 10 m) in Otsuchi Bay from 16 February to 24 May 2000 and from Aburatsubo Bay from 15 August to 5 September 2000. V_N : no treatment; V_T : thiuram treatment; V_A : NaN_3 treatment; ns: statistically significant increase was not observed ($p > 0.05$). $\pm\text{SE}$ is shown

Date	Transfer rate (particles $\text{ml}^{-1} \text{h}^{-1}$)			Ingestion rate (particles $\text{ml}^{-1} \text{h}^{-1}$)	V_N/V_A ($\pm\text{SE}$)
	V_n	V_T	V_A		
Otsuchi Bay					
16 Feb	74 \pm 2	48 \pm 15	12 \pm 2	ns	6.4 \pm 0.2
6 Apr	63 \pm 8	14 \pm 3	17 \pm 5	ns	3.7 \pm 0.3
7 Apr	65 \pm 11	ns	28 \pm 6	ns	2.3 \pm 0.3
8 Apr	30 \pm 2	ns	16 \pm 3	ns	1.9 \pm 0.2
9 Apr	55 \pm 11	28 \pm 3	18 \pm 4	ns	3.0 \pm 0.3
10 Apr	47 \pm 4	13 \pm 3	17 \pm 4	ns	2.8 \pm 0.2
11 Apr	63 \pm 8	22 \pm 2	9.6 \pm 2.5	ns	6.5 \pm 0.3
12 Apr	40 \pm 4	39 \pm 6	36 \pm 8	ns	1.1 \pm 0.2
24 May	750 \pm 50	ns	50 \pm 10	ns	10 \pm 0.2
Aburatsubo Bay					
15 Aug	4.3 \pm 0.2	0.86 \pm 0.08	0.37 \pm 0.12	ns	4.7 \pm 0.1
22 Aug	6.0 \pm 0.6	0.45 \pm 0.08	0.76 \pm 0.12	ns	6.5 \pm 0.6
29 Aug	14 \pm 1	1.6 \pm 0.2	2.1 \pm 0.1	ns	6.7 \pm 0.1
5 Sep	13 \pm 0.4	2.3 \pm 0.3	2.4 \pm 0.2	0.50 \pm 0.0	5.3 \pm 0.1

Effect of incubation temperature on transfer rate of fluorescent beads to re-suspended particles collected by sediment trap

The effect of incubation temperature on the transfer rates of fluorescent beads to the 5–20 μm particles was examined using the resuspended particles collected by the sediment trap deployed at Otsuchi Bay (Fig. 7). Among the samples, under 4 different incubation temperatures, differences in biological parameters (bacteria: 1.1 to 1.6 $\times 10^6$ cells ml^{-1} , free-living nanoflagellates: 3.7 to 4.0 $\times 10^2$ cells ml^{-1} , attached nanoflagellates: 3.9 to 6.8 $\times 10^2$ cells ml^{-1} , density of attached nanoflagellates: 0.21 to 0.40 cells particle $^{-1}$) during an incubation period of 18 h were less than 25% CV. The number of particles in the 5–20 μm

fraction (1.4 to $1.8 \times 10^3 \text{ ml}^{-1}$) did not change significantly ($p > 0.05$) and was at a similar level to those in the ambient water samples from Aburatsubo Bay in summer.

All of the transfer rates in the untreated incubation, under 4 different temperature conditions (3.0 to 20.0°C), were significantly higher than those in the NaN_3 -treatment incubations ($p < 0.01$; Fig. 7), and the transfer rates obtained from untreated incubation under 4 different temperature conditions had a peak at 14.0°C , while those in the NaN_3 -treatment (V_A) incubations showed an increase proportional to temperature ($V_A = 1.1 \times (\text{incubation temperature}) + 27$, $R^2 = 0.931$, $p < 0.05$, $n = 4$). The V_N/V_A ratio also peaked in the 14.0°C incubation. The contribution of ingestion rate by protozoa to transfer rate was less than 5%.

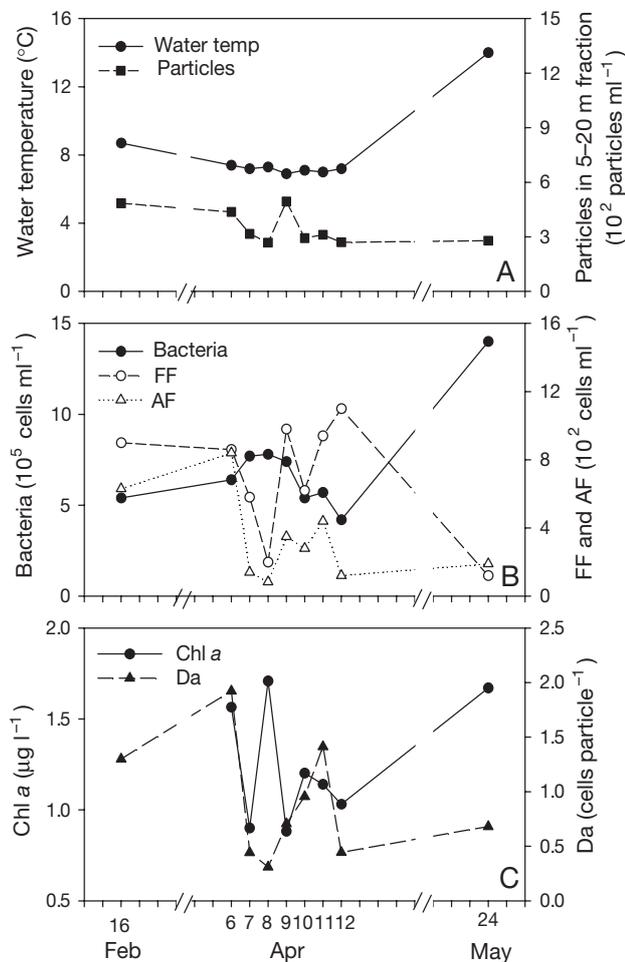


Fig. 5. Time series in (A) water temperature and total particle number in the 5–20 μm fraction, (B) bacterial abundance (Bacteria) and abundance of free-living and attached nanoflagellates (FF and AF, respectively), (C) concentration of chlorophyll *a* (chl *a*) and density of attached nanoflagellates per particle (Da) from 16 February to 24 May 2000 at the surface of Otsuchi Bay

DISCUSSION

Flow cytometry and experimental design for determination of particle flux of SMP to larger suspended particles

This study focuses on a new approach for quantitatively examining the microbial enhancement of aggregation by using flow cytometry with fluorescent beads and 2 types of metabolic inhibitors. Most previous studies on biological stimulation of aggregation were limited to qualitative analysis during the course of aggregation (reviewed in Biddanda 1985). By using fluorescent beads as a tracer particle, transport of a particle can be detected by flow cytometry without interference from suspended particles. More impor-

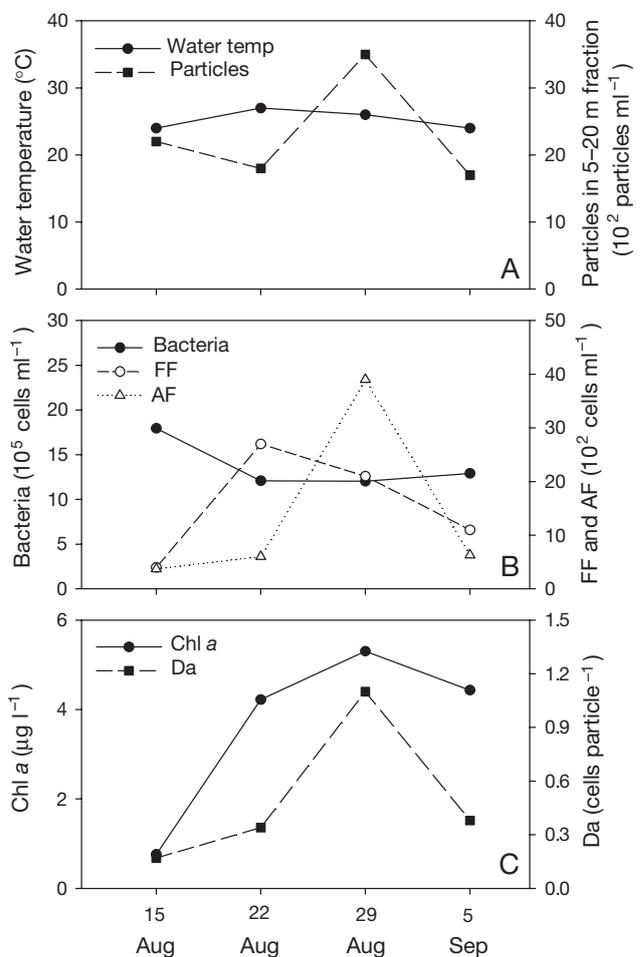


Fig. 6. Time series in (A) water temperature and total particle number in the 5–20 μm fraction, (B) bacterial abundance (Bacteria) and abundance and free-living or attached nanoflagellates (FF and AF, respectively), (C) concentration of chlorophyll *a* (chl *a*) and density of attached nanoflagellates per particle (Da) from 15 August to 5 September 2000 at the surface of Aburatsubo Bay

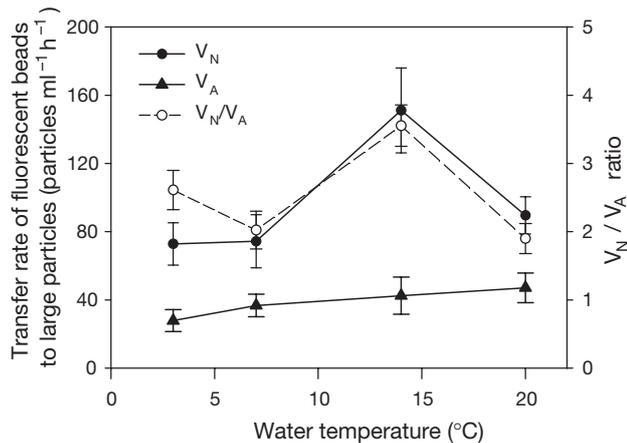


Fig. 7. Relationships between incubation temperature and the transfer rate of fluorescent beads to MSP in untreated culture (V_N), in the NaN_3 added culture (V_A) and the V_N/V_A ratio in the experiment using resuspended aggregates collected by sediment trap at Otsuchi Bay from 13 to 15 March 2000. Error bars indicate 1 SE

tantly, this flow cytometric technique does not need any pre-treatment such as particle collection; therefore, the creation of artifacts, such as change of particle size distribution and additional particle transfer during pre-treatment, is minimized. One possible restriction of this technique is that it requires more than 450 particles ml^{-1} in the 5–20 μm fraction to measure enough particles during the standard 1–2 min flow cytometer sampling period for statistical reliability. It may, therefore, be difficult to apply this technique to samples from oligotrophic waters.

The fluorescent beads used in this study may have different properties from natural marine colloids in specific gravity and surface stickiness. However, collision frequency by differential sedimentation is mainly determined by the settling velocity of the 5–20 μm sized particle rather than that of the fluorescent beads and the difference in stickiness is canceled in a V_N/V_A ratio of transfer rate in 2 different cultures (Fukuda & Koike 2000). Regarding the effect of inhibitors (thiuram and NaN_3) on beads stickiness, the size distribution of beads in filtered seawater did not change significantly with or without inhibitors during a 24 h incubation period (data not shown). NaN_3 chelates cations such as Mg^{2+} and Ca^{2+} , which in turn leads negatively charged material to stick to the beads, but the concentration of NaN_3 in our experiments (3.1 mM) was insufficient to chelate all of the Mg^{2+} and Ca^{2+} in the cultures (more than 32 and 6.2 mM, respectively, Wilson 1975). Therefore, we considered that the use of inhibitors did not directly change the stickiness of particles.

To compare our results with the aggregation processes in marine environments, the effects of laminar

or turbulent shear must be considered, since only Brownian motion and differential sedimentation occur in our experimental bottles. The effectiveness of advective transport induced by sinking or by shear compared with diffusive transport is represented as the Péclet number (reviewed in Karp-Boss et al. 1996). Because the size of SMP (0.4 to 1 μm) is in the upper bracket of colloidal particles (1 nm to 1 mm), diffusivity of SMP is calculated using Eq. (2) (see below). For encounters between SMP (0.4 to 1 μm) and particles in the 5–20 μm fraction by differential sedimentation, the Péclet number (Pe) is 2.4 to 73 assuming an average sedimentation velocity of 1 to 3 $\mu\text{m s}^{-1}$ (Bienfang 1985). On the other hand, the Péclet number for particles in steady shear (Pe_{shear} ; reviewed in Karp-Boss et al. 1996) is 1.7 to 69 and 17 to 690 with energy dissipation rates of 1×10^{-4} and $1 \times 10^{-2} \text{ cm}^2 \text{ s}^{-3}$, respectively (Oakey 1985). These Pe and Pe_{shear} numbers indicate that an increase in encounter rate under the existence of shear reaches a maximum about 2 times higher than that without shear (Karp-Boss et al. 1996).

Importance of biological processes for the aggregation process

In this study, transfer rates of fluorescent beads to particles in the 5–20 μm fraction in seawater samples collected from 3 coastal areas of Japan were determined. Several lines of evidence indicate that biologically mediated physical collision is involved in the transfer of fluorescent submicron-sized beads to particles in the 5–20 μm fraction. The first line of evidence is that comparison between the measured transfer rate of fluorescent beads to particles in the 5–20 μm fraction and the ingestion rate of the beads by protozoa associated with the particles showed that most of the transfer process was the result of physical processes, i.e. physical collision and subsequent sticking of the beads to particles in the 5–20 μm fraction (Tables 1 & 2). The second line of evidence is that most of the transfer rates obtained in untreated incubation were significantly higher than those in the NaN_3 -treatment incubation (Tables 1 & 2). This suggests that biological processes stimulate the aggregation process between submicron-sized beads and particles in the 5–20 μm fraction. In addition, temperature dependency of the transfer rate also supports the possible dominance of biological processes in untreated incubation, since transfer rate in untreated incubation had an optimum temperature, while that in the NaN_3 -treatment incubation increased proportionally with increase of incubation temperature (Fig. 7). The mean values of the V_N/V_A ratio at each sampling site were 4.3, 5.4 and 5.8 for Tokyo Bay, Otsuchi Bay and Aburatsubo Bay, respectively. Since

these mean values of the V_N/V_A ratio is greater than 1, even when V_A is doubled to estimate the physical encounter rate in a mixed layer (see above), these results suggest that biological stimulation of the transfer process of SMP into the 5–20 μm fraction exists in at least the upper layer of coastal waters.

Enhancement of the transfer process of SMP into the 5–20 μm fraction in the coastal waters was also inhibited by the addition of thiuram, except for a few occasions at Otsuchi Bay (Tables 1 & 2). Although thiuram has been reported to inhibit protein synthesis in eukaryotic cells (Taylor & Pace 1987), the exact mechanisms of the inhibitory effect of thiuram on the incorporation process of SMP into the 5–20 μm particles are difficult to identify. Taylor & Pace (1987) reported that thiuram inhibited the growth of protozoa and chlorophyll synthesis of phytoplankton. Thiuram also probably inhibits other metabolic pathways in eukaryotes. If thiuram is assumed to be a specific metabolic inhibitor for eukaryotes, the results of this observation suggest that the transfer process of SMP into the 5–20 μm fraction was caused by eukaryotes such as nanoflagellates separately from particle ingestion (Fukuda & Koike 2000).

Mechanisms for biological stimulation of particle aggregation

Several mechanisms are potential candidates for the biological stimulation of particle aggregation observed in this study. The first mechanism is the feeding current of attached nanoflagellates stimulating the collision rate between SMP and 5–20 μm sized particles (Fukuda & Koike 2000). This process is similar to the advection between SMP and settling large particles (Karp-Boss et al. 1996). The effect of this physical forcing increases proportionally with the increase of density of attached nanoflagellates on particles in the 5–20 μm fraction (Da) and increases with the average velocity of the feeding current of attached nanoflagellate assemblages, which may be affected by prey abundance, water temperature (Vaqué et al. 1994) or the species composition of nanoflagellates. The second mechanism is the increase in stickiness of particles in the 5–20 μm fraction due to bacterial, protozoa and phytoplankton metabolisms (Biddanda 1985, Koike et al. 1990, Kjørboe & Hansen 1993, Nagata 2000, Passow 2000). Sticky extracellular materials released from microbes stimulate the aggregation process by increasing the probability of particles sticking together after collision.

To examine the mechanisms stimulating SMP aggregation by biological processes, regression analysis between the V_N/V_A ratio and experimental condition and abundance of microorganisms was performed.

Significant correlation was observed with Da ($R = 0.630$, $p < 0.05$, $n = 22$), concentration of chlorophyll *a* ($R = 0.518$, $p < 0.05$, $n = 18$), bacterial abundance ($R = 0.489$, $p < 0.05$, $n = 22$) and incubation temperature ($R = 0.569$, $p < 0.01$, $n = 22$), but not with total particle number of particles in the 5–20 μm fraction and abundance of free-living or attached nanoflagellates ($p > 0.05$, $n = 22$ for these 3 variables). Positive correlation with Da suggests that the feeding current of attached nanoflagellates played an important role in experimental cultures. Also, positive correlation with chl *a* may indicate the change in particle stickiness in experimental cultures. As regards bacterial abundance, however, it is not clear why it correlates with the V_N/V_A ratio, since the biological stimulation observed was inhibited by the eukaryotic inhibitor, thiuram. Taylor & Pace (1987) reported that some bacterial activities were inhibited by addition of thiuram. Bacterial release of organic material and ectoenzymes or bacterial hydrolysis may stimulate the transfer process of SMP into the 5–20 μm fraction by changing particle stickiness. On the other hand, bacterial abundance may affect protozoan grazing rather than particle stickiness.

The correlation between the V_N/V_A ratio and the abundance of nanoflagellates was not significant ($p > 0.05$, $n = 22$). This suggests released materials produced by nanoflagellates did not contribute to the stimulation of the transfer process of SMP into the 5–20 μm fraction. Additionally, increased incubation temperature may affect these processes by stimulating protozoan grazing activity and release rate of extracellular materials by phytoplankton (Zlotnik & Dubinsky 1989, Rivkin et al. 1999). The increase of stickiness caused by the above processes would be cumulative during the incubation period. However, a non-linear time course of bead number on particles in the 5–20 μm fraction was not observed (e.g. Fig. 3).

Allredge & McGillivray (1991) reported the particle stickiness to be 0.60 for amorphous detrital aggregates and 0.88 for flocculated diatoms. Even if the stickiness of marine aggregates increases to their maximum value (=1), the coagulation rate will only be stimulated 1.1 to 1.7 times. The V_N/V_A ratios obtained in these experiments were significantly higher than those in most other experiments (the average of 3 locations ranged from 4.3 to 5.8) and the maximum V_N/V_A ratio reached 10 (Tables 1 & 2). The change in particle stickiness was not determined, but this high V_N/V_A ratio value suggests that the increase in stickiness would be a minor contributing factor to the observed biological stimulation of aggregation, even if the increase in stickiness occurs.

On the other hand, feeding currents of attached nanoflagellates could explain the stimulation of the transfer process of SMP into the 5–20 μm fraction to a significant extent. The effectiveness of both feeding

current of attached nanoflagellate on a particle and particle settling on particle encounter rate can be expressed as the Sherwood number (Sh), which indicates the relative enhancement of particle encounter due to advection against a purely diffusional encounter. The Sh number under uniform flow is given by:

$$Sh = \frac{1}{2} \left[1 + \left(1 + 2 \frac{Ur_1}{D} \right)^{\frac{1}{3}} \right] \quad (1)$$

where U is the settling velocity of 5–20 μm particles or velocity of SMP in the flow field of feeding current, r_1 is the radius of the 5–20 μm particles and D is Brownian diffusivity (Karp-Boss et al. 1996). Brownian diffusivity is determined by the equation:

$$D = \frac{KT}{6\pi\mu r_2} \quad (2)$$

where K is Boltzmann's constant, T is the absolute temperature, μ is dynamic viscosity, r_2 is the radius of SMP. Assuming that 1 attached nanoflagellate generating a steady flow of 220 $\mu\text{m s}^{-1}$, which is an average swimming speed of nanoflagellates (85 to 360 mm s^{-1} ; Goldman 1984), harbors on one 5–20 μm particle and that the average settling velocity of the particle is 1 to 3 $\mu\text{m s}^{-1}$ (Bienfang 1985), then the Sh number for feeding current (Sh_{fc}) or for particle settling (Sh_{st}) would be 6.0 to 9.2 or 1.5 to 2.6, respectively. As a first order approximation, the V_N/V_A ratio is regarded to be Sh_{fc}/Sh_{st} neglecting particle settling in V_N . The estimated V_N/V_A ratio is 3.3 to 4.7. In our observation, the mean value of V_N/V_A when 1 flagellate attaches to a 5–20 μm sized particle was 6.0 to 6.7 (95% CI), which is estimated from the regression line ($\text{Log } [V_N/V_A] = 0.86 \text{ Log}$

$Da + 0.80$; $R^2 = 0.398$, $p < 0.01$, $n = 22$). Therefore, feeding current of attached nanoflagellates could explain a significant portion of biological stimulation.

The assumed velocity of the feeding current (220 $\mu\text{m s}^{-1}$) may be an over-estimate, when the contribution of a raptorial feeder without feeding current to attached nanoflagellates population is high. Artolozaga et al. (2000) reported that attached nanoflagellates associated with the aggregate-water interface consisted mainly of suspension feeders. In addition, the results of backward stepwise regression analysis suggests that the feeding current is a more important mechanism to explain the observed transfer process of SMP into the 5–20 μm fraction than chl a (Table 3).

CONCLUSIONS

In previous studies on SMP aggregation, the primary focus of the discussion was the need of biological activity for the aggregation processes (reviewed in Bidanda 1985). However, this study is the first to attempt to examine the relative importance of physical forcing by biological and physico-chemical processes on the transfer process of SMP into the 5–20 μm fraction. Several lines of evidence obtained in this study indicate that the intensity of biological stimulation is as important as or even more so than the physical transfer process of SMP into the 5–20 μm fraction in coastal environments. Although it is not clear what kind of biological mechanism stimulates the particle aggregation process, the sensitivity to the eukaryotic inhibitor, thiuram, and regression analysis suggest that activity of attached nanoflagellates and phytoplankton play an important role in the aggregation process of marine particles. The number of attached nanoflagellates on marine snow in previous studies (reviewed in Kiørboe 2001) and sinking velocity of marine snow (Alldredge & Gotschalk 1988) suggest that the aggregation by feeding current is potentially effective below the euphotic zone, exceeding the encounter physical aggregation process, probably due to differential sedimentation (e.g. for 1 mm of marine snow, Da is 440 and the sinking velocity is 580 $\mu\text{m s}^{-1}$). Moreover, Wörner et al. (2000) showed that suspension- and filter feeders colonized more rapidly on aggregates than raptorial feeders. This suggests that the suspension- and filter-feeding attached nanoflagellates increase at the beginning of the decay phase of phytoplankton blooming. In addition, extracellular materials released by phytoplankton such as transparent exopolymer particles (TEP) are accumulated during phytoplankton bloom (reviewed in Passow 2002). Putting together these processes, microbial stimulation of colloidal aggregation plays important roles in oceanic carbon cycling.

Table 3. Results of backward stepwise multiple regression using data from water samples. The dependent variable was the V_N/V_A ratio, and independent variables were total particle number in the 5–20 μm fraction (PN), abundance of free-living nanoflagellates and attached nanoflagellates, density of attached nanoflagellates (Da), bacterial abundance (Bacteria), concentration of chlorophyll a (chl a) and incubation temperature (Temp). The limit of the F value was set to 2 to eliminate dependent variables. The lower line shows the result of similar regression using all of the data from incubations at *in situ* temperature (water samples and resuspended aggregates). Because of the lack of data with chl a , it was not used as an independent variable. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Independent variable	R^2
Using only water samples (n = 18)	
PN* Da*** Bacteria* Temp**	0.826
Using water samples and resuspended aggregates	
Da*** Temp***	0.776

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