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Simplified Method to Measure the Clearance Rate of Bivalve Fed on Microalgae

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A simple and rapid method to measure the clearance rate in the blue mussel *Mytilus galloprovincialis* was preliminary studied using various microalgae. The method is based on the measurement of the decrease of microalgal cell density due to the uptake by the mussel. For rapid measurement, cell densities of microalgae were determined by an evaluation of the *in vivo* chlorophyll *a* fluorescence. Good correlation coefficients ($r^2 > 0.99$) were obtained between the cell density and the *in vivo* chlorophyll *a* fluorescence over a wide range of concentrations of the microalgae. The clearance rates for six different phytoplankton species showed an almost similar range of values from 0.774 to 0.984 L $ind^{-1}h^{-1}$ except for the toxic dinoflagellate, *Alexandrium tamarense*. This method will be useful to evaluate the short-term response of *M. galloprovincialis* to microagae and may also be applicable to other filter-feeding bivalves.

Key word: Clearance rate, *in vivo* fluorescence, microalgae, *Mytilus galloprovincialis*, bivalve

Clearance rates in bivalves are influenced by environmental conditions such as temperature, salinity, pH, dissolved oxygen, and the size and shape of the suspended particles (Ali 1970, Winter 1978, Jørgensen 1990). Direct and indirect methods have been developed in order to measure the filtration rate and clearance rate (see Omori and Ikeda 1984) of bivalve in many previous studies (Jørgensen 1990). Indirect methods determine the water volume cleared of suspended particles which may be algal cells, or inorganic or organic particle, using photometric instruments, electronic particle counters or flow cytometry (Yentsch et al. 1983, Shumway et al. 1985, Jørgensen 1990). Indirect methods might be more suitable than direct ones considering the physiological stress on bivalves. However, measurements of particle densities using these instruments require complicated pre-treatments procedures for sample filtration, fixation, staining with immunofluorescence and extraction. In addition, measurements of particle densities by particle counters sometimes lead to an underestimation and overestimation of the particle number, because particle counters regards polymorphic or aggregated matters as a single particle. In the measurement of the chain-forming diatom *Skeletonema costatum*, there are further limits on the application of particle counters in feeding experiments, because the chains are easily broken into smaller chains by the animals activity (Omori and Ikeda 1984). Therefore, this method is hardly been adapted to determine densities for chain-forming phytoplankton.

We report here a simple and rapid measurement of the clearance rates of *Mytilus galloprovincialis* by estimating the decrease of microalgal cell densities using autofluorescence.

Materials and Methods

Mussel preparation Adult *Mytilus galloprovincialis* with a shell height 26.0 ± 1.2 (SD) mm, and whole weight 2.03 ± 0.28 (SD) g (n = 20), were used as the test organisms in the present study. The organisms were collected from Hiroshima Bay in the Seto Inland Sea, Japan, in March 1994. The collected mussels were maintained in running seawater at a temperature ranging from 17 to 19°C

and fed with cultured microalgae (*Isochrysis galbana*, *Skeletonema costatum*) over ten days. Prior to the experiments, each individual was placed into a container and gut contents purged in filtered seawater (Whatman GF/F, pore size 0.7 μ m) over 24h at 20°C ± 1.5°C. Filtered seawater was replenished at least once before each experiment. Only healthy individuals, which were judged by the production of byssus threads and the full extension of their mantle edge, were used for the experiments.

Culture of microalgae Clonal culture of *Isochrysis galbana* was provided by Dr. H. Iwasaki (Mie University). Other microalgal species used in the experiments were isolated from the surface water of Hiroshima Bay (Table 1). Clonal cultures of these species were obtained using the micropipette isolation method (Iwasaki 1967). All strains were cultured in modified SWM-3 medium (Chen et al. 1969, Itoh and Imai 1987) at 20°C under 100 μ $Em^{-2}s^{-1}$ of cool-white fluorescent illumination on a 12h light: 12h dark cycle. Cultures at the late exponential to early stationary phase (7 to 16 days after inoculation) were used in the experiments.

Measurements of clearance rate Cell densities of microalgae were determined by a fluorometer (Turner Designs Co., Model 110). The fluorometric method is based on the measurement of *in vivo* chlorophyll *a* fluorescence of the microalgae (hereafter termed "fluorescence") in the cuvette (Brand et al. 1980). Five milliliters of each culture supernatant was transferred into a cuvette and its fluorescence was measured by the fluorometer.

Prior to experiments, correlations between cell density and *in vivo* fluorescence units of microalgae were examined. Cultures of the microalgae species in the early stationary phase were diluted with filtered seawater to various concentrations (see Fig. 1). Cell densities of each sample were determined by counting the cells under a microscope concurrently with the fluorescence measurements. Same comparison was done in presence of *Mytilus galloprovincialis* to evaluate the effect of fluoremetric substances derived from both *I. galbana* cells and excrements of mussel on fluorescence measurement.

Clearance rate was measured by an indirect method which is based on the time course change of microalgal cell density caused by the filtration of the mussels. In the experiments, each microalgal species was inoculated to pre-aerated filtered seawater in which the final cell density was 60-90 relative units of fluorescence. Then, a healthy mussel was placed in the aerated semitransparent plastic beaker containing 50-100 ml of the microalgal culture. Time course measurements of fluorescence in the culture supernatant were conducted after the relaxation (shell opening and extension of mantle) of the mussel. When feces or pseudofeces of the mussel were observed during experiment, these were removed with Pasteur pipettes. The sample was not stirred or aerated to avoid any disturbance since this might affect the filtering-process of the mussel. Water temperature ranged from 21-23°C during the experiments. Clearance rates were calculated by the following equation (Coughlan 1969):

$$CR = \ln (F_0/F_t) \cdot M/t$$

where, CR is the clearance rate, F_0 is the initial fluorescence value of ambient water, F_t is the fluorescence at t, M is the total volume of ambient water and t is the time after the start of the experiment (10-20 minutes). Background fluorescence of filtered seawater was not detected in any of the

Table 1. A list of microalgae used in the present experiment. All except for *Isochrysis galbana* were isolated from Hiroshima Bay.

Species	Isolation record
Diatoms	
Skeletonema costatum	April 1993
Ditylum brightwellii	April 1993
Flagellates	
Isochrysis galbana	–
Scrippsiella trochoidea	March 1993
Heterosigma akashiwo	June 1992
Alexandrium tamarense	March 1994

experiments.

All experiments were conducted in triplicate and data sets were subjected to one-way analysis of variation.

Results and Discussion

Figure 1 shows the relationships between cell density and fluorescence for *I. galbana* and *S. costatum*. A linear relationship was clearly demonstrated over a wide range of cell densities for *I. galbana* and *S. costatum*. Correlation coefficients of $r^2 = 0.99$ for *I. galbana* and $r^2 = 0.99$ for *S. costatum* were determined. The experiments for *Ditylum brightwellii*, *Scrippsiella trochoidea*, *Heterosigma akashiwo*, and *Alexandrium tamarense* also demonstrated similar results (data not shown) indicating that this procedure is applicable to chain-forming species such as *S. costatum*. This is one of the advantages of this method compared to those using a particle counter or a flow cytometer.

Figure 2 shows the change of cell density and fluorescence of *I. galbana* in the presence of the mussel. Fluorescence is closely related to the cell number of *I. galbana* in the presence of the mussel, and the relationship between fluorescence and *I. galbana* cell density during this experiment corresponds to the linear plot shown in Fig. 1. Therefore, the measurement of fluorescence can be used to estimate the clearance rate of mussel although a low fluorescence value (< 0.3 units) were detected when all *I. galbana* cells disappeared from the culture supernatant (3h after). We considered that the fluorescence remaining in seawater is probably due to dissolved substances from algal cells and excrement from the mussel.

During the experiments, feces and pseudofeces excreted by mussels were removed to ensure the accurate measurements because these excrements cause overestimation of fluorescence. Especially, for *D. brightwellii* and *S. costatum*, pseudofeces began to be excreted immediately after the start of each experiment. These diatoms may eventually be recognized as an inadequate source of food for *M. galloprovincialis* (Sauriau and Baud 1994).

The decrease curve for *I. galbana* was not linear throughout experiment (see Figure 2). In batch condition, the mussel is continuously withdrawing particles and diluting the suspension with filtrate. The clearance rate of bivalves has been shown to be strongly influenced by the concentration of microalgae (i. e., Schulte 1975). We preliminary studied the

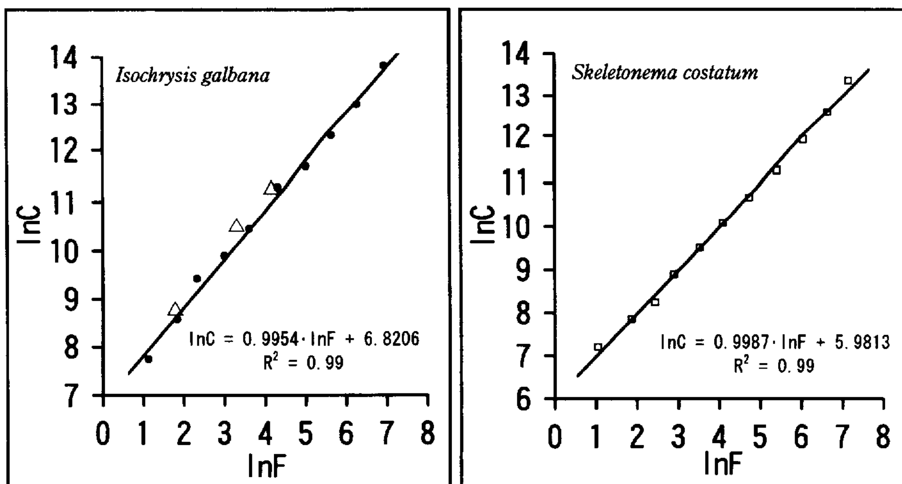

Fig. 1. Correlations between cell density (C; cells ml^{-1}) and *in vivo* fluorescence (F; Relative units). Open triangles show the lnC-lnF relationship when the mussel filters *Isochrysis galbana* as a food item (see Fig. 2).

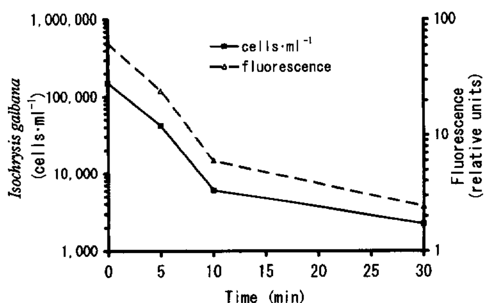

Fig. 2. Decrease in the cell density (cells ml^{-1}) and *in vivo* fluorescence (relative units) of *Isochrysis galbana* by uptake by a mussel. Clearance rate (0-10 min) was calculated as 0.845 L $ind^{-1}h^{-1}$.

effects of the initial concentration of *I. galbana* on the clearance rate of mussels used in this experiment. Results of these preliminary trials found the clearance rates of mussel are not variable in concentrations ranging from 5 to 300 relative units. Therefore this method, at least, is applicable for short-term measurements in the fluorescence range of 5-300 relative units (data not shown). However, the concentrations of microalgae measured in the present study are unusually observed in natural environments except during bloom. For the accurate measurement of the clearance rate especially in natural cell density, further examination is necessary.

Figure 3 shows the clearance rates for each microalgal species measured with the method mentioned above. The clearance rates calculated from the experiments using different microalgal species were of a relatively narrow range from 0.774 to 0.984 L $ind^{-1}h^{-1}$, with the exception of paralytic shellfish toxin producing dinoflagellate, *Alexandrium tamarense*. These results indicate that all microalgae tested except for *A. tamarense*, are filtered by *M. galloprovincialis* at almost the same rate regardless of their size and form.

Notable decrease in the clearance rates of *Mytilus edulis* exposed to *A. tamarense* has been observed in previous studies (Shumway and Cucci 1987, Lesser and Shumway 1993). This dinoflagellate is toxic not only to mammals but also to common phytoplankton

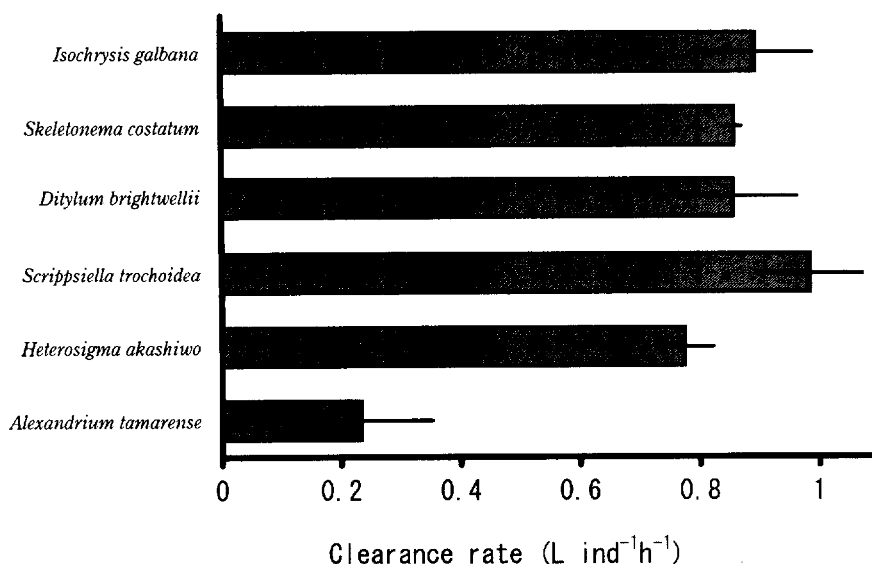

Fig. 3. Clearance rates of mussels for different species of microalgae. Bars denote the standard deviation. Initial cell densities of each microalgae were *Isochrysis galbana*; 67,000 cells ml^{-1}, *Skeletonema costatum*; 26,000 cells ml^{-1}, *Ditylum brightwellii*; 5,000 cells ml^{-1}, *Scrippsiella trochoidea*; 4,700 cells ml^{-1}, *Heterosigma akashiwo*; 12,000 cells ml^{-1}, and *Alexandrium tamarense*; 3,500 cells ml^{-1}, respectively. Clearance rate of mussels exposed to *A. tamarense* is significantly ($P < 0.01$) lower.

grazers i. e., zooplankton (Ives 1985), ciliate (Hansen 1989), and bivalves (Shumway and Cucci 1987, Gainey and Shumway 1988). On the other hand, the harmful fish killing phytoflagellate, *H. akashiwo* was readily taken up by the mussel without any toxic effects. Nagai et al. (1996) also reported that juvenile pearl oyster, *Pinctada fucata* survived in a dense culture (100,000 cells ml^{-1}) of this phytoflagellate, and even ingested them. In addition, *H. akashiwo* had no effect on the shell growth response of the mussel, *M. edulis* (Nielsen and Stromgren 1991).

Fluorometric measurements have been used to evaluate the growth of phytoplankton in culture (Brand et al. 1980, Imai et al. 1993, Yamaguchi 1991, Yamamoto et al. 1995) since this is a less labor intensive method than that by counting phytoplankton cells directly. Using the fluoremetric technique the present study has enabled the rapid and simple measurements of clearance rate by mussel.

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References

Ali, R. M., 1970: The influence of suspension density and temperature on the filtration rate of *Hiatella arctica*. *Mar. Biol.*, **6**, 291–302.

Brand L. E., R. R. L. Guillard and L. S. Murphy, 1980: A method for the rapid and precise determination of acclimated phytoplankton reproduction rates. *J. Plankton Res.*, **3**, 193–201.

Chen, L. C. M., T. Edelstein and J. McLachlan, 1969: *Bonnemaisonia hamifera* Hariot in nature and in culture. *J. Phycol.*, **5**, 211–220.

Coughlan, J., 1969: The estimation of filtering rate from the clearance of suspensions. *Mar. Biol.*, **2**, 356–358.

Gainey, L. F. Jr. and S. E. Shumway, 1988: A compendium of the responses of bivalve molluscs to toxic dinoflagellates. *Journal of Shellfish Research*, **7**, 623–628.

Hansen, P. J., 1989: The red tide dinoflagellate *Alexandrium tamarense*: effects on behaviour and growth of a tintinnid ciliate. *Mar. Ecol. Prog. Ser.*, **53**, 105–116.

Imai, I., Y. Ishida and Y. Hata, 1993: Killing of marine phytoplankton by a gliding bacterium *Cytophaga* sp., isolated from the coastal sea of Japan. *Mar. Biol.*, **116**, 527–532.

Itho, K. and I. Imai, 1987: Rafido so (Raphidophyceae), in "A Guide for Studies of Red Tide Organism", ed. by Japan Fisheries Resource Conservation Association, Shuwa, Tokyo, pp. 122–130.

Ives, D. J., 1985: The relationship between *Gonyaulax tamarensis* cell toxin levels and copepod ingestion rates. In: Anderson, D. M., White, A. W., Barden, D. G. (eds) Toxic dinoflagellates, Elsevier, New York, pp. 413–418.

Iwasaki, H., 1967: The isolation and culture of unicellular algae. Japan Fisheries Resource Conservation Association, Tokyo, 55 p.

Jørgensen, C. B., 1990: Bivalve filter-feeding: hydrodynamics, bioenergetics, physiology and ecology. Olsen & Olsen, Fredensborg, Denmark, 37p.

Lesser, M. P. and S. E. Shumway, 1993: Effects of toxic dinoflagellates on clearance rates and survival in juvenile bivalve molluscs. *Journal of Shellfish Research*, **12**, 377–381.

Nagai, K., Y. Matsuyama, T. Uchida, M. Yamaguchi, M. Ishimura, A. Nishimura, S. Akamatsu and T. Honjo, 1996: Toxicity and LD_{50} levels of the red tide dinoflagellate *Heterocapsa circularisquama* on juvenile pearl oysters. *Aquaculture*, **144**, 149–154.

Nielsen, M. V. and T. Stromgren, 1991: Shell growth response of mussels (*Mytilus edulis*) exposed to toxic microalgae. *Mar. Biol.*, **108**, 263–267.

Omori, M. and T. Ikeda, 1984: Methods in marine zooplankton ecology, John Wiley & Sons, New York, 332 pp.

Sauriau, P. G. and J. P. Baud, 1994: Artificial filament breakage of the diatom *Skeletonema costatum* intended for mollusc aquaculture. *Aquaculture*, **123**, 69–81.

Schulte, E. H., 1975: Influence of algal concentration and temperature on the filtration rate of *Mytilus edulis*. *Mar. Biol.*, **30**, 331–341.

Shumway, S. E., T. L. Cucci, R. C. Newell and C. M. Yentsch, 1985: Particle selection, ingestion, and absorption in filter-feeding bivalves. *J. Exp. Mar. Biol. Ecol.*, **91**, 77–92.

Shumway, S. E. and T. L. Cucci, 1987: The effects of the toxic dinoflagellate *Protoganyalux tamarensis* on feeding and behaviour of bivalve molluscs. *Aquatic Toxicol.*, **10**, 9–27.

Winter, J. E., 1978: A review on the knowledge of suspension-feeding in lamellibranchiate bivalves, with special reference to artifical aquaculture systems. *Aquaculture*, **13**, 1–33.

Yamaguchi, M., 1991: Effects of temperature, salinity and irradiance on the growth rates of noxious red tide flagellates *Chattonella antiqua* and *C. marina* (Raphidophyceae). *Nippon Suisan Gakkaishi*, **57**, 1277–1284.

Yamamoto, T., Y. Yoshizu and K. Tarutani, 1995: Effects of temperature, salinity and irradiance on the growth of toxic dinoflagellate *Alexandrium tamarense* isolated from Mikawa Bay, Japan. *Jpn. J. Phycol.*, **43**, 91–98.

Yentsch, C. M., P. K. Horan, K. Muirhead, Q. Dortch, E. Haugen, L. Legendre, L. S. Murphy, M. J. Perry, D. A. Phinney, S. A. Pomponi, R. W. Spinrad, M. Wood, C. S. Yentsch, and B. J. Zahuranec, 1983: Flow cytometry and cell sorting: a technique for analysis and sorting of aquatic particles. *Limnol. Oceanogr.*, **27**, 91–98.

微細藻類を用いた二枚貝濾水量の簡便な測定法

松山幸彦・内田卓志

ムラサキイガイの濾水量を簡便かつ迅速に測定するため，様々な植物プランクトンを用いて予備的な検討を行った。植物プランクトンの密度の変化は，クロロフィルaの自家蛍光を，蛍光光度計で測定した。細胞密度と蛍光値の間には幅広い濃度範囲にわたって強い正の相関が認められた。従って，蛍光値から細胞密度を正確に推定することが可能であった。いくつかの植物プランクトン（連鎖個体を含む）を用いて濾水量を測定した結果，*Alexandrium tamarense* を除いて高い再現性が得られた。二枚貝に対する短期間の暴露実験などを行う上で，本法は簡便かつ有効な方法と考えられる。

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