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Determination of Experimental Conditions for Measurement of the Clearance Rate of an Intertidal Bivalve, *Glauconome chinensis*

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ABSTRACT

To determine optimal conditions for measurement of the clearance rate in feeding experiment of an intertidal bivalve *Glauconome chinensis*, effects of starvation, extent of mixing at subsampling, and initial prey concentration were assessed. Experiments were conducted separately for each condition with different treatments. Two-way ANOVAs showed that there were significant differences in clearance rates among different starvation periods ($p < 0.001$), extents of mixing ($p = 0.005$), and prey concentrations ($p < 0.001$). Starvation for 1 or 2 days gave rise to 2 to 3-fold increase in the clearance rate. After starvation for 5 days, the clearance rate decreased seriously, implying loss of physiological status. It is suggested that animals should be fed during acclimation. The differences of the clearance rates between gentle and vigorous mixings were significant, but the differences were smaller than that among different incubation times. It was found that vigorous mixing is not necessary. The effect of initial prey concentration was great. However, optimal prey concentration could not be determined at any fixed value. Experiments with multiple concentrations of algal prey are recommended. Optimal incubation time for measurement of the clearance rate of *G. chinensis* was determined to be 2-4 hours.

Keywords: *Glauconome chinensis*, Clearance rate, Bivalve, Experimental conditions.

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INTRODUCTION

Clearance rate defined as the volume of water cleared per unit time (Bayne *et al.*, 1976) has been measured as an index of filtering activity in many studies concerning physiological and ecological aspects of suspension feeders. Clearance rate has been measured in various ways by direct or indirect methods with flow-through (Winter, 1973; Smaal and Twisk, 1997; Lassus *et al.*, 1999; Mills, 2000) or static systems (Werner and Hollibaugh, 1993; Rödström and Jonsson, 2000), and with short-term (Matsuyama *et al.*, 1997; Li *et al.*, 2001) or long-term (Smaal and Twisk, 1997; Laabirand Gentien, 1999) incubation periods. Since filtering activity is governed by a number of physiological conditions, many studies have focused on the effects of physical parameters such as temperature (Sicard *et al.*, 1999; Mills, 2000), salinity (Rödström and Jonsson, 2000), suspended particle (Strychar *et al.*, 1999) and/or biological parameters such as presence of toxic algae (Matsuyama *et al.*, 1997; Lassus *et al.*, 1999), kind of prey species (Werner and Hollibaugh, 1993), concentration of prey (Winter, 1973; Mills, 2000) on the clearance rate of bivalves. From these studies, it is well known that the clearance rates of bivalves can be greatly changed according to experimental conditions. In other words, poorly designed experiments can lead to production of inappropriate data. Therefore, it is essential to find the optimal conditions for measurement of the clearance rate in feeding experiment to better understand the physiological activity of bivalves.

Prior to feeding experiment, acclimation of test

organisms to experimental condition is necessary. In many studies, animals were not fed during acclimation to clear their gut contents (Matsuyama *et al.*, 1997; Laabir and Gentien, 1999). However, this starvation can change the physiological status of the animal, resulting in overestimation or underestimation of the clearance rate. Here, we established the first object of this study to know what is the effect of starvation on the clearance rate and how long will the effect continue as the starvation period increases. We selected *Glauconome chinensis* Gray (Bivalvia: Glauconomidae) and *Isochrysis galbana* (Prymnesiophyceae) as the predator and prey species, respectively. *G. chinensis* is a small bivalve (1-2 cm) inhabiting in upper intertidal zone of sandy silt near Kunsan with the density of more than 8,000 ind./m^2 (Lee *et al.*, unpublished data). We were able to obtain sufficient individuals for the experiments easily owing to their abundance, and to conduct experiments with small-scale (270-ml) static systems owing to their small size. *I. galbana* is considered as the standard prey for the experiments with marine bivalves (Werner and Hollibaugh, 1993; Bougrier *et al.*, 1997). We compared the change in clearance rates of *G. chinensis* among different starvation periods.

The second object of this study is related to pellets produced during the experiments. During experiments with bivalves, pellets are released in relatively short time of incubation. Many studies have revealed that there were undigested and even viable cells within fecal pellets (Laabir and Gentien, 1999; Bauder and Cembella, 2000). In practice, prior to subsampling, bottles should be mixed for the prey cells in suspension to be homogeneous, at which it is confusing that bottles were mixed to which extent whether pellets should be disintegrated completely or none of pellets should be disintegrated. To answer this question, we compared the clearance rates of *G. chinensis* between different extents of mixing (gentle vs. vigorous). We used *Lingulodinium polyedrum* (Dinophyceae) as prey species, because the size of *L. polyedrum* is relatively large (27-62 μm; Shin, 1999) so that pellets can be produced in relatively short time and be easily disintegrated completely when bottles are mixed vigorously.

It is well known that the clearance rates are estimated differently with different prey concentrations. Winter (1983) reported that there were 2 to 5-fold changes in the clearance rate of *Mytilus edulis* incubated with different algal concentrations. Here, we established the third object of this study to know the dependence of the clearance rate of *G. chinensis* on prey concentration. We compared the clearance rates of *G. chinensis* among different algal concentrations. We used *Prorocentrum minimum* (Dinophyceae) as prey species, because it grows rapidly and cultures with high concentration can be easily obtained.

Experimental data were assessed by statistical procedures and optimal conditions for feeding experiments of *G. chinensis* are suggested. This study will provide a basis for the experimental study of physiological and ecological aspects of bivalves.

MATERIALS AND METHODS

1. Test organisms

Approximately 200 individuals of *G. chinensis* were collected at Sura tidal flat near Kunsan (35°55'59" N, 126°36'56" E), west coast of Korea. Bivalves were separated from sediments by sieving with a 2-mm mesh screen and transported to the laboratory within 1 hour after collection. Bivalves were rinsed with 5-μm filtered seawater and acclimated to the experimental temperature (20℃) for 1 day except for Exp. 1 (Table 1), which tested the effect of starvation period and was conducted from the day of collection. Bivalves were maintained in a 20-liter aquarium with filtered seawater in a calm place under dim light (5 μ E/m^2/s). They were not fed during acclimation. Only individuals in size of 12 ± 1 mm were used for experiments.

The strains of prey species (*Isochrysis galbana, Lingulodinium polyedrum* and *Prorocentrum minimum*) were supplied from the Red Tide Research Center, Kunsan National University. They were grown at 20℃ in enriched f/2 seawater medium (Guillard and Ryther, 1962) without silicate, with continuous illumination of 100 μE/m^2/s provided by cool-white

fluorescent light. Only cultures in the exponential growth phase were used for experiments.

2. Effect of starvation period

Exp. 1 was designed to compare the clearance rates of *G. chinensis* among which were not starved, starved for 1, 2, and 5 days (Table 1). Thus, experiments were conducted on the day of collection, 1, 2, and 5 days after collection. Ten 270-ml polycarbonate bottles (Nalgene Co.) were used as feeding chambers. Experiments were conducted with a microflagellate *I. galbana* (6.1×10^5 cells/ml) as a prey. Bottles were filled with 250 ml of algal suspension, then 1 individual of *G. chinensis* was transferred to each bottle. A control bottle with *I. galbana* only (without *G. chinensis*) was also set up. The control was used to check changes in algal concentration due to growth, lysis from death, or attachment of the cells to the bottle. Bottles were incubated in a calm place at 20℃ under 5 $\mu E/m^2/s$ of cool white fluorescent light. Subsamples were taken at 30, 60, 90, and 120 min after the beginning of incubation. Five-ml aliquots of algal suspension were subsampled from each bottle and fixed with 5% Lugol's solution, and > 400 prey cells in triplicate 1-ml Sedgwick-Rafter chambers were enumerated. The clearance rate (CR) was calculated using the equations of Frost (1972) as:

$CR = V [\ln(C^*_t/C^*_0) - \ln(C_t/C_0)]/N_t$

where, V = volume; C^*_0 and C^*_t = the initial and final concentrations of prey in control bottles; C_0 and C_t = the initial and final concentrations of prey in experimental bottles; N = the number of *G. chinensis* t = incubation time.

3. Effect of extent of mixing

Exp. 2 was designed to compare the clearance rates of *G. chinensis* between gentle and vigorous mixing of chamber at subsampling (Table 1). Experiments were conducted with a dinoflagellate *L. polyedrum* (4.4×10^3 cells/ml) as prey. Bottles were filled with 250 ml of algal suspension, and 3 individuals of *G. chinensis* were transferred to each triplicate experimental bottle. Triplicate control bottles with *L. polyedrum* only were also set up. Experimental procedure was the same as described in Exp. 1 except that subsampling was done at 1, 2, 3, 4, 5, 6, and 24 hours after the beginning of incubation. We set total incubation time and subsampling interval longer than Exp. 1, because more than 2 hours' experiments should be required in order to observe sufficient amount of pseudopellets and fecal pellets. At each time of subsampling, bottles for gentle mixing were rotated by hand for prey in suspension to be mixed homogeneously to an extent that pellets should not be disintegrated. On the contrary, bottles for vigorous mixing were mixed vigorously so that all visible pellets in the bottle were completely disintegrated and resuspended into suspension. Enumeration of prey concentration at each incubation time and the calculation of the clearance rate were the same as described in Exp. 1.

4. Effect of prey concentration

Exp. 3 was designed to compare the clearance rates of *G. chinensis* among which were incubated with prey

Table 1. Design of experiments for comparison of clearance rates under different experimental conditions of *Glauconome chinensis*. Prey species, test variables and treatments for each experiment. The numbers in parentheses of prey column are initial concentrations of preys. Numbers of replicates for each treatment in Exp. 1, 2, 3 are 10, 3, and 3, respectively.

Exp. No.	Prey species	Test variables and treatments
1	*Isochrysis galbana* (6.1×10^5 cells/ml)	Starvation period: 0, 1, 2, 5 days Incubation time: 30, 60, 90, 120 min
2	*Lingulodinium polyedrum* (4.4×10^3 cells/ml)	Extent of mixing: gentle and vigorous Incubation time: 1, 2, 3, 4, 5, 6, 24 hours
3	*Prorocentrum minimum* (2.0×10^3-6.5×10^4 cells/ml)	Prey concentration (cells/ml): 2.0×10^3 (C1), 4.9×10^3 (C2), 1.3×10^4 (C3), 3.0×10^4 (C4), 6.5×10^4 (C5) Incubation time: 1, 2, 3, 4 hours

concentrations of 2.0×10^3, 4.9×10^3, 1.3×10^4, 3.0×10^4, and 6.5×10^4 cells/ml of *P. minimum* (Table 1). Bottles were filled with 250 ml of algal suspension, and 4 individuals of *G. chinensis* were transferred to each triplicate experimental bottle. Triplicate control bottles with P. minimum only with 6.5×10^4 cells/ml were also set up. Experimental procedure and enumeration of prey concentration were the same as described in Exp. 1 except that subsampling was done at 1, 2, 3, and 4 hours after the beginning of incubation. We set total incubation time and subsampling interval as such, because we found from Exp. 1 and 2 that incubation time less than 1 hour could cause overestimation of the clearance rate and that 4 hour was sufficient to obtain reliable and consistent results. The calculation of the clearance rate was the same as described in Exp. 1.

5. Statistical analyses

To test the effects of experimental conditions on the clearance rate of *G. chinensis*, we performed both two-way (model I) and one-way analyses of variance (ANOVA) on SPSS package. Two-way model I ANOVAs on the clearance rate data were applied to test for starvation period and incubation time (Exp. 1), extent of mixing and incubation time (Exp. 2), and prey concentration and incubation time (Exp. 3), all as fixed variables. We used Type I sum of squares in all cases because it could be assumed that the variables for each experiment gave little random effects. In case of the interactions between two variables were significant, one-way ANOVAs were applied separately to examine the effects of one variable on the clearance rate for each treatment of the other variable. Comparison of the clearance rates between extents of mixing at subsampling (Exp. 2) for each incubation time was conducted by the independent sample t-tests. Before statistical analyses, clearance rate data were tested for normality (Shapiro-Wilk's test) and homogeneity of variance (Bartlett's test). If one of the above ANOVA requirements was not met, the data were log10 transformed, then ANOVA was repeated. For all analyses, a significance level of $\alpha = 0.05$ was used. If significant F values were observed in any ANOVA tests, multiple comparisons were conducted using Tukey's HSD (Zar, 1984) to determine which means were significantly different from one another.

RESULTS

1. Effect of starvation period

Clearance rate of *Glauconome chinensis* when feeding on *Isochrysis galbana* with different conditions of starvation period and incubation time ranged from 12.4 to 110.4 ml/h/ind. (Fig. 1). Clearance rate increased from 29.3-39.6 when clams were not starved (0 day) to 46.4-69.2 ml/h/ind. when clams were starved for 1 day. It further increased to 54.9-110.4 ml/h/ind. when starved for 2 days, but decreased rapidly to 12.4-17.9 ml/h/ind. when starved for 5 days. Clearance rate at 5 days' starvation was much lower than that of 0 day. As incubation time increased, clearance rate decreased. But, there were little changes in the clearance rates with the incubation time when bivalves were starved for 5 days. Two-way ANOVA on the clearance rate showed that there were significant differences in clearance rates of *G. chinensis* among

Table 2. Two-way ANOVA (model I) of the effects of starvation period and incubation time on the clearance rate of *Glauconome chinensis* when feeding on *Isochrysis galbana* (starvation period: 0, 1, 2, and 5 days incubation time: 30, 60, 90, and 120 min; $^{***}p < 0.001$)

Source of variation	df	MS	F	p
Starvation period	3	26532.094	332.756	$< 0.001^{***}$
Incubation time	3	3062.642	38.411	$< 0.001^{***}$
Starvation period × Incubation time	9	1487.941	18.661	$< 0.001^{***}$
Error	144	79.734		
Multiple comparison (Tukey's HSD)				
Starvation period	$\mu_{0day} \neq \mu_{1day} \neq \mu_{2days} \neq \mu_{5days}$			
Incubation time	$\mu_{30min} \neq \mu_{60min} \neq \mu_{90min} = \mu_{120min}$			

different treatments of the starvation period ($F = 332.8$, $p < 0.001$) and incubation time ($F = 38.4$, $p < 0.001$; Table 2). Starvation period was the most important factor contributing to total variation. The interaction between the starvation period and incubation time was also significant ($F = 18.7$, $p < 0.001$) indicating that the change of clearance rate with the incubation time was dependent on the starvation period. Multiple comparison showed that clearance rate (ml/h/ind.; mean ± sd) were significantly different between unstarved and starved for 1 day (34.3 ± 10.0 vs. 55.9 ± 12.0; $p < 0.001$), between 1 and 2 days (55.9 ± 12.0 vs. 74.1 ± 24.7; $p < 0.001$), and between 2 and 5 days (74.1 ± 24.7 vs. 14.9 ± 4.2; $p < 0.001$). As for incubation time, clearance rates were significantly different between 30 and 60 min (56.6 ± 39.2 vs. 45.9 ± 22.6; $p < 0.001$) and between 60 and 90 min (45.9 ± 22.6 vs. 39.3 ± 19.3; $p = 0.005$), but clearance rates were not significantly different between 90 and 120 min (39.3 ± 19.3 vs. 37.1 ± 16.1; $p = 0.688$). One-way ANOVA on the clearance rate also showed that there were significant differences among the starvation periods at each incubation time and among the incubation times with each starvation period (Table 3). The starvation period affected strongly on the clearance rate in all cases of incubation time, while the effect of incubation time on the clearance rate of unstarved bivalves was not significant ($F = 2.0$, $p = 0.135$).

2. Effect of extent mixing

Clearance rate of *G. chinensis* when feeding on *Lingulodinium polyedrum* with different extent of mixing at subsampling and the incubation time ranged from 0.7 to 17.4 ml/h/ind. (Fig. 2). On the whole, clearance rates of gentle mixing (1.6-17.4 ml/h/ind.) were higher than those of vigorous mixing (0.7-9.5 ml/h/ind.). But, differences of clearance rates between extents of mixing were smaller than those among incubation times. The highest clearance rate (9.5-17.4 ml/h/ind.) was observed at incubation for 1 hour. As the incubation time increased, the clearance rate decreased to less than half of that at 1 hour and fluctuated within 2.2-6.2 ml/h/ind. until 6 hours. After 24 hours, the clearance rate slightly decreased to

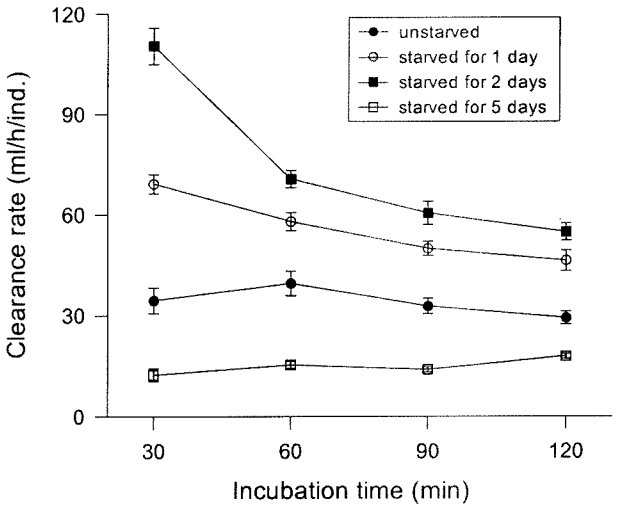

Fig. 1. Changes in clearance rates of *Glauconome chinensis* when feeding on Isochrysis galbana with different conditions of starvation period and incubation time. Symbol represents treatment mean ± SE.

Table 3. One-way ANOVA of the effects of starvation period and incubation time on the clearance rate of *Glauconome chinensis* when feeding on *Isochrysis galbana* ($^{*}p<0.05$; $^{***}p<0.001$; ns: not significant).

Variable	df	MS	F	p
Starvation period				
Incubation time = 30 min	3	18302.950	129.132	$< 0.001^{***}$
Incubation time = 60 min	3	5768.595	80.867	$< 0.001^{***}$
Incubation time = 90 min	3	4154.293	73.936	$< 0.001^{***}$
Incubation time = 120 min	3	2770.079	55.761	$< 0.001^{***}$
Incubation time				
Starvation period = 0 day	3	184.599	1.975	0.135 ns
Starvation period = 1 day	3	1021.052	14.166	$< 0.001^{***}$
Starvation period = 2 days	3	6265.899	45.177	$< 0.001^{***}$
Starvation period = 5 days	3	54.916	3.743	0.019^{*}

0.7-1.6 ml/h/ind. Two-way ANOVA on the clearance rate showed that there were significant differences in clearance rates of *G. chinensis* among different treatments of extent of mixing ($F = 9.3$, $p = 0.005$) and the incubation time ($F = 14.0$, $p < 0.001$; Table 4). Incubation time was more important factor contributing to total variation than extent of mixing. The interaction between extent of mixing and incubation time was not significant ($F = 1.8$, $p = 0.141$) indicating that extent of mixing and incubation time affected on the clearance rate independently. Multiple comparison showed that clearance rates (ml/h/ind.; mean ± sd) among incubation times were significantly different only between 1 and 2 hours (13.4 ± 6.2 vs. 4.4 ± 3.2; $p < 0.001$). Clearance rates were not significantly different between the incubation times for 2 and 3 hours (4.4 ± 3.2 vs. 6.2 ± 2.4; $p = 0.897$), between 3 and 4 hours (6.2 ± 2.4 vs. 2.2 ± 2.0; $p = 0.175$), between 4 and 5 hours (2.2 ± 2.0 vs. 5.4 ± 3.0; $p = 0.398$), between 5 and 6 hours (5.4 ± 3.0 vs. 2.8 ± 1.0; $p = 0.634$), and between 6 and 24 hours (2.8 ± 1.0 vs. 1.2 ± 0.6; $p = 0.931$). Comparisons (t-test) of clearance rates between gentle and vigorous mixing showed that the clearance rate was significantly different in only two cases when incubation time was 5 hours ($p = 0.013$) and 24 hours ($p = 0.030$; Table 5). There were no significant

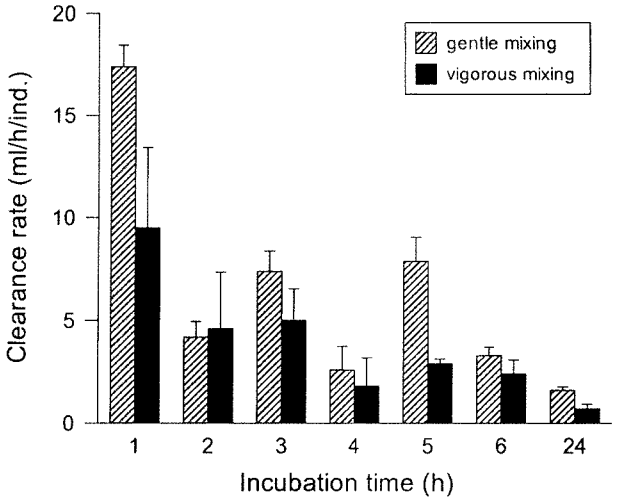

Fig. 2. Comparison of clearance rates of *Glauconome chinensis* when feeding on *Lingulodinium polyedrum* between gentle and vigorous mixing of bottle at each incubation time. Vertical bar represents SE.

Table 4. Two-way ANOVA (model I) of the effects of extent of mixing at subsampling and incubation time on the clearance rate of *Glauconome chinensis* when feeding on *Lingulodinium polyedrum* (extent of mixing: gentle and vigorous; incubation time: 1, 2, 3, 4, 5, 6, and 24 hours $^{**}p < 0.01$; $^{***}p < 0.001$; ns: not significant).

Source of variation	df	MS	F	p
Extent of mixing	1	66.264	9.264	0.005^{**}
Incubation time	6	100.049	13.988	$< 0.001^{***}$
Starvation period × Incubation time	6	12.675	1.772	0.141 ns
Error	28	7.153		
Multiple comparison (Tukey's HSD)				
Incubation time	$\mu_{1hour} \neq \mu_{2hours} = \mu_{3hours} = \mu_{4hours} = \mu_{5hours} = \mu_{6hours} = \mu_{24hours}$			

Table 5. Comparison (t-test) of clearance rates (ml/h/ind., mean ± sd, n = 3) of *Glauconome chinensis* when feeding on *Lingulodinium polyedrum* between gentle and vigorous mixing of incubation chamber at subsampling ($^{*}p < 0.05$; ns: not significant).

Incubation time (hours)	Clearance rate		t	p
	Gentle mixing	Vigorous mixing		
1	17.4 ± 1.8	9.5 ± 6.8	1.941	0.176 ns
2	4.2 ± 1.3	4.6 ± 4.8	-0.132	0.902 ns
3	7.4 ± 1.7	5.0 ± 2.7	1.318	0.258 ns
4	2.6 ± 2.0	1.8 ± 2.4	0.475	0.660 ns
5	7.9 ± 2.0	2.9 ± 0.4	4.245	0.013^{*}
6	3.3 ± 0.7	2.4 ± 1.2	1.174	0.305 ns
24	1.6 ± 0.3	0.7 ± 0.4	3.313	0.030^{*}

differences in the cases of incubation times being 1 hour ($p = 0.176$), 2 hours ($p = 0.902$), 3 hours ($p = 0.258$), 4 hours ($p = 0.660$), and 6 hours ($p = 0.305$).

3. Effect of prey concentration

Clearance rate of *G. chinensis* when feeding on *P. minimum* with different prey concentrations and incubation times ranged from 2.2 to 30.2 ml/h/ind. (Fig. 3). Clearance rate increased from 14.0-16.5 ml/h/ind. (prey concentration of 2.0×10^3 cells/ml; C1) to 18.6-30.2 ml/h/ind. (prey concentration of 4.9×10^3 cells/ml; C2). However, as prey concentration increased further, the clearance rate continuously decreased down to 2.2-8.3 ml/h/ind. when prey concentration was 6.5×10^4 cells/ml (C5). Differences of clearance rates among incubation times were less than those among prey concentrations. Two-way ANOVA on the clearance rate showed that there were significant differences in the clearance rate of *G. chinensis* among different treatments of prey concentrations ($F = 177.9$, $p < 0.001$) and the incubation times ($F = 7.0$, $p < 0.001$; Table 6). Prey concentration was the most important factor contributing to total variation. The interaction between the prey concentration and the incubation time was also significant ($F = 5.4$, $p < 0.001$) indicating that the change in the clearance rate with incubation time was dependent on prey concentration. Multiple comparison showed that clearance rates (ml/h/ind.; mean ± sd) were significantly different between C1 and C2 (14.8 ± 3.0 vs. 24.8 ± 4.7; $p < 0.001$), between C2 and C3 (24.8 ± 4.7 vs. 15.3 ± 3.8; $p < 0.001$), and between C3 and C4 (15.3 ± 3.8 vs. 5.3 ± 1.3; $p < 0.001$), but clearance rates were not significantly different between C4 and C5 (5.3 ± 1.3 vs. 4.2 ± 2.7; $p = 0.737$). As for incubation time, clearance rates were not significantly different between 1 and 2 hours (11.6 ± 5.7 vs. 13.7 ± 9.0; $p = 0.059$), and between 2 and 3 hours (13.7 ± 9.0 vs. 14.6 ± 10.0; $p = 0.679$), but clearance rates were significantly different between 3 and 4 hours (14.6 ± 10.0 vs. 11.6 ± 8.0; $p = 0.004$). One-way ANOVA on the clearance rate also showed that there were significant differences among prey concentrations at each incubation time and among incubation times with each prey concentration (Table 7). Prey concentration affected strongly on the clearance rate in all cases of incubation times, while the effects of incubation time on the clearance rate with prey

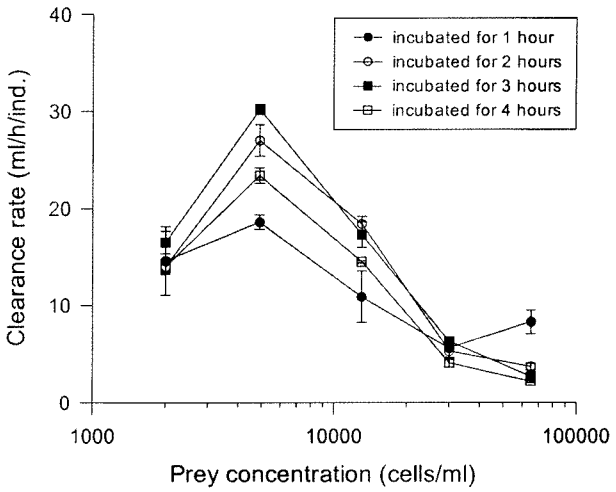

Fig. 3. Changes in clearance rates of Glauconome chinensis when feeding on Prorocentrum minimum with different conditions of prey concentration and incubation time. Symbol represents treatment mean ± SE.

Table 6. Two-way ANOVA (model I) of the effects of initial prey concentration and incubation time on the clearance rate of *Glauconome chinensis* when feeding on *Prorocentrum minimum* (prey concentration: C1, C2, C3, C4, and C5, see Table 1 for each concentration incubation time: 1, 2, 3, and 4 hours $^{***}p < 0.001$).

Source of variation	df	MS	F	p
Prey concentration	4	849.894	177.882	$< 0.001^{***}$
Incubation time	3	33.589	7.030	$< 0.001^{***}$
Starvation period × Incubation time	12	25.796	5.399	$< 0.001^{***}$
Error	40	4.778		
Multiple comparison (Tukey's HSD)				
Prey concentration	$\mu_{c1} \neq \mu_{c2} \neq \mu_{c3} \neq \mu_{c4} = \mu_{c5}$			
Incubation time	$\mu_{1hour} = \mu_{2hours}$, $\mu_{2hours} = \mu_{3hours}$, $\mu_{3hours} \neq \mu_{4hours}$			

concentrations of C1 and C4 were not significant ($F = 0.3$, $p = 0.781$ and $F = 1.8$, $p = 0.230$, respectively).

DISCUSSION

Data from this study show that starvation has great effects on the clearance rate (Table 2, 3). Clearance rate of unstarved *G. chinensis* was relatively stable with the incubation time (coefficient of variation = 13.6%). However, those starved for 1 or 2 days greatly increased (2-3 folds), especially within first 30 min of incubation. This indicates that filtering activity of starved bivalves is enhanced when they detect prey particles again in suspension. In many studies, bivalves were starved to clear their gut contents before feeding experiments (Matsuyama *et al.*, 1997; Laabir and Gentien, 1999). In these cases, short-term experiment could have lead to overestimation of clearance rate. Longer period of starvation gave rise to more serious problem. Clearance rate of *G. chinensis* starved for 5 days decreased to less than 50% of that of unstarved bivalve. Moreover, enhancement of clearance rate within first 30 min was not found (Fig. 1). This implies that starvation longer than 5 days can cause loss of physiological activity of *G. chinensis*, which cannot be recovered. Therefore, starvation during acclimation followed by a short-term feeding experiment is not recommended. Instead, supply with small amount of algal prey of concern during acclimation seems to give more reliable estimate of clearance rate.

There were little differences of clearance rates between gentle and vigorous mixing (Table 5). Vigorous mixing had caused resuspension of fecal pellets and pseudopellets formed in the course of the experiment, consequently resulting in reduction of clearance rates. Actually we have observed visible amount of pellets after 1 hour of incubation. However, the reduction in clearance rates in this study was not large, and variation in clearance rates due to different extents of mixing was smaller than that due to different incubation times (Table 4). Comparisons of means resulted in inconsistent results showing significant differences in only 2 cases (Table 5). Strychar and MacDonald (1999) stated that the percentage of particles rejected as pseudofeaces by *Crassostrea virginica* ranged from 1 to 2%. Thus, even if these pseudofeaces had been completely disintegrated into suspension, there would be little change in cell concentration and clearance rate. Percentage of 1 to 2% can be lower than the range of counting error. Vigorous mixing can give rise to another problem to reduce the clearance rate. When mixed vigorously, bivalves can be physically damaged or cannot maintain their physiological status. If this will be the case, mixing itself will act as a cause of underestimation of clearance rate. Therefore, it is not necessary to mix vigorously in order to resuspend fecal pellets and pseudopellets. Gentle mixing will be better to obtain precise estimates of clearance rates. The effects of initial prey concentration on clearance rates of *G. chinensis* were great (Table 6, 7).

Table 7. One-way ANOVA of the effects of initial prey concentration and incubation time on the clearance rate of *Glauconome chinensis* when feeding on *Prorocentrum minimum* (see Table 1 for each concentration of prey; *p < 0.05; ***p < 0.001; ns: not significant).

Variable	df	MS	F	p
Prey concentration				
Incubation time = 1 hour	4	79.289	5.914	0.011*
Incubation time = 2 hours	4	276.057	97.055	< 0.001***
Incubation time = 3 hours	4	347.549	169.287	< 0.001***
Incubation time = 4 hours	4	224.386	278.108	< 0.001***
Incubation time				
Prey concentration = C1	3	4.054	0.364	0.781 ns
Prey concentration = C2	3	73.416	23.779	< 0.001***
Prey concentration = C3	3	33.402	4.751	0.035*
Prey concentration = C4	3	2.528	1.773	0.230 ns
Prey concentration = C5	3	23.372	19.187	< 0.001***

Clearance rate increased with increasing prey concentration at certain range around low concentration, but it continuously decreased when prey concentration increased further (Fig. 3). This indicates that clearance rate can be either overestimated or underestimated according to at which concentration of prey the experiment was carried out. In general, the clearance rate is undetectable when the prey is very scarce in water column (Bayne *et al.*, 1976), and the maximum clearance rate is found at low prey concentration. However, maximum clearance rate is not the ultimate estimate of physiological activity of a bivalve, but is regarded as the filtration potential of the species. Instead, average or natural value of physiological estimate of bivalve can be more important. In most studies on feeding of bivalves, the ingestion rate is also calculated with the clearance rate (Sicard *et al.*, 1999; Strychar and MacDonald, 1999; Sar *et al.*, 2000; Li *et al.*, 2001). Ingestion rate, in general, increases at low concentration, and is saturated over a certain range of prey concentration showing an asymptotic curve (Frost, 1972 Sprung, 1984 Hansen *et al.*, 1997 Li *et al.*, 2001). Therefore, the maximum ingestion rate is found at relatively high prey concentration. In addition, concentrations of prey at which the maximum clearance rate and the maximum ingestion rate are found are known to be species-specific. These mean that there is no general rule in determining the optimal prey concentration for every prey-predator combinations. In conclusion, it is recommended that experiments should be conducted with multiple concentrations of prey with the range that can cover concentrations at which both the maximum clearance rate and the maximum ingestion rate can be found.

In this study, we set up incubation time differently in each experiment, since experiments were not conducted simultaneously. Instead, we conducted experiments step-by-step from Exp. 1 to Exp. 3. So, we could adjust the incubation time appropriately. Clearance rates in Exp. 1 continuously decreased with the incubation time. Statistical analyses told us that there were significant differences among the incubation times (Table 2, 3). So, we could not determine optimal incubation time with data of only 2 hours. In Exp. 2, we, therefore, extended the incubation time up to 24 hours to find the range of the incubation time in which there was no statistical significance in clearance rate. As a result, incubation time ranging from 2 to 24 hours gave no significant differences (Table 4). From the results of Exp. 1 and Exp. 2, we concluded that 4 hours would be sufficient for estimation of clearance rate of *G. chinensis*. But, all of our data showed that incubation time less than 1 hour was not appropriate. Therefore, it is recommended that incubation time should be within the range of 2 4 hours.

To calculate the clearance rates, we used the equation of Frost (1972), which considers the change in prey cell concentration in control bottles. However, in practice, change in control bottle was negligible in all of our experiments (less then 5%), because the incubation time was not long enough for cell number to change. Therefore, equation of Coughlan (1969) will also be good when the incubation time is 2 4 hours as suggested above.

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