

Application of enzyme activity test for the diagnosis of rotifer mass culture

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Early detection of stress in rotifer mass cultures is important for aquaculturists because it allows preventative action to be taken. We applied measurement of rotifer enzyme activity for evaluating the status of rotifer mass cultures in 1 kl tank at 28°C. Two tests were conducted; one was to directly measure the enzyme activity of mass cultured rotifers, and the other was to monitor the enzyme activity of neonates from resting eggs after exposing them to a filtrate of rotifer mass culture water. From trials using three substrates (cFDAam, PLA2, FDGlu) against neonates from resting eggs, esterase (cFDAam as a substrate) activity increased one day prior to the decline of rotifer population growth, indicating that this enzyme can be used as a tool for early warning for detection of instability of the mass culture practices conducted at a hatchery in Nagasaki. The daily change of rotifer population density was most parallel to that of glucosidase activity. When direct measurement of enzyme activity was conducted for mass cultured rotifers, however, esterase activity did not reveal a relationship with rotifer population growth. It was suggested that the enzyme activity test is appropriate when used for rotifers with the same size and age.

Key words: Rotifera, marine *Brachionus*, batch culture, enzyme activity, rotifer density, culture diagnosis

Introduction

Euryhaline rotifers *Brachionus plicatilis* and *B. rotundiformis* are used world-wide as live feed for marine fish larvae. Rotifer cultures occasionally crash, reducing larval food production and leading to larval starvation. Such collapses could conceivably lead to a loss of an entire year's production (Lubzens 1987). Therefore, it would be useful to develop indicators that can assess the status of rotifer mass cultures daily and serve as an early warning of culture instability.

Several approaches have been attempted in order to minimize the likelihood of a crash and lessen its impact. These include adjusting the feeding rates or changing the food type to reduce the amount of organic material available for bacterial degradation, and lowering culture pH and using temperature to shift the chemical equilibrium of ammonia away from the unionized form (Bower and Bidwell, 1978; Yu and Hirayama, 1986). Droop (1975) tried to prevent the occurrence of rotifer crashes by growing axenic populations of *B. plicatilis*. He concluded, however, that this system is probably impractical for commercial practice.

Early detection of stress in mass cultures is important for aquaculturists because it allows preventative action to be taken. Snell *et al.* (1987) suggested culturists to monitor the physiological state of rotifers by measuring egg ratios and/or swimming activity as an early warning of culture collapse. However, egg ratio is not appropriate for a real-time assessment, because it reflects the rotifer reproductive ability in previous days. Swimming activity is more appropriate for real-time diagnosis of the culture. Koiso and Hino (1999) reported that ingestion rate was not sensitive enough to provide an assessment of the growth potential of the rotifer

Brachionus plicatilis in mass culture environment. Monogonont rotifers occasionally stop swimming to attach substrates even when their physiological status is viable. Other stress indicators more amenable to these factors are useful in managing rotifer mass cultures.

In ecotoxicology, the sensitivity of any toxicant test is a critical factor in evaluating its usefulness for toxicity assessment. A variety of test methods have been published using reproductive rate (Snell and Moffat 1992; Janssen *et al.* 1994a), ingestion rate (Ferrando *et al.* 1993; Juchelka and Snell 1994), swimming activity (Janssen *et al.* 1993; Janssen 1994b), and enzyme activity (Burbank and Snell 1994; Moffat and Snell 1995) as endpoints. The advantages and disadvantages of a variety of methods are shown in Table 1. Behavioral responses such as swimming activity and ingestion rate are usually rapid, occurring in minutes as opposed to the traditional endpoints such as mortality and reproduction, which require days for analysis. The disadvantages of behavioral endpoints, however, is that it is often unclear whether the effect is truly adverse. To demonstrate toxicity, an adverse effect must be shown (Rand and Petrocelli 1985). However, some behavioral responses might be temporary, with test animals for example, which make a complete recovery after toxicant exposure has been terminated. Behavioral endpoints are particularly useful when they can be directly related to widely accepted adverse effects like reduced survival or reproduction. Swimming activity has been measured visually by counting the number of squares entered by a tested rotifer during a 30 sec observation period (Snell *et al.* 1987). This method was used by Janssen *et al.* (1993, 1994b) to assess the effects of toxicants on the swimming behavior of *B. calyciflorus*. Rotifer feeding behavior is an endpoint used in

Table 1. Advantages and disadvantages of methods used as stress indicators in ecotoxicological tests

Stress indicators	Advantages	Disadvantages	References
Egg ratio	easy, cheaper	less reliable, less sensitive, 1-2 days delayed response	Snell <i>et al.</i> (1987)
Survival	easy, sensitive, cheaper	labor intensive (2-10 days), time consuming, less reliable	Snell and Janssen (1995)
Ingestion rate	easier; repeatability, more rapid, more reliable (image analysis), direct measurement	time consuming (5 hrs), estimation error (hemacytometer), less sensitive to chronic tests	Ferrando <i>et al.</i> (1993) Juchelka and Snell (1994)
Swimming activity	more rapid, repeatability sensitive	intensive labor (1hr), less reliable (frequent change of swimming direction, occasional attachment to substrate)	Janssen <i>et al.</i> (1993, 1994b) Snell <i>et al.</i> (1987)
Enzyme inhibition	more rapid, easier, repeatability cheaper, more sensitive, more reliable direct measurement, sensitive, visual observation (image analysis)	no visual observation (fluorometry), intensive labor (2 hrs)	Burbank and Snell (1994) Moffat and Snell (1994)

toxicity studies that can be rapidly and easily quantified, and it can also be directly related to reproduction. Feeding behavior is typically measured by the subtraction method that quantifies microalgae concentration in the beginning and end of a feeding interval (Fernandez-Casalderrey *et al.* 1992; Ferrando *et al.* 1993). The problem with this approach is that the exposure period to toxicant (5 hrs for this author) far exceeds the gut passage time for *B. calyciflorus* (Ferrando *et al.* 1993). Furthermore, when algal cells are individually counted, it is possible that some algal cells pass through the alimentary canal intact, resulting in an underestimation of the ingestion rate. In addition, the high variance associated with hemocytometer counts, deems this method inappropriate. Juchelka and Snell (1994) reported that ingestion rate using fluorescently labelled 2 µm microspheres and image analysis was useful in acute toxicity tests in *B. calyciflorus* exposed to heavy metals.

One approach that holds promise as a stress indicator is enzyme inhibition assays. These are general indicators of stress that have been effectively employed in aquatic toxicology with cladocerans (Janssen and Persoone 1993, Janssen and Coen 1994) and rotifers (Snell and Janssen 1995, Janssen *et al.* 1994a, b, Moffat and Snell 1995, Burbank and Snell 1994). When using rotifers as test organisms, the general procedure is to hatch neonates from resting eggs and expose them to a toxicant for a period of time. A substrate is then added which releases a fluorescent product upon cleavage by intracellular enzymes. Rotifers readily take up the substrate from the medium and fluorescence localizes at sites in the rotifer where there is corresponding enzyme. The fluorescence produced by the enzyme

can be quantified using a fluorometer or by image analysis. Freshwater species such as *Brachionus calyciflorus* Pallas and *B. rubens* Ehrenberg, have been used as test animals for measuring acute and chronic toxicity of metals, organics and pesticides (Snell and Janssen 1995, Janssen *et al.* 1994a).

To assess the status of rotifer cultures, enzyme activity measurement was applied to small scale rotifer cultures by Araujo *et al.* (2000, 2001), where the effect of stressors was tested with rotifer enzyme activity, as well as with reproductive variables of the euryhaline rotifer *Brachionus plicatilis* and *B. rotundiformis*. This methodology proved to be reliable for detecting the physiological condition of rotifers cultured individually. Responses to stressors varied among tested enzymes. Figure 1 shows the schematic pattern of rotifer enzyme activity change against the

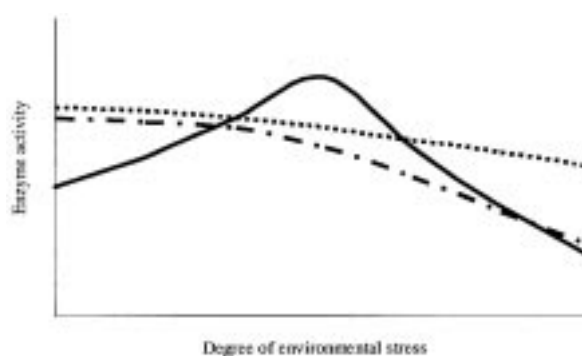


Fig. 1 Features of rotifer enzyme activity change against stress. Substrates for measuring esterase (—), glucosidase (—•—) and phospholipase (···) activity were cFDAam, FDGLu and PLA2, respectively.

degree of environmental stress, which was illustrated based on the results reported by Araujo *et al.* (2000, 2001). Glucosidase activity was highest under optimal environmental condition and decreased with the increase of stress. Phospholipase activity responses varied according to stressors type and/or slightly changed with increase of stress. Esterase activity increased with an early increase of stress and declined at higher levels. Similar trends have also been observed in esterase activity of the freshwater rotifer *Brachionus calyciflorus* in toxicity tests conducted by Burbank and Snell (1994), and esterases were reported to be involved with detoxification.

In Araujo *et al.* (2000, 2001), tests were undertaken under laboratory conditions, where rotifer density was low and test animals were physio-morphologically uniform. In mass culture practices however, rotifer density is high and rotifers are of different age and size. High densities can lead to depletion of the culture environment and make it inappropriate for the rotifer growth. With respect to this paper, trials were conducted by applying enzyme activity measurement, to investigate whether this method is applicable to rotifer mass culture practices for evaluating environmental stress and predicting culture instability or collapse.

Material and Methods

Enzyme activity measurement

The enzyme activity measurement was conducted as following. Tested rotifers were exposed to one of three fluorogenic substrates (Molecular Probes Inc). These substrates, including 5-carboxyfluorescein diacetate acetoxy-methyl ester (cFDAam), 2-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-hexanoyl-1-hexadecanoyl-sn-glycerol phosphocoline (PLA2), and fluorescein di-D-glucopyranoside (FDGlu) were cleaved by endogenous esterase, phospholipase, and glucosidase, respectively, and yielded a fluorescein derivative that was highly fluorescent. Each substrate solution was prepared according to Burbank and Snell (1994) and divided into several 50 μ l aliquots and stored at -80 °C until use.

An aliquot of 1.3 μ l of cFDAam, FDGlu and PLA2 solution was added to each of the three replicated test wells containing 100 rotifers, and incubated at 25°C in darkness for 15 min. The concentration of these substrates was 0.12, 1.9 and 15.2 mM, respectively. At the end of the 15 min incubation, 20 μ l of sodium dodecyl sulfate (SDS) was added to stop the enzyme reaction. Samples were then vortexed for 15 sec and centrifuged at 9,000 rpm for 5 min. Approximately 450 μ l of the supernatant was transferred to a 6 x 50 mm borosilicate microcuvette cylinder which was placed into a fluorometer (Turner TD-700) to measure the enzyme activity of fluorescent produced. Fluorescence emission was read at 515 nm with an excitation wavelength of 490 nm (Moffat and Snell 1995).

Rotifer culture

Rotifer mass cultures were conducted at the Larval Rearing Center at Nagasaki Prefectural Institute of Fisheries, Japan, from July to September of 1998. This institute aims to develop techniques to produce economically important fish larvae in large quantities and high consistency. *B. rotundiformis* was mass cultured using a batch culture method. Three-day batch cultures were conducted in 2 kl tanks by continuously feeding condensed *Chlorella vulgaris* (Chlorella Industry Co.) at 2×10^8 cells/ml. Water temperature and salinity were adjusted at 28 °C and 26 ppt, respectively, with pH regulation at 7.0. Rotifer density in each tank was determined daily during the experiment. In this facility, rotifer cultures are started at 1000~1500 ind./ml to aim at 2000~3000 ind./ml for harvesting and re-inoculation after 2 days.

Culture diagnosis

Two assays were conducted. The first assay was conducted directly with mass cultured *B. rotundiformis* population that consists of rotifers with different size and age. In the second assay, the filtrate of rotifer mass culture medium was collected and exposed to *B. plicatilis* (NH3L strain) neonates, which hatched within 6 hrs from resting eggs. Resting egg hatchlings of NH3L strain have been used as standard in the laboratory for a series of rotifer diagnosis study (Araujo *et al.*, 1998, 2000, 2001).

Rotifer culture methods are numerous and are not consistent among hatcheries. Furthermore, the types of environmental stressors are specific to each hatchery. Culture temperature also varies among hatcheries or seasonally in a hatchery, which strongly affect the speed of enzymatic reaction. Thus, prior to two assays, we conducted screening of enzyme species with the method of the first assay, to find out which enzyme should be tested for diagnosing the mass culture status conducted in the institute. Since esterase was found to be the most promising enzyme for the culture diagnosis in this hatchery (see results section and Fig. 2), we employed esterase substrate for the diagnosis of culture trials.

Both tested rotifers or culture water filtrate were sampled from mass culture tanks after 0, 1 and 2 day from the culture start. For the first assay, 100 rotifers were collected from mass culture tanks and their enzyme activities were measured directly by replicating measurement of the same sample for three times. For the second assay, 45 μ m mesh plankton net was used to separate the culture water from rotifers. The culture water was then filtered GF/C (Whatman) to remove *Chlorella* that affect rotifer enzyme activity measurement. One hundred hatched neonates from resting eggs were then exposed to a 1 ml culture filtrate, in a total of three replicates, at 25 °C for 2 hrs. After exposure, enzyme activity measurement was conducted. The control was the enzyme activity of the newly hatched neonates exposed to filtered and autoclaved natural seawater with salinity at 22 ppt (as in Araujo *et al.* 2000).

For enzyme screening, first and second assays, two, five and four mass culture trials were conducted. The enzyme activity data was compared with the change of rotifer density in the cultures, and with a control. Analysis of variance was conducted to determine the difference in enzyme activity with culture progress, and Tukey or Dunnett test was performed for multiple comparisons of enzyme activity data between different stages of the culture or between data of each culture stage and control.

Results

Enzyme Screening

Figure 2 shows the result of enzyme screening test. The highest rotifer density and enzyme activities were observed on day 1 and the lowest were found to be on day 2. For all of the tests, enzyme fluorescence increased with higher rotifer density. Esterase activity on day 0 was higher but not significantly different from that of the control. It was higher than the control on day 1 and close to significant ($p=0.055$), and significantly lower than the control on day 2 ($p=0.005$). Phospholipase activity on day 0 and 1 significantly increased compared with control ($p=0.038$; $p<0.001$, respectively), and it decreased significantly on day 2 ($p<0.001$). Glucosidase activity was higher on day 0 and 1 (each $p<0.001$), and lower on day 2 ($p=0.001$). Among the patterns of the enzyme activities change during the culture, only the increase of esterase activity (day 1) can explain the decline of rotifer population in the next day (day 2), which was consistent with the trend reported by Araujo *et al.* (2000, 2001)

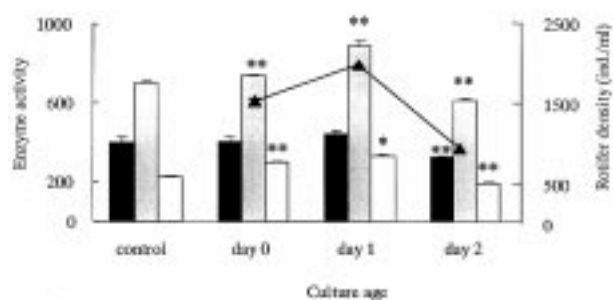


Fig. 2. Screening test of three enzymes for assessing the rotifer mass cultures. Columns represent esterase (cFDAam, black), glucosidase (FDGlu, gray) and phospholipase (PLA2, white) activities (amount of fluorescence) measured on newly hatched *B. plicatilis* neonates from resting eggs exposed to culture filtrate from a batch culture. First columns represent enzyme activities of control rotifers. Line graphs are *B. rotundiformis* density in culture tanks. Vertical bars indicate standard deviation of three replicates. Significant differences between the control and enzyme activity data of each culture stage are indicated as * ($p<0.05$) and ** ($p<0.01$) (Dunnett test).

Culture diagnosis

Figure 3 shows esterase activity that was measured directly with mass cultured rotifers. Rotifer density increased with culture proceeds in trials I-3 and I-4, and rotifer population continued to decline in trial I-1. However, responses of esterase activity were not consistent with different patterns of rotifer population growth among four trials. For example, esterase activity trend was similar in I-2, I-3 and I-4, but a decline of the rotifer population was observed in I-2, while rotifers continued to grow in trials I-3 and I-4. Figure 4 indicates rotifer population growth in two mass culture trials and esterase activity change of *B. plicatilis* resting egg hatchlings exposed to the filtrate of rotifer mass culture. Results of two trials out of four are shown. In trial II-1, both rotifer density and esterase activity became higher as culture progressed. In trial II-2, the highest rotifer density and esterase activity were observed on day 1 and the lowest was found to be on day 0. Multiple comparison data shows a significant difference between enzyme activity data of different culture stages in trial II-1, and between data of each culture stage and the control ($p<0.001$). Similar result was obtained in trial II-2, except between the control and esterase activity on day 0, where significant difference was not observed. Table 2 depicts a summary of the results of mass culture diagnosis using esterase. With the first assay using mass cultured rotifers, we conducted five trials. Among them, culture instability on day 1 or day 2 was observed in three cases, and in only one case, increase of rotifer esterase activity was observed on the previous day. With regard to the second assay using filtrate of mass cultured rotifers, the increase of esterase activity was always followed by declines of rotifer population growth on the next day.

Table 2. Occurrence of culture instability in trials I-1 to 5 and II-1 to 4, and increase of esterase activity on the previous day of culture instability

Trial	Culture instability	Esterase increase
I-1	YES	NO
I-2	YES	YES
I-3	NO	
I-4	NO	
I-5	YES	NO
II-1	NO	
II-2	YES	YES
II-3	NO	
II-4	YES	YES

Discussion

The presence or contamination of certain bacteria or protozoans can cause a rapid and sudden crash of rotifer population (Yu *et al.* 1990; Cheng *et al.* 1997). However, in most cases, the instability of rotifer cultures is caused by a gradual increase of environmental stressors, such as an increase of free ammonia and

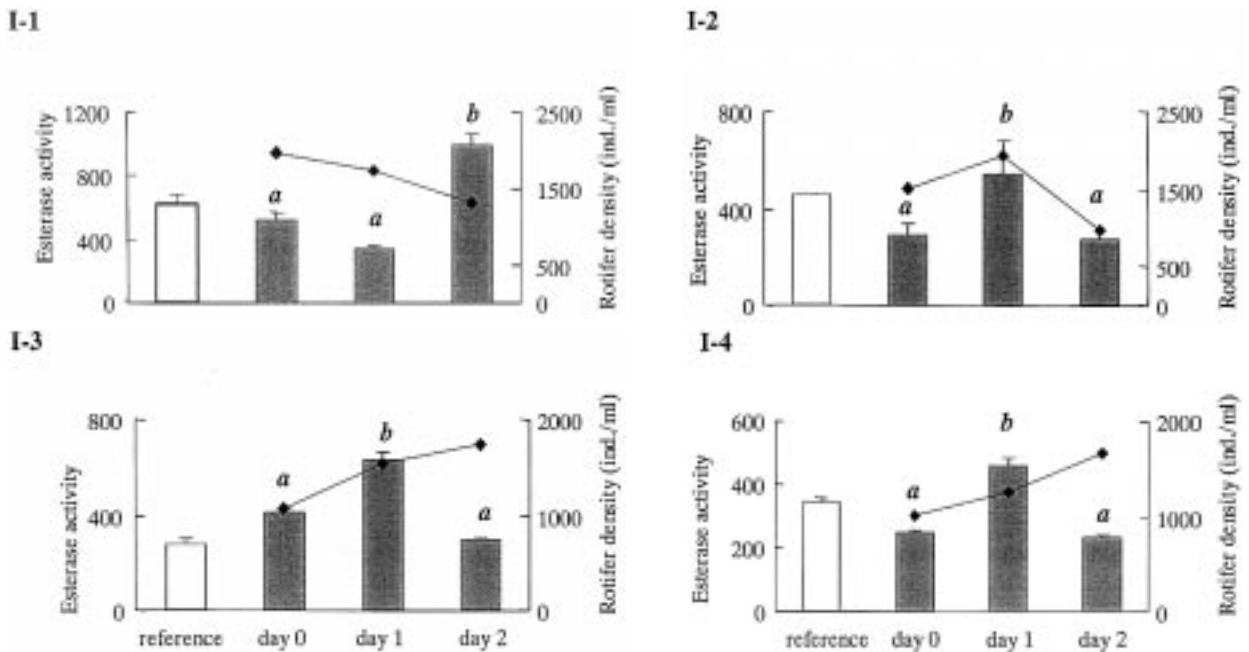


Fig. 3. Esterase activity measurement using mass cultured rotifers. Columns (light) represent esterase activity (amount of fluorescence) of *B. plicatilis* neonates from resting eggs exposed to seawater (as reference). Columns (dark) represent esterase activity of randomly sampled rotifers from tank. Line graphs represent *B. rotundiformis* density in mass cultures. I-1, I-2, I-3 and I-4 indicate different trials. Vertical bars indicate standard deviation of three replicates. Significant differences (p < 0.05) between esterase activity data of different culture stages are indicated as *a* and *b*, where *a* < *b* (Tukey HSD test).

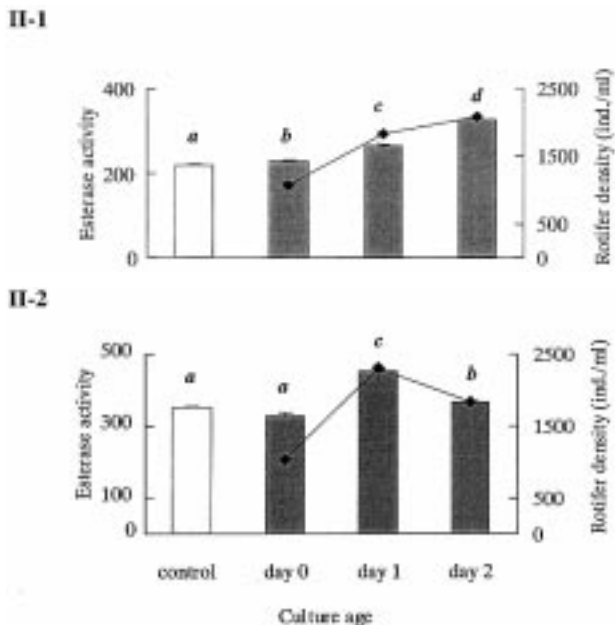


Fig. 4. Esterase activity measurement using filtrate of culture water. Columns (dark) represent esterase activity (amount of fluorescence) measured on newly hatched *B. plicatilis* neonates from resting eggs exposed to culture filtrate from a batch culture. Columns (light) represent esterase activity of control rotifers. Line graphs are *B. rotundiformis* density in culture tanks. II-1 and II-2 indicate different trials. Vertical bars indicate standard deviation of three replicates. Significant differences (p < 0.05) between esterase activity data are indicated as *a*, *b*, *c* and *d* where *a* < *b* < *c* < *d* (Tukey HSD test).

contamination of bacteria and protozoans. This process includes, 1) increase of environmental stress, 2) decrease in physiological activity in rotifers, 3) decrease in reproductive ability and 4) decrease of rotifer population growth. The activities of three enzymes in the enzyme-screening test changed parallel with the change of rotifer density during the culture period (Fig. 2). Generally, glucosidase and phospholipase activities of rotifers show a parallel change to a degree of physiological stress in rotifers (Fig. 1, Araujo *et al.* 2000, 2001). This implies that the activity of these two rotifer enzymes on day 1 (Fig. 2) did not indicate the physiological deterioration of rotifers that leads to the population decline on day 2. This may be because the decrease in physiological activity occurred rapidly due to the higher temperature (28°C) for rotifer cultures, and because of observation at 24-hrs intervals. Another possibility is that the enzymatic response of glucosidase and phospholipase against stress may be slower. Among the three enzymes tested in this study, responses of glucosidase was most highly correlated with reproductive variables (fecundity and lifespan) of rotifers exposed to environmental stress (Araujo *et al.* 2000, 2001). The use of glucosidase may be appropriate for rotifer cultures conducted at lower temperatures, where rotifer population growth rate and changes in culture environment are slower. Koiso and Hino (1999) observed that the decline in percent of swimming rotifers among eggs-bearing amictic females after 3 hrs exposure to 70 ppt seawater correlated with daily population growth rate. However, this does not indicate that swimming activity measurement could serve as an early

warning of culture collapse. When the percent of swimming rotifers was detected as low, the rotifer population was found to have already declined.

Since esterase activity of rotifers generally increases with a slight increase of physiological stress (Fig. 1), the increase of esterase activity on day 1 may have explained the rotifer population decline on day 2. Thus, we conducted subsequent trials of culture diagnosis by using only esterase.

With respect to the first assay, the increase of esterase activity was not always followed by declines of rotifer population growth on the next day (Table 1, Fig. 3). The problems that may be involved in this assay are; 1) sampled rotifers were of different age and size, which were not physiologically uniform, 2) enzyme activity data may have been reflected by that of contaminated protozoans because of the difficulty of isolating only rotifers when sampling.

Before the experiments, we anticipated the less sensitivity of the second assay (rotifers exposed to filtrate), because we needed to remove the contaminated protozoans by GF/C filtration in order to avoid the presence of rotifer food (*Chlorella*) during the assay test. When rotifers are fed, intense esterase activity is observed in the rotifer digestive organs (Araujo *et al.* 1998, Hagiwara *et al.* 2001). Nevertheless, the result of the second assay suggest that the measurement of esterase activity with the culture water filtrate is useful for assessment of rotifer mass culture status, and can serve as an indicator of the upcoming culture instability or collapse. The mechanism of interference of rotifer population by protozoans has not been clarified (Jung *et al.* 1997), but chemical substances secreted by protozoans or associated bacteria may have primary roles. During the second assay experiment, only a limited quantity of *B. rotundiformis* resting eggs were available in our laboratory stock, so that the rotifer species used for esterase activity tests (*B. plicatilis*) and for culture trials (*B. rotundiformis*) were not identical. Although the patterns of enzyme activity change against environmental stress were similar between two species (Araujo *et al.* 2000, 2001), further experiments by using same rotifer species may provide sufficient information for the application of the method.

There are various methods of rotifer mass culture among hatcheries (Fulks and Main 1991). Further research in various methods is necessary to confirm the effectiveness of an enzyme activity test for the diagnosis of cultured rotifer condition, including semi-continuous (or thinning out) and continuous cultures.

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酸素活性試験を応用したワムシの大量培養診断

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大量培養中のワムシのストレスを検出できれば、培養不良の事前の回避に役立つ。ここでは、酸素活性測定によるワムシ培養診断を試みた。ワムシの培養には1kl水槽を用い、培養水温を28℃とした。本研究では、まず、バッチ式で大量培養中のワムシを直接材料として酸素活性を測定したが、ワムシ個体群の増殖経過との間に明瞭な関係は認められなかった。これは個体群内のワムシの齢やサイズの組成が一定でないことに基づくものと推測された。そこで、耐久卵から孵化したばかりのワムシをあらかじめ用意し、これらをワムシ培養槽から採取した培養濾液に曝露して、耐久卵孵化ワムシが示す酸素活性を求めた。その結果、グルコシダーゼ活性（使用した基質はFDGlu）は、大量培養ワムシの個体群サイズと関連しながら変化することが分かったが、培養水温が高かったせいもあり（28℃）、一日一回の測定によって、大量培養ワムシ増殖率の低下を事前に検知することはできなかった。一方、環境中のストレス因子の増大ともななってエステラーゼ活性（基質、cFDAam）が上昇し、大量培養ワムシの増殖率が低下するとエステラーゼ活性も下降した。耐久卵孵化ワムシを用いたエステラーゼ活性の測定はワムシ培養不良を事前に検出する一手段になると考えられた。