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Zooplankton Sorting by Sedimentation in Gradient Solutions*

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Abstract

For rapid separation of fish larvae from invertebrates, 2 *l* simple designed sorting chamber was made and three gradient layers (0, 15 and 25% of Ludox Am) modified with 1.0 % agent were prepared. Separation examined with Kagoshima Bay samples preserved in 5 % formalin proved to be significant: more than 70 % of fish larvae and invertebrates were recovered from 25 % and 0 % layers respectively, in all of the best conditions. The best conditions for recovery of fish larvae from 25 % layer were obtained as follows: 1.0 % Trimetaphosphate as agent, distilled water as dilutant, less than 5 g as amount of samples, between 6 and 30 min as settling time, pretreatment of samples in 70 % alcohol, and samples preserved more than two weeks. It was shown that this method greatly reduced time of all-manual sorting by about one half. Fish eggs were not constantly sedimented in any fixed layer, whose reason could not be explained.

Introduction

One problem in ichthyoplankton study is the long time required to separate larval fishes from bulk of invertebrates present in each plankton tow. Techniques such as use of small subsample have been employed to reduce the sorting time, but they usually generate unreliable data for true population parameters (BOWEN *et al.*, 1972). Moreover, rare ichthyoplankton present in samples are often neglected and results are mainly focused on abundant species.

A technique being investigated here takes advantage of differences in specific gravity of plankters, allowing them to sink to layer of their density and to be collected separately. At first this method was used to separate calcareous and silicious organisms (BE, 1959; MCGOWAN and FRAUNDORF, 1961) but recently tested to separate higher members of zooplankton (BOWEN *et al.*, 1972).

The present experiments were based on the works of BOWEN *et al.* (1972), ST. ONGE and PRICE (1975) and PRICE *et al.* (1977), who employed isopycnic sedimentation technique for separating groups of zooplankton, particularly fish eggs and larvae from bulk of invertebrates. Their works proved to be effective in sorting semi-automatically organisms required but we found their methodology to be complex. An example was the "Rho Spectrometer" (PRICE *et al.*, 1977) which together with limitation of use by patent, seemed too difficult to make and handle. Moreover, their gradients were composed of ten layers which required extra time for preparation and harvest.

The purposes of this study are to simplify the methodology employed by the previous authors and to examine the best conditions for separation of fish larvae from other plankters.

Materials and Methods

Plankton samples used in our experiments were taken from monthly cruises of a small boat "Shiranami" (1.5 tons) of our laboratory in Kagoshima Bay with large plankton net (diameter 1.3 m; length 4.5 m; mesh size 0.51 mm) towed in step hauls to the depths of about 30 and 15 m for 10 min in total at a speed of about 2 knots. They were preserved in seawater with 5 % formalin.

A simple sorting chamber (2.0 *l* cap. cylinder; 24.0 cm H, 11.8 cm D) was constructed as follows. The base of 2.0 *l* transparent cylindrical container

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Reprint and "Manual for use of sorting chamber" are available for request to the second author, T. OZAWA.

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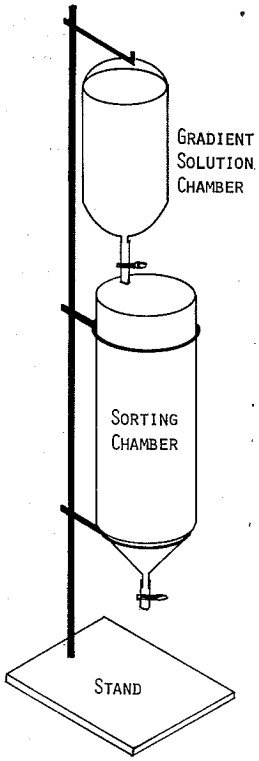

Fig. 1. The sorting chamber used in this study.

made of Polyvinyl chloride was cut and attached to glass funnel (12.0 cm D). Gradient solutions were kept in 1.0 *l* irrigator bottle hung above the chamber and were flowed down along the inner wall of the chamber through rubber tube fitted with a pinch cock for volume regulation (Fig. 1). Recovery of samples was controlled by a pinch cock attached to rubber tube at the narrow end of the base of sorting chamber.

Gradient solutions were prepared by mixing pure Ludox Am (a commercial preparation of silica sol, E.I. du pont de Nemours, Inc., Wilmington, Delaware, USA) and fresh water to make desired percent concentrations (v/v). Modification of the solutions was done by adding one of the following agents: Dextran sulfate (Pharmacia, New Market, New Jersey, USA), Polyvinyl alcohol (Wako Pure Chemical Industry, Ltd., Japan) and Trimetaphosphate (Sigma Chemical Co., St. Louis, Missouri, USA). Concentration of agent (1.0 %) was based on the works of BOWEN *et al.* (1972). Mixing with magnetic stirrer was done for less than 30 min depending on agent added.

Except the experiment on reduction of gradient layers, volumes of solutions were 500 m*l* for each of 25 % and 15 % gradient layers and 1,000 m*l* for 0 %. Drained plankton samples after washing with tap water were directly placed on top of the uppermost layer and were allowed to settle. An L-shaped wire was used to loosen entangled samples during settling process.

Harvesting of sedimented plankton was done layer by layer, collecting first 500 m*l* of 25 % layer followed by 500 m*l* of 15 % and then 1,000 m*l* of 0 %. Contents of each layer were filtered through a fine mesh plankton net and placed into a petri dish. Filtered plankton was then sorted manually. Fish eggs and larvae were counted while amount of invertebrates inclusive rarely of phytoplankton was determined by volume displacement after draining.

Speculating that one of the purposes of this study, i. e. reduction of number of gradient layers would work well, we considered the following items to cause significant difference of recovery of organisms: agents, amount of samples, settling time and period of preservation. Because of large combination of experiments to get the best conditions of these items for recovery of organisms, we examined first the effect of settling time on recovery under the supposition that best conditions of other items would be as follows: Trimetaphosphate as agent, 5 g of samples and three months old or over samples. After determining the best condition of settling time, effect of other item was examined under the same supposition as before on the remaining items. By this procedure, the best conditions were set on all items. If supposed condition was proved not to be best, preceding experiments were repeated.

Under the best conditions of the above items, we tried to improve recovery with choice of dilutants and pretreatment of samples. Further experiments were made to reconfirm the effect of an item in question under the best conditions of other five items. Therefore, it can be considered that in all of the experiments, conditions are always best except those to be compared. The results of this run will be referred to in the next section. Because three replicates were usually done in each experiment, results are

shown with averages and ranges.

Results and Discussions

1. Reduction of number of gradient layers.

First of all, some similar experiments with PRICE *et al.* (1977) on number of gradient layers were made. Instead of 10 layers in the previous authors, we made 8 gradient layers because of small chamber. However, the results were very similar between them. Majority of invertebrates were found in 5–10 % layers, fish larvae in layers of more than 20 %, and fish eggs in 10 and 15 % layers (Fig. 2). This suggested that the same result might be obtained for fish larvae and invertebrates when gradient layers were reduced to about 3: 0 % layer for zooplankton, 25 % for fish larvae, and 15 % for buffer zone. We examined difference between 0 % and 5 % layer as the uppermost gradient. Since the results were the same, we used 0 % layer in all of our following experiments. As will be shown below, separation of fish larvae from invertebrates was satisfactory with reduced layers.

2. Agents

The previous authors (ST. ONGE and PRICE, 1975) tested the following three agents: Dextran sulfate, Polyvinyl alcohol and Trimetaphosphate, and obtained good results with the last one. In our experiments these agents were also compared.

Preliminary experiments using Dextran sulfate showed plankton specimens to be damaged or distorted, rendering them unrecognizable for identification. This test made this agent unsuitable for further experiment. The results of sedimentation with Polyvinyl alcohol showed high quantity of fish larvae in 15 % layer, resulting to poor separation of ichthyoplankton from others which were abundant in 0 % layer (Fig. 3). Moreover, preparation of gradient solutions with this agent took a longer time (>ca 30 min) due to their low solubility. On het other hand, we found better separation with Trimetaphosphate than Polyvinyl alcohol. The results with this agent (Fig. 3) showed high abundance of invertebrates in 0 % layer and of fish larvae in 25 %, resulting to good separation. Moreover, preparation of gradient solutions with Trimetaphosphate was shorter (5–10 min) than Polyvinyl alcohol.

According to the results by the previous authors and the present ones, Trimetaphosphate was found to be best agent for the separation of fish larvae from other plankton.

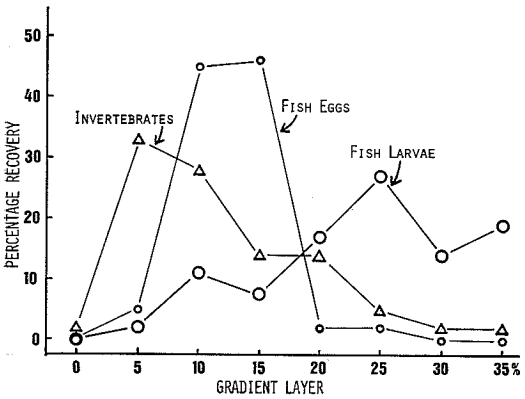

Fig. 2. Percentage recoveries of plankton organisms with eight gradient layers. For the experimental conditions, see "Materials and Methods" of the text.

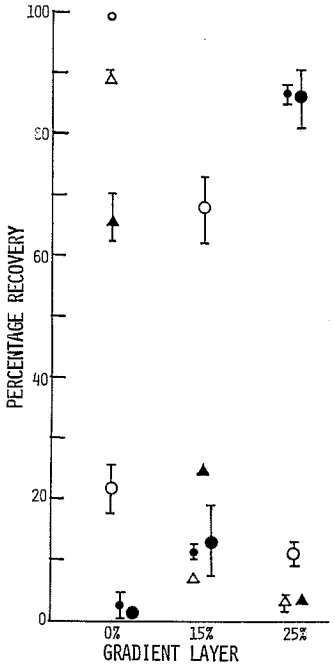

Fig. 3. Difference of percentage recoveries of plankton organisms between Polyvinyl alcohol (open symbols) and Trimetaphosphate (closed symbols) agents. Symbols and vertical bars indicate the averages and the ranges, respective of 3 experiments. For the symbols see Fig. 2, and for the experimental conditions see "Materials and Methods" of the text.

Fish eggs were abundant in 0% layer with Polyvinyl alcohol but in 25% with Trimetaphosphate (Fig. 3). This clear difference was not necessarily considered to be due to that of agents. For example, high recovery of eggs from 0% layer in the experiment on 5 g of samples (Fig. 4) was observed under the same conditions with the experiment on Trimetaphosphate of Fig. 3. Because we could not find out the reason of this uncertainty shown also in some other experiments, we will focus our attention only on fish larvae and invertebrates in the following results.

3. Amount of samples

Since recovery of samples is influenced by capacity of sorting chamber, an appropriate amount of samples for our chamber was determined by getting percentage recovery of 5 and 10 g (wet weight) of plankton (Fig. 4). With 5 g samples, separation was satisfactory: high percentage recovery of fish larvae from 25% layer and that of invertebrates from 0%. Ten gram samples showed somewhat poorer separation in that about 30% of fish larvae was recovered from 0% layer. Therefore, we opted to use 5 g in all of our experiments.

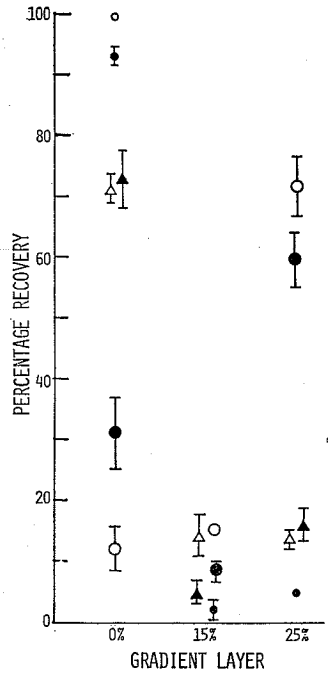

Fig. 4. Difference of percentage recoveries between 5 g (open symbols) and 10 g (closed symbols) samples. For the symbols see Fig. 2, and for the experimental conditions see "Materials and Methods" of the text.

However, an amount of samples seemed also affected by kinds of fish larvae as well as invertebrates. As shown in the results of previous authors (ST. ONGE and PRICE, 1975: fig. 3) this difference of organisms was supposed to be inevitable factor to produce difference of recovery. In our experiments the order of recoveries of fish larvae was usually as follows: highest recovery (>70%) from 25% layer followed by 15% and least from 0%. However, there was sometimes reversion between recoveries from 0 and 15% layers (for example see Fig. 5). In those cases, fish larvae from 0% were composed of elongated type, such as engraulid fishes which were entrapped in networks made by filamentous phytoplankton and zooplankton.

As above, types of fish larvae are inevitable factor to influence recovery. Therefore, samples from the same bottle were used for comparison of conditions in each item throughout our experiments. Although 5 g were used in our experiments from the result on amount of samples where short bodied fish larvae dominated (Fig. 4), it may be possible to change an amount per sample.

4. Settling time

As settling of plankton was supposed to change with time, it was needed to define the time of recovery. In our experiments (Fig. 5), samples

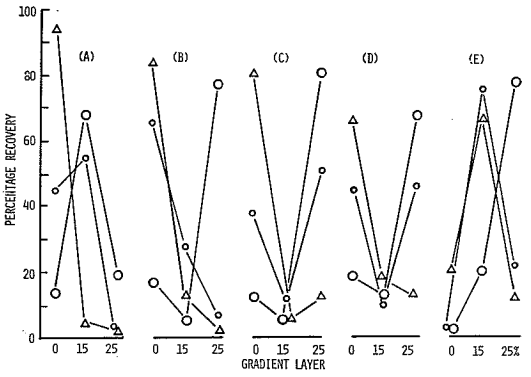

Fig. 5. Percentage recoveries of plankton organisms during the time elapsed after settling. A: 3 min, B: 6 min, C: 12 min, D: 30 min, E: 60 min. For the symbols see Fig. 2, and for the experimental condition see "Materials and Methods" of the text.

which were allowed to settle for 3 min showed high concentration of fish larvae in 15 % layer and abundant invertebrates in 0 %. After 6 min of settling, majority of fish larvae was shifted to 25 % layer while invertebrates remained abundant at 0 %, showing good separation between them. Until 30 min after, plankton organisms were in steady state and in good separation. Usually, invertebrates concentrated at the bottom of 0 % layer and fish larvae were evently floating in 25 % layer except the densest ones at the bottom. Sinking of mass of invertebrates to 15 % layer followed after about an hour, resulting to poor separation from fish larvae. From these experiments, it was known that settling of organisms could be devided into three phases: the first of rapid sinking of dense organisms, the second of no sinking and good separation and the last of sinking of lighter organisms. It is easily understood that recovery of samples is best during the steady period, from 6 to 30 min in these experiments.

5. Eflect of duration of sample preseruation

As one of the possible conditions to affect separation of organisms, it was tested to determine the effect of time elapsed for preservation (Fig. 6).

On average, in four days old samples, about 84 % of invertebrates was recovered from 0 % layer, being the same results stated above. However, settling of fish larvae differed greatly, showing nearly even distribution in all layers: 27 % recovery from 0 % layer, 46 % from 15 % and 27 % from 25 %. One week old samples showed almost the same pattern of distribution as four days old ones for both invertebrates and fish larvae: for the former, 76 % recovery from 0 % layer, 18 % from 15 % and 6 % from 25 %, and for the latter, 25 % from 0 %, 31 % from 15 % and 44 % from 25 %. Two weeks old samples yielded significant recoveries (64–81 %) of fish larvae from 25 % layer, but recoveries of invertebrates from 0 % layer decreased a little (57–69 %). One month old samples showed 71–73 % and 72–78 % recoveries for fish larvae and invertebrates respectively, being nearly the same results as two weeks old ones.

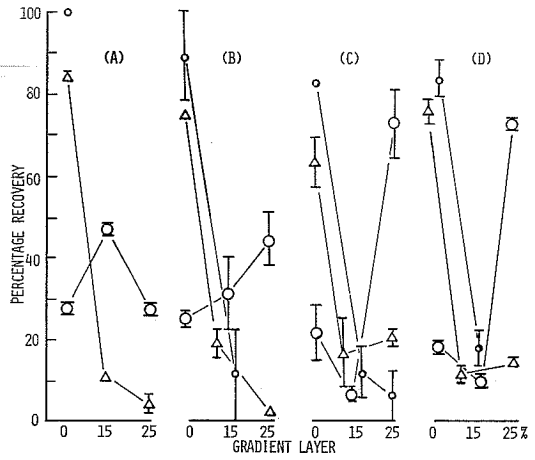

Fig. 6. Difference of percentage recoveries of plankton between samples of different preservation duration. A: 4 d, B: 1 wk, C: 2 wks, D: 1 mth. For the symbols see Fig. 2, and for the experimental conditions see "Materials and Methods" of the text.

Invertebrates occupied 0 % layer abundantly regardless of preservation period, while fish larvae occupied 25 % layer densely only beyond two weeks preservation. Therefore, plankton samples beyond this duration were used in all other sedimentations.

Though density analysis of samples was not made in these experiments, it can be supposed that fish larvae preserved for less than two weeks might not yet reached their stable density, resulting to banding evenly in 3 layers.

Fish eggs showed 100 % recovery from 0 % for four days old samples. After this period up to one month, their recovery from 0 % layer decreased because some of them also banded in 15 % and 25 % layers. However, the majority (82–89 %) were found in the uppermost layer.

6. Diflerence of diluant

As a dilutant, quality of water (distilled and tap) was compared (Fig. 7). Percentage recoveries with distilled water medium ranged from 74–86 % in 25 % layer for fish larvae and 76–83 % in 0 % for invertebrates. With tap water medium they ranged from 73–83 % in 25 % for fish larvae and 64–77 % in 0 % for invertebrates.

Though the small difference was found only in recoveries of invertebrates between these experiments, distilled water if available was considered to be better as dilutant than tap water.

7. Pretreatment of samples

When thick plankton were used in experiments,

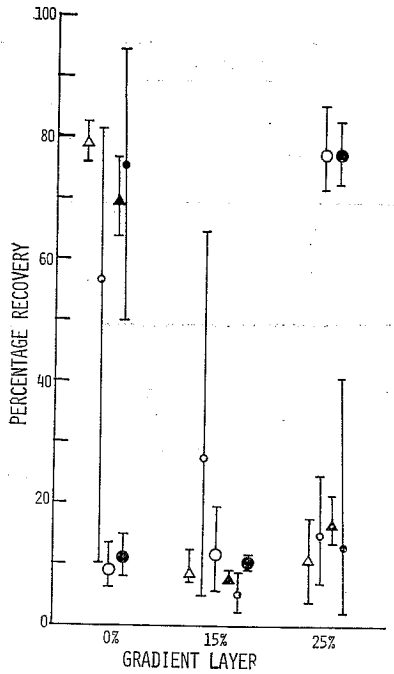

Fig. 7. Difference of percentage recoveries of plankton organisms between tap water (closed symbols) and distilled water (open symbols) dilutants. For the symbols see Fig. 2, and for the experimental conditions see "Materials and Methods" of the text.

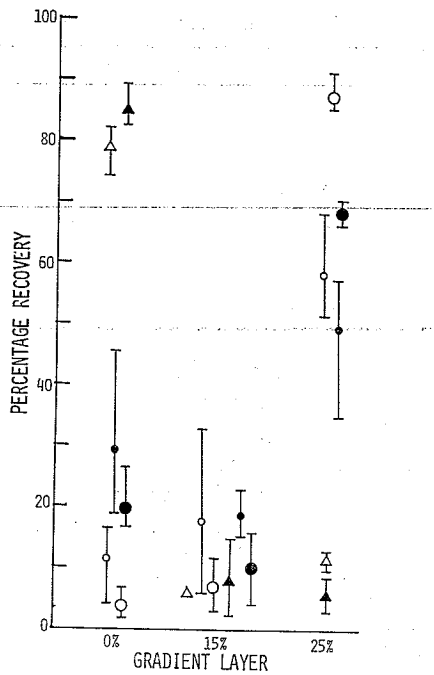

Fig. 8. Difference of percentage recoveries of organisms between pretreated with 70 % Ethyl alcohol (open symbols) and untreated (closed symbols) samples. For the symbols see Fig. 2, and for the experimental conditions see "Materials and Methods" of the text.

they were entangled with each other too tightly to be separated even with use of L-shaped wire. It is usually seen that when samples preserved in 70 % alcohol are placed in water, they disperse quickly on surface of water. It was tested how soaking plankton in 70 % alcohol (5 min in our experiments) before settling to the chamber might affect separation of fish larvae from others (Fig. 8).

Percentage recoveries in pretreated samples ranged from 86-92 % in 25 % layer for fish larvae and from 75-83 % in 0 % layer for invertebrates while in untreated samples, they ranged from 67-71 % for fish larvae and from 83-90 % for invertebrates, respectively in the same layer. The lower recoveries for fish larvae in untreated samples were due to occurrence of some larval fishes in 0 % layer. They were found to be attached to or entrapped with other plankton. On the other hand, it was observed that once placed on the uppermost layer, pretreated samples dispersed freely on surface, facilitating settling of dense fish larvae downwards, and stayed there for a time, allowing use of L-shaped wire efficiently. It was assumed that this phenomenon due to alcohol soaking was the main reason for high separation of fish larvae from others. Distribution of fish eggs was not affected by presoaking with alcohol. They were found in all layers with the majority in 25 %.

Though pretreatment was obseved to be effective for separation, small amount of fish larvae, especially elongated ones, remained entrapped in almost all experiments.

8. Comparison between all-manual sorting and use of chamber

The effectiveness of use of sorting chamber can be judged from reduction of time required for sorting. And, if reduction is significant, it is easily understood that sorting error may be decreased.

Time required for all-manual sorting and use of chamber was compared. The time included in the sedimentation method was measured from

Table 1. Time consumed in sorting 5 g samples with all-manual and sorting chamber methods.

Sample	Performer	All-manual (a)	Sorting Chamber (s)	Ratio (s/a)
A	1	5 h 1 min	2 h 36 min	0.52
	2	5 h 9 min	2 h 51 min	0.55
	3	6 h 11 min	3 h 13 min	0.51
Average				0.53
B	1	1 h 1 min	31 min	0.50
	2	1 h 36 min	51 min	0.53
	3	2 h 27 min	1 h 25 min	0.57
Average				0.53

layering of samples through harvesting to end of sorting fish larvae and eggs. Manual method only included actual sorting process.

Table 1 shows the total time consumed in two methods for sorting 5 g samples from two typical collections where number of fish larvae contained was significantly different, resulting to shorter or longer sorting time. For all of three persons engaged in sorting, the times required were significantly different between both methods, on average being about a half time shorter with the use of chamber than all-hand sorting. Thus we can conclude that the use of this method greatly reduces sorting time needed.

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比重勾配液を用いた動物プランクトンの選別方法

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要旨: 無脊椎動物から仔魚を早く選別する方法として 2 *l* の簡単な選別容器と Trimetaphosphate 1.0% の試薬で修正した 0,15 及び 25% Ludox Am の 3 比重勾配液を用いた。5% フオルマン保存の鹿児島湾産標本で 25% 液と 0% 液から各々 70% 以上の仔魚と無脊椎動物が最良条件下で回収された。25% 液からの仔魚の最良回収条件は希釈液として蒸留水，5 g 以下の試料，5～30 分の沈積時間，70% アルコール浸漬による前処理，及び 2 週間以上の保存試料であった。この方法は手作業による選別時間を約半分に減じた。魚卵は特定の勾配液に沈積せず，その理由も明らかでなかった。

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