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Comparative analysis of *ex situ* zooplankton hatching methods

Análise comparativa de métodos de eclosão de zooplâncton *ex situ*

Daniel Nino Flores-Mendez[1]* and María Florencia Gutierrez[1,2]

[1]Instituto Nacional de Limnología, Universidad Nacional del Litoral – UNL, Consejo Nacional de Investigaciones Científicas y Técnicas – CONICET, Ciudad Universitaria, 3000, Santa Fe, Argentina

[2]Escuela Superior de Sanidad "Dr. Ramón Carrillo", Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral – UNL, Ciudad Universitaria, 3000, Santa Fe, Argentina

*e-mail: d.floresm@santafe-conicet.gov.ar

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Abstract: Aims: This study aims to analyze the efficiency of two novel methods for *ex situ* zooplankton hatching experiments, compared with a traditional one. Both proposed methods were specifically designed to minimize sediment resuspension during the sampling of hatched individuals when no previous egg isolation is performed. **Methods:** Sediment samples were collected from shallow lakes, homogenized, and incubated for 18 days under stable laboratory conditions. The traditional method (1M) involved simple water filtration from incubated sediments. The so called "inverted funnel filtering" method (2M) includes an inverted funnel located above the sediment to trap zooplankton that passes through the funnel aperture, and the "levels filtering" method (3M) involves perforated plates above the sediment. The efficiency of each method was evaluated by analyzing the cumulative abundance and number of taxa in hatched total zooplankton, rotifers, and microcrustaceans, as well as the overall composition. **Results:** The new proposed methods significantly favored higher abundances than 1M for total zooplankton and rotifers. Even more, 3M outperformed 2M in the case of microcrustacean hatching abundances. **Conclusions:** Our findings suggest that despite all analyzed methods being suitable for studying zooplankton hatchings, the newly proposed methods incorporating internal structures to minimize sediment resuspension displayed increased capture efficiency.

Keywords: methods; *ex situ* experiments; resistance eggs; passive zooplankton; sediment.

Resumo: Objetivo: Este estudo tem como objetivo analisar a eficiência de dois novos métodos para experimentos de eclosão de zooplâncton *ex situ*, comparados com um método tradicional. Ambos os métodos propostos foram especificamente projetados para minimizar a ressuspensão de sedimentos durante a amostragem de indivíduos eclodidos quando não há isolamento prévio dos ovos. **Métodos:** Amostras de sedimentos foram coletadas de lagos rasos, homogeneizadas e incubadas por 18 dias em condições laboratoriais estáveis. O método tradicional (1M) envolveu uma simples filtração da água dos sedimentos incubados. O método chamado "filtragem por funil invertido" (2M) inclui um funil invertido localizado acima do sedimento para capturar zooplâncton que passasse pela abertura do funil, e o método "filtragem por níveis" (3M) envolveu placas perfuradas acima do sedimento. A eficiência de cada método foi avaliada analisando a abundância cumulativa e o número de táxons no zooplâncton total eclodido, rotíferos e microcrustáceos, bem como a composição geral. **Resultados:** Os novos métodos propostos favoreceram significativamente uma maior abundância do que 1M para zooplâncton total e rotíferos. Além disso, 3M superou 2M no caso das capturas de eclosão de microcrustáceos. **Conclusões:** Nossos resultados sugerem que, apesar de todos os métodos analisados serem adequados para estudar eclosões de zooplâncton, os novos métodos propostos que incorporam estruturas internas para minimizar a ressuspensão de sedimentos apresentaram maior eficiência de captura.

Palavras-chave: métodos; experimentos *ex situ*; ovos de resistência; zooplâncton passivo; sedimento.

1. Introduction

Zooplankton "egg banks" have been a target of several studies aiming to explore past communities, assess temporal patterns, population dynamics and seasonal succession among many other processes within this community (Hairston et al., 2000; Brendonck & De Meester, 2003; Gyllström & Hansson, 2004; Nevalainen et al., 2011; Vehmaa et al., 2018). In this regard, the study of passive zooplankton has been useful in diversity studies, paleolimnology, fundamental and applied ecology, climate change effects on aquatic systems, and other related field of research (Jeppesen et al., 2001; Vandekerkhove et al., 2005; Gaikwad et al., 2008; Nielsen & Brock, 2009). More recent investigations have analyzed the relationship of eggs banks with harsh environmental conditions such as pollution, and eutrophication as well as their implications in environmental restoration practices (Nevalainen et al., 2011; Piscia et al., 2016; Gutierrez et al., 2017; Rogalski et al., 2017; Portinho et al., 2018; Coelho et al., 2021).

Although hatching ecology of passive zooplankton is a relatively old field of research, there are still many methodological gaps for the *ex situ* experiments involving hatching tests. More specifically, the selection of an appropriate hatching method for those experiments that use the whole sediment instead of resting eggs previously isolated is an important challenge. In this regard, there is no consensus on how to collect recently hatched zooplankton organisms avoiding alterations on the sediment structure (e.g., resuspension), water characteristics (e.g. turbidity), and sample quality (e.g. containing suspended particles or even non-hatched resting eggs).

Direct visualization and manual separation of newborns have been frequently reported, which is a useful technique for studying easily visible individuals such as microcrustaceans (Vandekerkhove et al., 2004; Liefferink et al., 2014; Vargas et al., 2019). However, this technique is inadequate for small individuals such as rotifers for which the filtering method seems to be the most convenient. This consists of removing and filtering the water from the incubation tray by using hoses, pipettes or even tilting the container to pour the water over the filter (Battauz et al., 2015; Silva Bandeira et al., 2020; Souza Santos et al., 2021; Brazil et al., 2022). Another similar technique consists of sweeping the water with a manual net directly inside the tray (Vendramin et al., 2023). Nonetheless, all these techniques have disadvantages already mentioned, primarily related to the sediment's resuspension during the water collection phase or when returning the water after the filtering process. Another important problem that emerges is the possibility that some hatched organisms remain retained in the upper layer of the substrate during the water collection process. This failure may cause an underestimation of the number of hatchings and the possibility that such organisms be reproduced before the next sampling stage, causing subsequent overestimations.

To overcome these limitations, a recent alternative technique was used involving the inclusion of a series of perforated plates at different levels inside the incubation trays (Gutierrez et al., 2017). This method could be based on the assumption that hatching organisms ascend in the water column, but in any potential descent to the bottom do not reach the sediment. However, the efficiency of this technique has not been experimentally validated yet.

Based on methodologies previously used in other *in situ* and *ex situ* hatching experiments, we designed and tested two incubation methods that allow minimizing sediment resuspension during the hatched individuals sampling. Both methods were compared with a traditional one in terms of capture efficiency (measured as individual abundance and number of taxa) and composition differences.

2. Material and Methods

2.1. Methodological designs

The main characteristics of the capture methods of zooplankton hatching are visualized in Figure 1.

The first method is the traditional "free filtering technique" (1M). This classic filtering technique consists of extracting and filtering the supernatant water from the containers through a small hose or pipette.

The second method is called "inverted funnel filtering" (2M) which is a modification of the frequently used *in situ* hatched method (Whiteside & Williams, 1975; Brakke, 1976). Inverted funnel traps have been widely used in *in situ* hatching studies, mainly for cladocerans and copepods (De Stasio, 1990; Compte et al., 2016) but also it has been successfully employed in laboratory experiments (Gutierrez et al., 2020). This consists of using an inverted funnel above the sediment, which works as a trap for the hatching zooplankton that passes the funnel opening, becoming trapped in the upper level. For this, we used a plastic inverted funnel with an opening diameter of 2.2 cm which was put on 1.5 cm of sediment level.

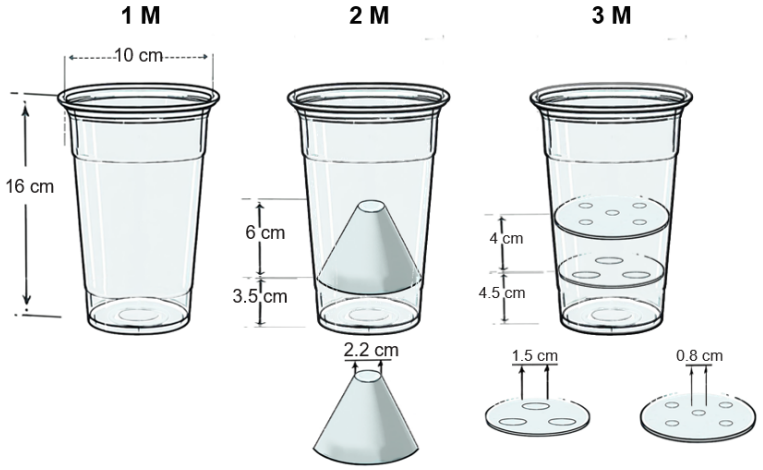

Figure 1. Characteristics of the containers used in three methods for capturing dormant states of zooplankton: 1M = free filtering technique; 2M = inverted funnel filtering technique; 3M = levels filtering technique.

The third capture method is called the "levels filtering" (3M) which is a modification of the filter method with perforated trays (Gutierrez et al., 2017). This technique consists of using perforated trays placed on different levels above the sediment. Similarly, to the second method, the trays work as a trap for the hatching zooplankton that passes the perforations. For this, we used two perforated trays and put them into 4 cm and 8 cm of sediment level. The lower level tray had 3 perforations of 1.5 cm in diameter while the upper one had 5 perforations of 0.8 cm in diameter.

In the three cases, we used clear plastic cups of similar characteristics and dimensions (volume of 1000 ml, height of 17 cm, upper diameter of 10.5 cm, and lower diameter of 7 cm). Our decision to use taller than wide containers was based on preliminary experiments that displayed higher hatchings in the former than in the latter.

2.2. Sediment sampling and treatments

Sediment samples from two neighboring shallow lakes were collected between May and July 2023. Both lakes were located in the ecological reservoir of the University Campus of "Universidad Nacional del Litoral" (Santa Fe, Argentina 31°37′ S, 60° 41′ W). The lakes belong to the alluvial plain of the Paraná River and receive inputs from groundwater and other water bodies close to the Paraná River mainstream during flood periods.

The samples were collected from the surface sediment (<10 cm deep) with an 8 cm diameter sediment corer. A total of 20 sediment samples were taken at different points of the littoral zone of both lakes. Subsequently, the sediment was dried on trays in an environment climatized laboratory (22 °C) for a week. After this time, the samples were mixed, homogenized, and stored in the dark at 4 °C for 3 weeks, to stimulate the hatching process of zooplankton (Vandekerkhove et al., 2005).

2.3. Experimental setup and hatching collection

After the storage period, 30 g of homogenized sediment was distributed into 18 containers, which were randomly assigned to one of the three capture methods (i.e. 6 replicates per method). Then, 700 ml of 24-h aerated and dechlorinated tap water was added to each container. The sediment level inside each container reached 1.5 cm and the water level reached 13.5 cm. During the hatching assessment period, the containers were maintained in controlled conditions of temperature (25 °C) and photoperiod (16 h light: 8 h dark).

The hatchings of zooplankton resting stages in the sediment of all containers were monitored for 18 days. Samples of hatched organisms were collected from the containers by filtering the entire water content using a 60 μm plankton net. After filtration, the water was returned to the containers, and dechlorinated water was added to maintain the initial volume. Sampling occurred every two days during the first two weeks and every three days during the final week.

All the collected samples were fixed with 10% formalin. The samples were identified and counted under an optical microscope at the magnification of 20-400x, in 1 mL Kolkwitz chamber. For taxonomy identification, we used specific keys (Ringuelet, 1958; Reid, 1985; Voigt & Koste, 1978; Paggi, 1979; Smirnov, 1992).

2.4. Data analyses

To compare the efficiency of the capture methods, we calculated the cumulative abundance and number of taxa. The cumulative data were used to constrain the daily variation in zooplankton hatching. We employed generalized linear models (GLM) to assess three capture methods' influence on the cumulative abundance and number of taxa. Error distributions and link functions were chosen for each variable based on the base of outlined by Buckley (2015). The binomial negative distribution and logarithm link function were best adjusted to cumulative abundance, and the Poisson distribution and logarithm link function to cumulative numbers of taxa. Consequently, Likelihood Ratio Tests (LRT) were conducted to determine the significance of the models and Bonferroni post hoc tests by contrast among capture methods. All analyses were made in R software (version 4.0.2).

We analyzed the community structure to analyze the temporal dynamics among the capture methods. The community structure of captured zooplankton was analyzed through Monte Carlo permutation tests in constrained ordination, calculated by Principal Response Curves (PRC) analyses using *prc* {vegan} in R. PRC is a multivariate analysis grounded in redundancy analysis (RDA), which is adjusted to account for overall changes in community response over time. This analytical approach allows for both graphical and quantitative interpretations of effects at the species level over time (Van Den Brink & ter Braak, 1999). PRC employed diagrams to illustrate treatment deviations over time relative to a control treatment (1M) and species weights about the pattern in the PRC. Permutation tests are carried out by sampling date using treatment levels as explanatory variables to determine treatment significance, with $p < 0.05$ denoting significance. PRC has been employed in many studies involving Treatments with Repeated Observations in zooplankton (Van Den Brink et al., 2000; Hanson et al., 2007; López-Mancisidor et al., 2008; Moreira et al., 2021).

The physicochemical parameters were assessed using the Wilks test of Multivariate Analysis of Variance (MANOVA) using *manova* {stats} in R. MANOVA is an extension of ANOVA tailored for two or more continuous response variables. It combines these variables linearly to optimize the separation of groups defined by the independent variable. MANOVA is applied by researchers in water quality assessment (Basu & Lokesh, 2014; Medeiros et al., 2013).

3. Results

A total of 30 taxa of hatched zooplankton were identified throughout the experiment (Table 1). Rotifers exhibited the highest taxa number, (n=22), followed by microcrustaceans, of which 7 taxa and one larval stage were observed, enclosing 6 cladoceran species and a single copepod.

There was a significant difference in the abundance among the three methods (LTR tests: $p < 0.05$); however, no variations were observed in the number of taxa (LTR tests: $p > 0.05$, Figure 2). 2M and 3M resulted in significantly higher abundances compared to 1M, both in the total and rotifers counts. In microcrustacean counts, 3M exhibited a greater abundance compared to 2M. Conversely, the number of taxa remained consistent across all three methods, inclusive of total, rotifers and microcrustaceans counts.

The composition of hatched zooplankton was unaffected by the capture methods (Monte Carlo test, $p = 0.25$). The PRC showed a similar zooplankton taxonomy composition among the three capture methods (Figure 3).

Physicochemical parameters of water were comparable across the three capture methods (MANOVA: $\text{Wilks}_{(8:94)} = 0.828$; $p = 0.328$). However, they varied significantly concerning sample days (MANOVA: $\text{Wilks}_{(4:47)} = 0.0507$; $p < 0.001$). Temperature, pH, and dissolved oxygen increased over time, while conductivity decreased (Table 2).

4. Discussion

Our data demonstrate that the capture methods 2M and 3M with barriers that create two or three internal levels, respectively, in the water column could be more efficient to collect hatched individuals than the traditional ones in terms of organism abundance. Both methods significantly improved the number of rotifers, while 3M significantly improved the number of both, rotifers and microcrustaceans. However, the number of taxa and taxonomic composition were similar among the three evaluated methods.

The differences observed among the three methods could be mainly associated with the effects of sediment resuspension during water filtration and then returning. On the one hand, sediment disturbances have the potential to alter the vertical distribution of eggs and modify sediment thickness (Marcus & Taulbee, 1992; Radzikowski et al., 2016), consequently impacting the hatching

Table 1. List of zooplankton taxa in capture methods.

Taxa	1M	2M	3M
Rotifera			
Bdelloidea	X	X	X
Brachionus angularis (Gosse, 1851)	X	X	
Brachionus rubens (Ehrenberg, 1838)			X
Cephalodella serrata (Wulfert, 1937)		X	X
Dipleuchlanis propatula (Gosse, 1886)	X		
Epiphanes sp. (Ehrenberg, 1832)			X
Filinia terminalis (Plate, 1886)	X		
Lecane bulla (Gosse, 1851)	X	X	X
Lecane closterocerca (Schmarda, 1859)	X	X	X
Lecane cornuta (Müller, 1786)			X
Lecane hamata (Stokes, 1896)	X	X	X
Lecane leontina (Turner, 1892)			X
Lecane pyriformis (Daday, 1905)	X	X	X
Lecane quadridentate (Ehrenberg, 1830)			X
Lecane undulata (Hauer, 1938)		X	
Lepadella ovalis (Müller, 1786)	X	X	X
Lepadella patella (Müller, 1773)	X	X	
Lepadella rhomboides (Gosse, 1886)	X	X	
Mytilina ventralis (Ehrenberg, 1830)	X		
Platyias quadricornis (Ehrenberg, 1832)	X	X	X
Trichocerca stylata (Gosse, 1851)	X		
Trichocerca tenuior (Gosse, 1886)	X		
Cladocera			
Ceriodaphnia cornuta (Sars, 1885)	X	X	X
Ceriodaphnia dubia (Richard, 1894)	X		X
Chydorus pubescens (Sars, 1901)	X		
Daphnia obtuse (Kurz, 1874)			X
Leberis davidi (Richard, 1895)		X	
Neonates	X	X	X
Simocephalus vetulus (Müller, 1776)		X	X
Copepoda			
Cyclopoida	X		X

1M = free filtering technique; 2M = inverted funnel filtering technique; 3M = levels filtering technique.

Table 2. Mean and standard deviation (SD) values of water physicochemical parameters.

Method	Day	pH		Conductivity (µS cm-1)		T (°C)		DO (ppm)	
		mean	SD	mean	SD	mean	SD	mean	SD
1M	0	8.20	0.16	227	17	20.7	0.0	9.23	0.28
2M	0	8.15	0.07	211	7	20.8	0.1	9.23	0.18
3M	0	8.19	0.12	218	11	20.8	0.1	9.36	0.25
1M	2	7.54	0.07	303	51	20.4	0.0	8.30	0.12
2M	2	7.51	0.09	240	7	20.4	0.1	8.55	0.11
3M	2	7.50	0.17	264	19	20.4	0.0	8.32	0.12
1M	18	7.17	0.05	401	106	19.8	0.0	6.84	0.62
2M	18	7.28	0.18	398	23	19.8	0.1	7.14	0.98
3M	18	7.25	0.12	398	26	19.8	0.1	6.18	0.92

1M = free filtering technique; 2M = inverted funnel filtering technique; 3M = levels filtering technique; DO = Dissolved oxygen.

success of the eggs. On the other hand, some individuals that prefer to hide near the bottom (Tavşanoğlu et al., 2012) can be retained and trapped in sediment meanwhile the water media is being filtered. Therefore, the presence of barriers, creating internal compartments in the water column

Table 3. Practical characteristics of the *ex situ* zooplankton hatching methods.

Practical conditions	1M	2M	3M
Number of captures	Low	High	High
Sediment resuspension	High	Intermediate	Low
Sampling time *(means ± SD)* in min.	Low *(3.96±0.13)*	Intermediate *(4.52±0.10)*	High *(5.14±0.14)*
Manipulation complexity during the filtering process	Low	Intermediate	High

1M = free filtering technique; 2M = inverted funnel filtering technique; 3M = levels filtering technique.

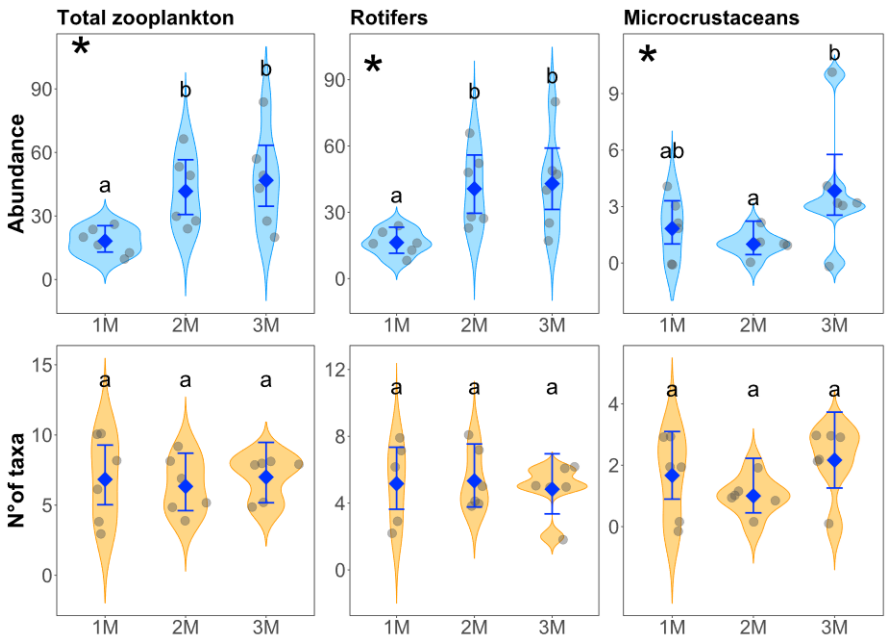

Figure 2. Abundance and number of taxa in the total zooplankton, rotifer, and microcrustaceans groups. 1M = free filtering technique; 2M = inverted funnel filtering technique; 3M = levels filtering technique. Asterisks (*) show the models that significatively differ from the null one and letters a, b, and ab indicate homologous groups among capture methods based on Bonferroni *post hoc* test.

has the double role of diminishing sediment resuspension and increasing individual captures.

The difference observed in microcrustaceans between both proposed methods (i.e. lower capture rate in M2 compared to M3) may be attributed to their swimming behavior patterns. Some cladocerans typically swim by hopping and sinking (Dodson & Ramcharan, 1991; O'Keefe et al., 1998; Uttieri et al., 2014), which could be a disadvantage to ascend to the upper level when colliding on the inclined surface of the cone at 2M.

The similarity in the number of taxa, and composition, among the capture methods suggests that all three methods are appropriate for studying zooplankton hatchings. However, each method may also present practical complications. For this reason, we provide a comparative table of practical conditions that can help using each capture method according objective of the study (Table 3).

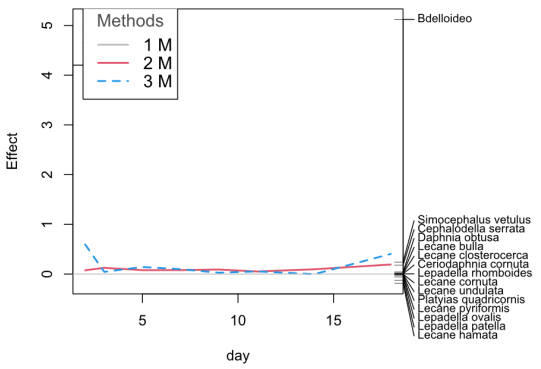

Figure 3. Principal Response Curves of the zooplankton composition among the three capture methods. 1M = free filtering technique; 2M = inverted funnel filtering technique; 3M = levels filtering technique.

The structures of the inverted funnel and perforated trays, while effective in preventing sediment

resuspension, also pose challenges for water filtration manipulation. Consequently, this necessitates greater caution in the filtration process.

Up to date, this study represents the first comparative analysis of *ex situ* capture methods for dormancy stages in zooplankton. We emphasize the need for further research on other methodological gaps in studies concerning dormancy stages in zooplankton, including issues such as sampling periodicity, light intensity, temperature, and other factors that trigger the hatching process.

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