Culture Techniques for *Acartia clausi* from the Romanian Black Sea coastal waters *(George-Emanuel Harcotă, Elena Bișinicu, Florin Timofte, Aurelia Țoțoiu, Cristina Tabarcea, Geta Rîșnoveanu)*	"Cercetări Marine" Issue no. 52 Pages 91-105	2022
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CULTURE TECHNIQUES FOR *ACARTIA CLAUSI* FROM THE ROMANIAN BLACK SEA COASTAL WATERS

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ABSTRACT

As copepods are an important food source for most fish larvae, there is a continuing interest in developing techniques for culturing marine copepods as live food in aquaculture. Studies have shown that several species of calanoid copepods can be used successfully in aquaculture, acclimatized and grown in the laboratory over several generations and serve as testing organisms in acute toxicity tests. However, significant difficulties in cultivating calanoid copepods in high quantities were reported. They are related to their low tolerance to changes in water quality and reduced production capacity compared to other taxonomic groups. Therefore, working methods for obtaining viable cultures of calanoid copepods are dependent highly on the local environmental context. Until now, such a method has not been implemented for calanoid species from the Romanian Black Sea coast. This paper details the methodology we adapted and used for achieving a viable *Acartia (Acartiura) clausi* (Giesbrecht, 1889) culture using specimens collected along the Romania Black Sea coast Reproduction and egg hatching occurred under laboratory-controlled conditions, and viable individuals of *A. clausi* were obtained. Our results open the possibility of integrating this species in toxicity tests and food production for the marine aquaculture industry.
Keywords: laboratory, *Acartia clausi*, copepod, Black Sea, culture

AIMS AND BACKGROUND

Copepods dominate the marine environment, and most development stages are generally planktonic (Alajmi *et al.*, 2015).

Marine copepods are a rich source of essential fatty acids, free amino acids and other micronutrients compared to traditional food, rotifers or *Artemia* sp. (Conceição *et al.*, 2010; Støttrup *et al.*, 1986). Moreover, the small size of the nauplii is essential for feeding fish larvae, being easy to digest in the early development stages. Live food (copepods) stimulates the feeding

responses of fish larvae through their distinctive swimming mode (Buskey *et al.*, 1993).

More than 11,500 species of copepods have been classified to date (Humes, 1994), but the number of species relevant to aquaculture and harvested on a large scale is very small. Of the ten taxonomic orders of copepods, only three are relevant aquaculture and used for feeding: Calanoida, Harpacticoida and Cyclopida. The most studied are the species belonging to the genera Acartia and Calanus, but also Temora, Paracalanus, Pseudocalanus or Centropages. They have high abundances in the pelagic and coastal environments, so sampling is more accessible (Støttrup, 2019). Due to their importance as a food source for most fish larvae, there is an ongoing interest in developing techniques for the culture of marine copepods to be used as live food in aquaculture (Alajmi *et al.*, 2015).

Previous studies have shown great difficulties in obtaining calanoid copepod cultures in high abundance due to their low tolerance to water quality changes and low production capacity compared to other taxonomic groups (Støttrup, 2019). Recent studies have demonstrated that several calanoid species such as *Acartia (Acanthacartia) tonsa* (Dana, 1849), *Acartia (Acanthacartia) sinjiensis* (Mori, 1940) and *Parvocalanus crassirostris* (Dahl F., 1894) can be successfully used in aquaculture, acclimatized and cultured in the laboratory over several generations.

The cryptophyte *Rhodomonas sp.* was used as a food source for copepods in culture. It represents a high-quality food with a high efficiency of carbon absorption by pelagic copepods. According to Peter Thor and Ida Wendt (2010), the rate of ingestion and absorption of the *Rhodomonas baltica* Karsten, 1898 (current name *Cryptomonas baltica* (Karsten) Butcher, 1967) is higher than those of other microalgae species (*Dunaliella tertiolecta* Butcher, 1959; *Thalassiosira weissflogii* (Grunow) G. Fryxell & Hasle, 1977) considered important sources of food for herbivorous copepods. Higher densities have been observed in the cultures where the organisms were fed with *Rhodomonas* sp. algae (Thor & Wendt, 2010). ISO 14669 (1999) recommends *R. baltica* algae as the primary food for the species *A. tonsa*. This is why this algal species is used as the main food source for laboratory-grown cultures.

Experiments use marine copepods acclimatized in the laboratory for long periods (ISO 14669, 1999) or kept in the laboratory for a relatively short time after collection (Finiguerra *et al.*, 2013; Sun *et al.*, 2015). Both methods can influence the results of experiments since the long-term maintenance of organisms in the laboratory can decrease genetic variability over time (Lopes *et al*., 2021), and freshly collected individuals need to be acclimatized.

The main objective of this study is to demonstrate a working method in a laboratory-controlled environment using *Acartia (Acartiura) clausi*

Giesbrecht, 1889, under characteristic conditions of the Romanian Black Sea waters to obtain viable offspring.

Sample Collection

Organisms were collected from the natural environment using the Juday net along the Mamaia Pier (Fig. 1).

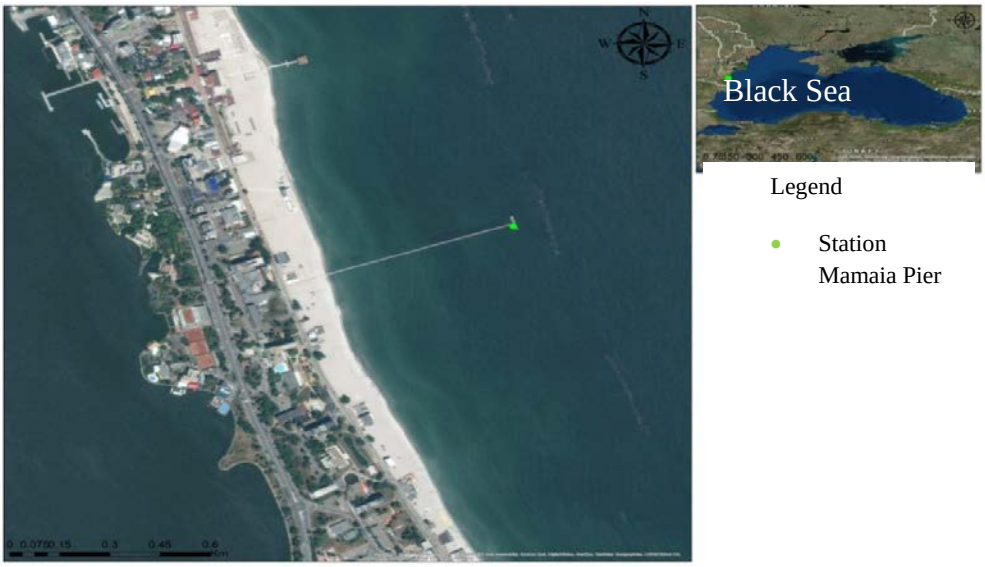

Fig. 1. Sampling station (original, GIS map)

The Juday net (Fig. 2) is recommended by the Zooplankton Manual "Black Sea Monitoring Guidelines Mesozooplankton" (Harris *et al.*, 2000) and has the following characteristics:

• the opening/diameter of the net (upper ring) has of 36 cm;

• the middle ring has Ø = 50 cm, forming a truncated cone with a height of 120 cm made of non-filtering material;

• the lower part, forming another truncated cone of 150 cm, has a 150 μm sieve mesh filtering sieve;

• the collecting has a 150 μm filter sieve;

• to maintain the net verticality when it is pulled towards the surface, a weight of at least 5 kg will be used;

• a flowmeter attached to the net inside the upper ring to determine the filtered volume.

The net was completely submerged in water and towed horizontally (Fig. 3). Suppose the operation of collecting organisms takes place at a fixed point; the depth from the sampling station must be at least 5 meters. In that case, it can be towed vertically (in the water column) (Fig. 4). A flow meter can be attached to calculate the filtered volume.

Fig. 2. Juday net (*original photo*)

The biological material from the collection cup was placed in a container filled with seawater from the sampling location (Fig. 5). The samples were taken to the laboratory as soon as possible to avoid being subjected to other environmental factors that could negatively influence the organism's behaviour.

Fig. 3. Towing the Juday net-horizontal (*original photo*)

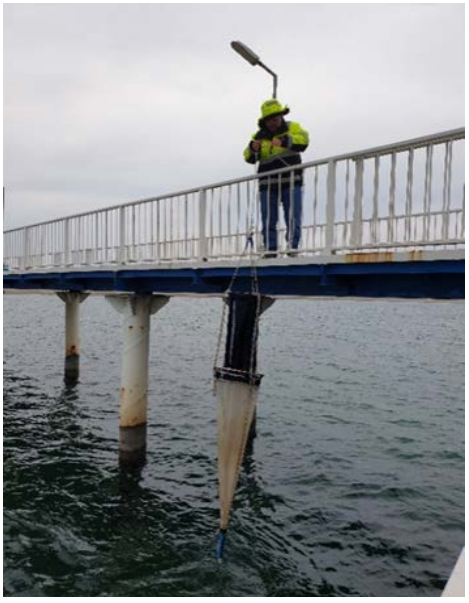

Fig. 4. Towing the Juday net-vertical (*original photo*)

Fig. 5. Collection of individuals and their transfer in a container which was transported to the laboratory (*original photo*)

RESULTS AND DISCUSSION

A. Acartia clausi characteristics

The species of interest in this experiment is *A. clausi*, a marine copepod belonging to the Acartiidae family. It has an elongated and thin body, having a single eye on the dorsal side of the head and no lenticular orbit.

- Male size 1 – 1.5 mm (Muller & Godeanu, 1995) (Fig. 6);
- Female size 1.17-1.75 mm (Muller & Godeanu, 1995) (Fig. 7);
- Egg size 70-80 µm (Muller & Godeanu, 1995) (Fig. 8).

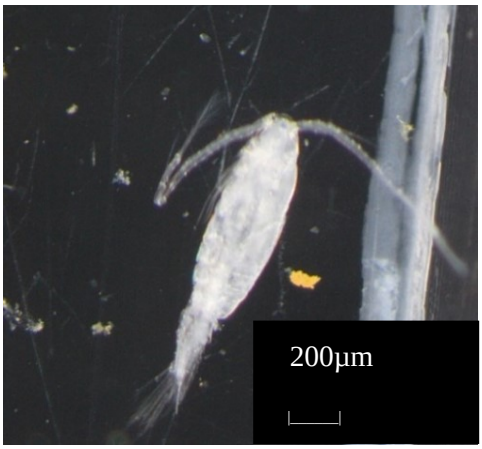

Fig. 6. *A. clausi* male (*original photo*)

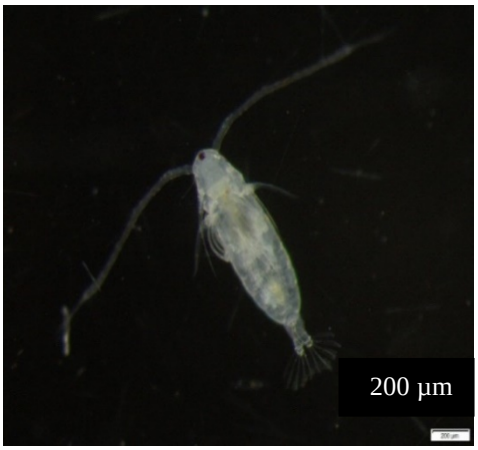

Fig. 7. *A. clausi* female (*original photo*)

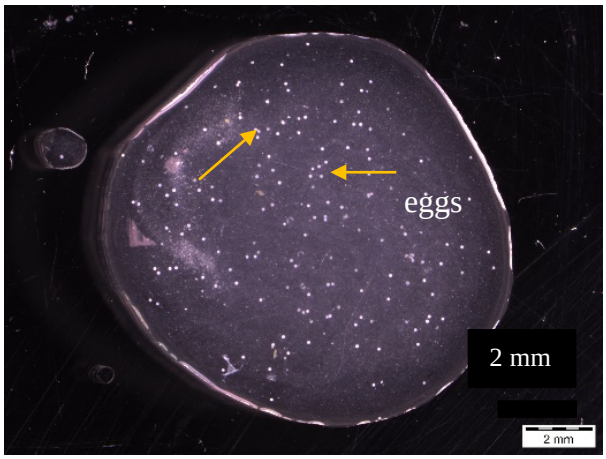

Fig. 8. *A. clausi* eggs (*original photo*)

This species was thought to be distributed worldwide. Still, recent research has shown that the distribution area is restricted to the coastal regions of the North-East Atlantic Ocean, the Mediterranean Sea and the Black Sea (Onciu *et al.*, 2006).

A. clausi, a pelagic species, lives in marine waters, in the water column, from the surface to 200m depth. It performs both horizontal and vertical migrations every day. During the night, it reaches the surface layers. *A. clausi*, dominant in the Black Sea plankton, a species with a rapid development cycle, achieves high abundances and biomasses in a short period, being a widespread species with maximum development in the warm season (summer) (Onciu *et al.*, 2006).

Feeding takes place by filtering the water and consuming phytoplankton and also zooplankton. Reproduction is achieved through internal fertilization. Eggs are denser than water, so they sink to the bottom of the sea.

B. Set up the culture of A. clausi

The samples were brought to the laboratory and sorted to remove gelatinous organisms that represent a major risk (predation) for copepods. The recommendation is to filter through a 5000 μm sieve (opening of the filter mesh) to eliminate large gelatinous organisms. The sample was again filtered through sieves of 60, 150, 300 and 500 μm (Fig. 9) to sort the organisms according to their size and developmental stage (Table 1).

This method makes it much easier to select copepods of interest. Freshly collected samples were placed in glass containers with water from the collected environment and left for 48 hours, with light aeration (1 bubble/second) continuously, for acclimatization to the laboratory

environment (Fig. 10). After acclimatization, *A. clausi* individuals were extracted and filtered using 300/150 μm filter sieves (Table 1) according to the required sizes or stages. The copepods' adult stages were selected; in this way, the eggs were obtained faster to carry out future cultures in the laboratory.

After filtering, the organisms were sorted using the Bogorov counting chamber or a Petri dish and placed under the magnifying glass (Fig. 11) to eliminate other uninteresting organisms. In this way, a culture with only *A. clausi* individuals was obtained.

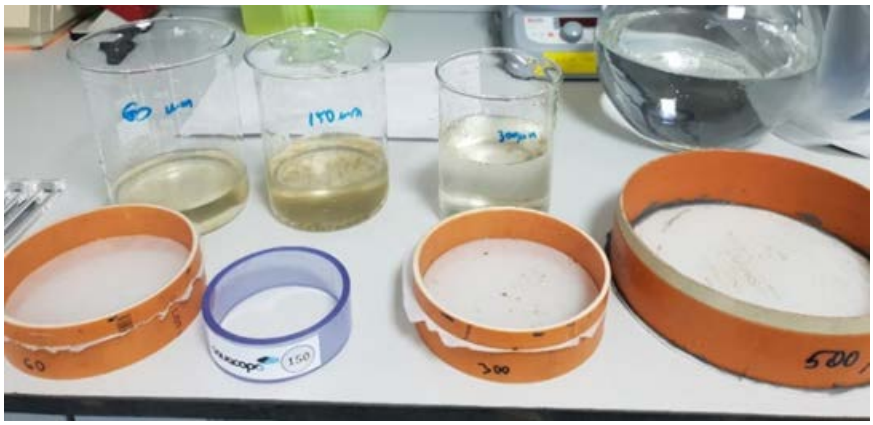

Fig. 9. Sieve types- 60, 150, 300 and 500 μm (*original photo*)

Fig. 10. The procedure for distributing organisms in culture dishes (*original photo*)

Table 1. Types of sieves used to filter the organisms adapted from ISO 14669, 1999

Sieve dimension (μm)	Specifications
60	Retains all stages, including eggs
100	Retains all stages except the 0-2 day nauplii
140	Retains copepods and adults
180	Retains only adults
500	Retains small gelatinous organisms (*Pleurobrachia pileus*)
5000	Retains gelatinous organisms (ctenophores, scyphozoans)

The proposed filtering method modifies the ISO 14669, 1999 method and considers the recent literature (Santhosh *et al*., 2018). Specifically, two filtering steps were added to the ISO protocol to remove gelatinous organisms that inevitably end up in samples collected from the natural environment:

-a 500 μm sieve that removes tiny jellyfish such as *Pleurobrachia pileus*, and

-a 5000 μm sieve that retains the larger jellyfish and prevents them from reaching the experimental vessels.

After the isolation of the organisms, they were carefully placed in a container (4 L round flat-bottomed glass flask) with 3.5 L of filtered seawater.

Fig. 11. Bogorov counting chamber, Petri dish and a binocular (*original photos*)

Water used as a culture medium in the laboratory should be collected from an unpolluted natural environment. Water was filtered through filters (Millipore) with a porosity of 0.45 μm (or smaller if applicable) using a vacuum pump through a recirculation system to remove harmful organisms and pathogens. Afterwards, water was treated with a UV lamp (Fig. 12). Filters with a porosity of less than 0.45 μm were used whenever there was a possibility of water contamination with microalgae, microsediment particles, or microplastics is susceptible. For the complete elimination of microalgae from the used water, water sterilization in an autoclave is recommended.

The ideal conditions to maintain the cultures of *A. clausi* were tested, and ISO 14669 (1999) standard was modified to meet the salinity requirement of species adapted to the Black Sea environmental conditions as follows:

- Salinity – 11-18 PSU (similar to the salinity of the Black Sea, according to the data provided by NIMRD)
- Temperature – 20°C ± 3°C
- Lighting – up to 2200 lux
- Oxygen saturation > 80%
- pH 8 ± 0.5

Before initiating cultures, the above conditions were checked and adjusted for compliance so as not to endanger the organisms' survival, growth and prolificity.

Fig. 12. Filters (Millipore) with different porosities used for the vacuum pump (*original photos*)

C. Set up the culture of Rhodomonas sp.

Production of microalgae cultures *Rhodomonas sp.*, as a food source was also ensured. *Rhodomonas sp.* was produced in a sterile culture medium to exclude contamination with bacteria of other microalgae (Fig. 13).

To obtain the microalgae culture, *Rhodomonas sp.* was acclimatized to the environmental conditions of the Black Sea. Seawater from the Black Sea, obtained through a pumping system from the natural environment, was filtered (filters with a porosity of 0.22 μm) and autoclaved for 40 minutes at a temperature of 121 °C.

Microalgae culture was carried out in sterile glass containers with volumes between 50 and 4000 ml (Fig. 14).

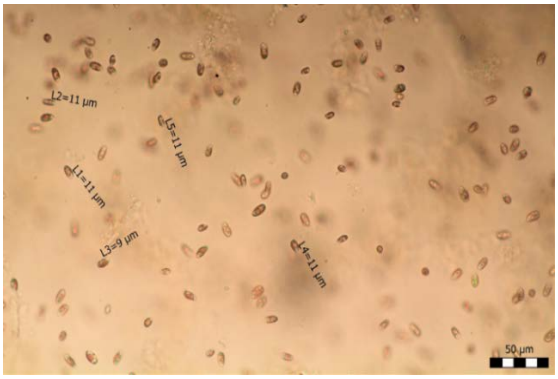

Fig. 13. *Rhodomonas sp.* (*original photo*)

Fig. 14. Microalgae culture with *Rhodomonas sp.*(*original photo*)

The ideal conditions for the optimal development of the *Rhodomonas sp*, according to ISO 14669, 1999, are:

• Temperature 20°C ± 3°C
• Lighting - up to 10,000 lux (preferably cool white light)
• Controlled photoperiod 16:8 light/dark
• Strong oxygenation – to produce a continuous movement of microalgae
• pH 8 ± 0.5

The microalgae culture was started from a small volume (50 ml) to which 0.05 ml of Walnes culture medium was added (1 ml of Walnes medium per 1 litre of culture). Production of microalgae proved to work better on the Walnes medium than on other culture mediums mentioned in the literature (e.g., F/2 culture medium). Microalgae culture medium can be purchased directly from a manufacturer or prepared in the laboratory following a recipe. As *Rhodomonas sp* is recommended as the best food source for *Acartia sp.* (Thor & Wendt, 2010) and is not a native species in the Black Sea, it was bought commercially. In the laboratory, *Rhodomonas sp*. was acclimatized to the Black Sea's salinity values characteristics. Under the Black Sea characteristic salinity, the algae grow better on Walnes medium; therefore, we modify the ISO14669 (1999) standard by using Walnes medium instead of the F/2 medium as the standard recommends.

Culture stocks were replanted at the time of maximum density by subsequent replanting, increasing the volume with sterile seawater and culture medium up to the desired volume. The culture of *Rhodomonas* sp. was administered as copepod food when the concentration of the micro-algae culture had a density of about 2 × 10 ^7 cells/ml of *Rhodomonas sp.*

Feeding of *A. clausi* individuals was carried out daily, taking into account the microalgae culture density (the number of microalgae cells) relative to the number of individuals in the culture.

The microalgae were concentrated by centrifugation and resuspended in the copepod culture as food. The biological material was centrifuged in 50 ml test tubes with threaded caps at 2,000 rpm for 20 minutes using the laboratory centrifuge (Fig. 15). After centrifugation, the supernatant was removed, and the centrifuged biomass was carefully transferred to a sterile container. The food is recommended to be stored in the refrigerator, with a validity term of one week.

The density of microalgae resulting from centrifugation was determined using a hemocytometer under a microscope.

The calculation formula of the food volume for the organisms in the culture is: 2 ml ÷D.a. ×N. ÷50

Where:

D.a = density of microalgae in the culture;
N. = number of individuals in the copepod culture;
50 = maximum number of copepods in one litre of culture.

Fig. 15. The concentration of *Rhodomonas* sp. culture by centrifugation (*original photos*)

D. Monitoring the A. clausi culture

Monitoring the food supplied, the copepod development stages, and the culture's density is essential to keep the copepod culture under control.
Copepod cultures were monitored as follows:

- the copepod culture was filtered through a 60 µm sieve to retain all organisms once every three days;
- the sieve was washed with a pestle with filtered sea water;
- the organisms were placed in a container with a volume of at least 200 ml of sea water;
- the organisms were counted and observed in the Bogorov counting chamber under the magnifying glass;
- the status of the organisms was noted on the monitoring sheet;
- a water sample was taken from the culture, and the parameters (dissolved oxygen, pH, salinity and temperature) were measured and noted in the monitoring sheet;
- after that, the water was put back into the glass container, and then the organisms were placed back very carefully.

Reproduction of organisms and eggs collection

Adults of *A. clausi* were filtered from the initial culture using a 300 µm sieve

and placed in a 5 L container filled with seawater, separated in half by a removable sieve of 150 µm. The adult organisms were placed on top of the 150 µm sieve, which was submerged in water. After 24 hours, the organisms were removed from the collection container. The eggs produced by copepods settled at the bottom of the container due to their greater density.

Separating eggs from adults eliminates cannibalism in copepod culture and largely eliminates produced residues (faecal, food particles and detritus).

Given that the eggs are larger than 80 µm, a 60 µm sieve was used to collect them. This method of breeding and collecting eggs is an entirely new approach not found in ISO14669 (1999).

Egg hatching procedure

The collected eggs were placed in the Bogorov counting chamber under the magnifying glass, and the entire sample content was counted. If the number of eggs is large (more than 1000), the sample is divided into subsamples of a smaller volume (at least three subsamples), and the eggs are counted in each subsample. The total sample volume, the subsample volume and the number of eggs identified in the subsamples are considered to assess the hatch rate.

After counting, all eggs were added to a 250 ml flat-bottomed flask with filtered seawater (200 ml water). Aeration (1 bubble/second) was used for a higher hatching rate during the hatching period, providing the necessary oxygen for the organisms in the nauplia stage.

The flask with eggs was stored in a thermostatic chamber or an oven that ensured a constant temperature (20 °C ±3) and constant light of a maximum of 2,200 lux (light falling perpendicular to the flask).

Hatching occurs after 24 hours or later, up to 72 hours, depending on egg quality. The average time for egg hatching was 48 hours.

After the eggs were hatched, the flask`s contents were transferred to a Berzelius beaker with a volume of 250 ml for easier handling. The organisms in the nauplia stage were counted in the Bogorov counting chamber, and the hatching rate was assessed.

Abiotic parameters

The temperature was recorded daily, and salinity, pH, and dissolved oxygen were recorded weekly. The amount of feed added throughout the experiment influenced salinity, pH and dissolved oxygen. All parameters were maintained at the recommended values, and when exceedance was identified, necessary adjustments were made.

Sodium chloride (1 molar) was used to increase the salinity, and distilled water was added to decrease the value. Sodium hydroxide (1 molar) was added to increase the pH, and hydrochloric acid was added to decrease the value. The temperature was adjusted via thermostatic chamber settings,

and the aeration pressure was adjusted to maintain dissolved oxygen concentration. All these operations were performed only in the culture medium, without organisms. The organisms were added to the flasks only after the culture medium stabilized at optimal parameters.

E. Results obtained in the culture

The developed methodology (adapted ISO 14669, 1999) was applied to *A. clausi* to check if the organisms obtained in cultures using this method could survive and produce viable offspring.

This experiment took place over three months. The temperature varied between 19.6°C and 25°C, salinity between 15.98‰ and 17.69‰, pH 7.6 and 8.74, and oxygen was over 7.56 mg/l. The food was provided according to the number of individuals in experimental vessels (Fig. 16).

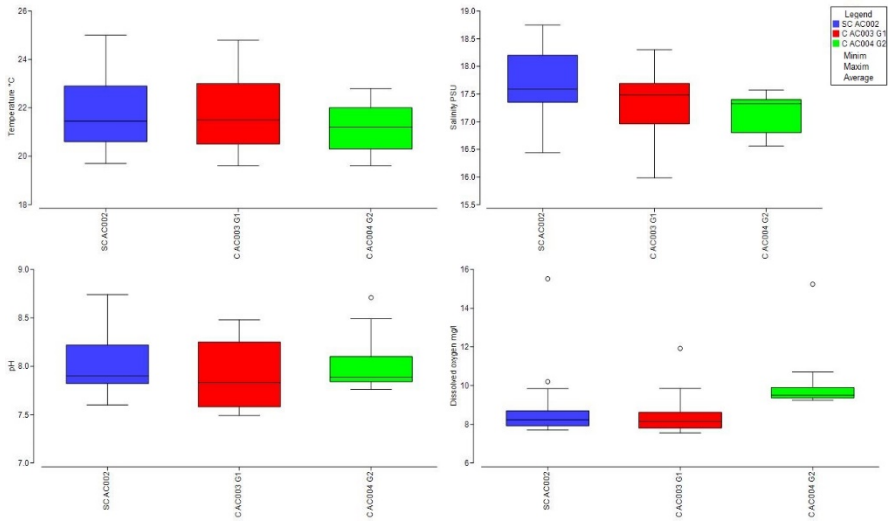

Fig. 16. Variation of the physical and chemical parameters during the *A. clausi* experimental culture; SC AC002 – stock culture, C AC003 G1- culture 1st generation, C AC004 G2- culture 2nd generation

The first generation of *A. clausi* eggs (Culture code AC003) was obtained from 40 females. The eggs hatched after 48h. The live nauplii were counted from subsamples of 1 mL each, and 375 nauplii were produced with sea water in 3.5 L of culture. From July to September, the copepod culture reached a density of 4,933 individuals in 3,5 L of culture with sea water.

Twenty females from the first generation produced the second generation of eggs (Culture code AC004). During 48h, 50 nauplii were produced with seawater in 3.5 L of culture. From July to September the copepod culture reached a number of 1,680 individuals (Table 2).

For the third generation, only qualitative observations were made (e.g., the presence of nauplii viable offspring).

CONCLUSIONS

The protocol designed for *A. clausi* collection from the natural environment, its separation and acclimatization in the laboratory under the water characteristics of the Black Sea allows for obtaining viable cultures.

A stable culture of *A. clausi* in the laboratory could supply individuals in the early stages of development for conducting toxicity tests at any time of the year. It will allow the development of toxicity test protocols adapted to the particular conditions of the Black Sea.

This study paves the way for experimentation on marine copepods from the Romanian Black Sea coast, which are the least studied on the Romanian coast. Future experiments with laboratory-produced individuals may contribute to understanding the context-based behavior of marine copepods - particularly *A. clausi* hatching, nauplii survival rates, and the development period from nauplia to the adult stage under the impact of pollution of the marine environment and climate changes.

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