(04-011) - Daphnia breeding and reproduction protocol for its application as a bioindicator in the Katari Basin, Bolivia.

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The current concern about the degradation of water quality in Bolivia has driven the need to identify and apply bioindicators as a rapid and low-cost evaluation tool in water management. This research focuses on adapting breeding and reproduction protocols for an aquatic macroinvertebrate isolated from Lake Titicaca, Daphnia sp., seeking to simplify existing methodologies and optimize costs. The species Daphnia sp. It has a unique adaptability to variable conditions, which makes it eligible to be used as a biological indicator in different ecological zones. The specific patterns of the life cycle of this organism in relation to different factors such as temperature and radiation typical of the study area, biome of the Andean highlands, allow the validation of the organism for the development of biological assays. The impact of this study will provide an instrument that contributes to the comprehensive management of water in the Katari Basin, specifically to determine the degree of aquatic contamination.

Keywords: safe water;breeding and reproduction protocols;life cycle;aquatic macroinvertebrate

Protocolo de crianza y reproducción de Daphnia para su aplicación como bioindicador en la Cuenca Katari, Bolivia.

La actual preocupación por la degradación de la calidad del agua en Bolivia ha impulsado la necesidad de identificar y aplicar bioindicadores como herramienta de evaluación rápida y de bajo costo en la gestión del agua. Esta investigación se centra en adaptar protocolos de crianza y reproducción para un macroinvertebrado acuático aislado del lago Titicaca, Daphnia sp., buscando simplificar las metodologías existentes y optimizar costos. La especie Daphnia sp. tiene una adaptabilidad única ante condiciones variables, lo que la convierte en elegible para ser usado como indicador biológico en distintos pisos ecológicos. Los patrones específicos del ciclo de vida de este organismo con relación a diferentes factores como temperatura y radiación propios del área de estudio, bioma del altiplano andino, permiten validar el organismo para el desarrollo de ensayos biológicos. El impacto de este estudio permitirá dotar de un instrumento que aporte a la gestión integral del agua en la Cuenca Katari, específicamente para determinar el grado de contaminación acuática.

Palabras clave: agua segura;protocolos de crianza y reproducción;ciclo de vida;macroinvertebrado acuático

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1. Introduction

Water quality is essential for human health and the conservation of aquatic ecosystems. In recent decades, concern about water quality degradation has significantly increased (Guadarrama et al., 2016; Martínez & Villalejo, 2018). This concern has been exacerbated by the consequences of increased urbanization, industrialization, mining, agriculture, and livestock farming activities. The Katari Basin, located in the Andean highlands of Bolivia, is a particularly susceptible area in terms of water quality (Molina et al., 2017). The increasing anthropogenic activity and ineffective water management have exacerbated the situation (Ede & Fuentes, 2019). The search for effective, validated, and economical tools to assess water quality has become essential to sustain water quality management (Meruvia et al., 2019; Olarte & Gonzáles, 2018).

Bioindicators, living organisms whose biological integrated responses help determine the quality of the environment, have emerged as an option for assessing existing contamination (Narcís & Munné, 2014). The particular sensitivity of these organisms to pollution quickly and accurately makes them adequate tools for monitoring water quality changes (de la Lanza, Hernández, & Carbajal, 2000; Vásquez et al., 2006). Among the various bioindicators used for detecting contamination of aquatic environments (Gamboa, Reyes & Arrivillaga, 2008), the species *Daphnia sp.*, an aquatic macroinvertebrate, has been widely used in aquatic toxicology studies. It exhibits characteristics and responses that make it ideal for this purpose. Some of the characteristics that make the species *Daphnia sp.* a promising bioindicator include its ability to adapt to variable environmental conditions (Colbourne, Hebert & Taylor, 1997), its wide geographic distribution (Edmondson, 1987), its importance in the zooplankton community (Carpenter et al., 1987), its reproduction by parthenogenesis (Hebert, 1987), and its ability to detect and respond quickly and proportionately to physical and chemical changes in water (Díaz-Báez, Bustos López, & Espinosa Ramírez, 2004). *Daphnia sp.* organisms have a short life cycle, lasting from a few weeks to sometimes months. This allows for studies of the effects of various pollutants in the short term (González et al., 2014; Dodson & Hanazato, 1995; Hosmer et al., 1998).

In the laboratory, Daphnia sp. is easy to maintain under controlled conditions. They can feed on cyanobacteria, yeasts, or microscopic algae (Olmstead & LeBlanc, 2003). With the appropriate conditions, they can reproduce rapidly (Díaz-Báez et al., 2004). Their reproduction can be sexual or asexual. The combination of these characteristics makes *Daphnia sp.* a good specimen to use in bioassays for the determination of the toxicity of environmental contaminants or to evaluate the toxic potential of effluents. These assays involve exposing the macroinvertebrates to different levels of the substance or aquatic samples in question and monitoring the effects on their survival, behavior, and reproduction (Díaz-Báez, Bustos López, & Espinosa Ramírez, 2004).

The life cycle of Daphnia sp. comprises several stages from egg to adulthood. The duration of these stages can vary depending on environmental conditions. It begins its life cycle with the egg stage (Gándara, Leite, & Caraballo, 2013). Adult females lay eggs and can hatch in the presence of males (sexual reproduction) or in their absence (parthenogenesis). After hatching, the neonates emerge, which are highly vulnerable to changes in water conditions and require optimal and stable conditions (Hebert & Ward, 1972). As they grow, they enter the juvenile stage, which lasts for a variable duration depending on conditions (usually several weeks). Once they reach sexual maturity, the organisms enter the adult stage, where they can begin to reproduce (Díaz-Báez et al., 2004). Asexual reproduction, parthenogenesis, is much more common during the cultivation of these individuals in the laboratory. This has several advantages, such as having uniform populations for

experimental studies because eggs develop and hatch without fertilization, meaning that individuals will be genetically identical (Castiglioni & Collins, 2010).

The laboratory's cultivation and reproduction process of aquatic macroinvertebrates requires strict control of management and optimal environmental factors that mimic their natural habitat (Peters, 1987). Temperature control is essential because it influences macroinvertebrates' growth rate, development, and reproduction (US EPA, 2002). Similarly, lighting, with an appropriate light-dark cycle, helps regulate individuals' biological rhythms, reproduction, and behavior (Díaz-Báez et al., 2004). After capture, *Daphnia sp.* must gradually adapt to the laboratory culture medium to ensure the survival of the majority of specimens. This is achieved through progressive water dilution from their natural habitat into the culture water.

Daphnia sp., is a genus of planktonic crustaceans commonly known as water fleas. To cultivate this macroinvertebrate, conditions must be prepared to mimic the natural habitat from which the samples. In this study, the habitat is Lake Titicaca located at a high altitude (approximately 4000 meters) and in a cold climate region with a constant temperature between 10-18°C and intense solar radiation. Regarding feeding, *Daphnia sp.* is an active filter feeder whose diet is based on phytoplankton, microscopic algae, and other aquatic organisms. In the laboratory, a wide range of foods can be provided to them (unicellular algae, yeasts, and commercial preparations such as spirulina) (Cid, 2018). This research focuses on adapting and optimizing breeding and reproduction protocols for a specific local macroinvertebrate.

2. Objective

This research aims to develop a simplified and low-cost protocol for cultivating and reproducing Daphnia sp. in the laboratory. This will allow for the identification of optimal conditions for applying the local macroinvertebrate to assess the Katari Basin's water quality. This could be replicable in other regions with similar climatic conditions, complementing traditional monitoring programs.

3. Methodology

The present research on cultivating of *Daphnia sp.* has taken an inductive experimental approach. An experimental design was conducted in the laboratory to develop a breeding and reproduction protocol for *Daphnia sp.* based on other protocols and guidelines from Latin American countries to create an applicable protocol in the study area. During the analysis and comparison of the different cultivation methodologies used in different cities in Latin America, similarities and differences between the protocols were identified, allowing for conclusions regarding the key factors influencing the cultivation of *Daphnia sp.* This inductive comparison served as the basis for developing the protocol adapted to the local conditions of the study basin located in the department of La Paz, Bolivia.

For the literature review, a selection of relevant and updated sources related to the cultivation and reproduction of Daphnia sp., limnology, aquatic ecology, and water quality has been carried out. Various scientific databases such as PubMed, Scopus, Google Scholar, and Web of Science have been utilized. Scientific studies, reviews, books, and articles from specialized journals that provide significant, updated, and interdisciplinary information on the cultivation of Daphnia sp. and its use as a water quality bioindicator have been selected. Priority has been given to peer-reviewed publications and those specifically addressing aspects relevant to the research objective, such as optimal cultivation conditions, feeding, reproduction, and adaptation to the laboratory environment. Data discrimination based on geographic relevance has been conducted, selecting sources that address the

cultivation of *Daphnia sp.* in regions with climatic and environmental conditions like La Paz, Bolivia, and other countries in Latin America. This discrimination has allowed for the contextualization of the findings of the literature review and adaptation to the local study conditions.

The experimental part was conducted at the CINAES laboratory of the Universidad Católica Boliviana San Pablo, located in La Paz, Bolivia. The capture of *Daphnia sp.* macroinvertebrates was carried out in Lake Titicaca following a standard procedure (Dodson & Hanazato, 1995; Gándara, Leite & Caraballo, 2013), which was adapted to ensure the collection of healthy individuals from the natural population. The adaptation process to the laboratory culture medium was conducted following established guidelines, including preparing culture water and selecting suitable containers for organism maintenance (Castiglioni & Collins, 2010) (Monterrubio Palma et al., 2022). For the development of the cultivation protocol in the laboratory, critical aspects described in previous studies were considered, such as appropriate temperature and lighting to replicate the natural environment of *Daphnia sp.* (Hosmer, Warren & Ward, 1998; Cid Martínez, 2018).

Specific guidelines were established for feeding the organisms at different cycle stages, considering the optimal quantity and frequency of food supply to ensure their health and development (Núñez & Hurtado, 2005). The development and reproduction of the organisms in the laboratory were systematically monitored. Regular reviews were conducted to assess their status and adapt the protocol. Environmental conditions and organism behavior were recorded throughout the experimental process, allowing for the following guidelines, the frequency of water changes and cleaning of culture containers was established to ensure optimal maintenance conditions (Díaz-Báez et al., 2004; Monterrubio Palma et al., 2022). Special attention was paid to technical details and the standardization of procedures to minimize potential errors and ensure the reproducibility of results (Dodson & Hanazato, 1995; Gándara, Leite & Caraballo, 2013).

4. Results

4.1. Comparison of breeding and reproduction protocols

After the literature review in countries with conditions similar to those in the study area, a comparison of the protocols found is presented in Table 1.

Table 1: Comparison of protocols.

Variables	Puebla, México	Lima, Perú	Sucre, Colombia
Temperature (ºC)	18-20	18-22	26-30
Photoperiod	12:12	16:8	Natural
Containers	50 mL breakers	3 L fish tanks	100-250 mL breakers
Culture water	Autoclaved sterile médium water	Dechlorinated water	Filtered Aquarium water
Food	Spirulina	Fresh yeast and alfalfa juice	Collections from fish ponds
Cleaning	Weekly	Every 10 days	-
References	Monterrubio Palma et al. (2022)	Núñez & Hurtado. (2005)	Gándara, Leite & Caraballo (2013)

Table 1 shows similarities among the temperatures of the different protocols, except for the protocol developed in Sucre, Colombia, which used much higher temperatures. The containers are all different, with containers of less than 1 L capacity being used (except in Lima, Peru). Spirulina was chosen as a feeding based on the result of the review and its availability of in the local market. Cleaning has generally been carried out differently in each protocol.

4.2. Breeding and reproduction system

The breeding and reproduction system was set up considering studies of Monterrubio Palma et al. (2022), Núñez & Hurtado. (2005), and Gándara, Leite & Caraballo (2013). The materials used to assemble the breeding and reproduction system included beakers of different capacities (1000 mL for adults, 500 mL for juveniles, 250 mL for neonates), an aquarium oxygenator, Pasteur pipettes, Parafilm, and a thermometer for controlling water temperature. Commercial Spirulina was used to feed the macroinvertebrates. Finally, to create the aquatic environment, distilled water, and reconstituted hard water were used, prepared in the laboratory according to the data from Table 2.

Tabla 2. Composition of reconstituted hard water per liter of solution.

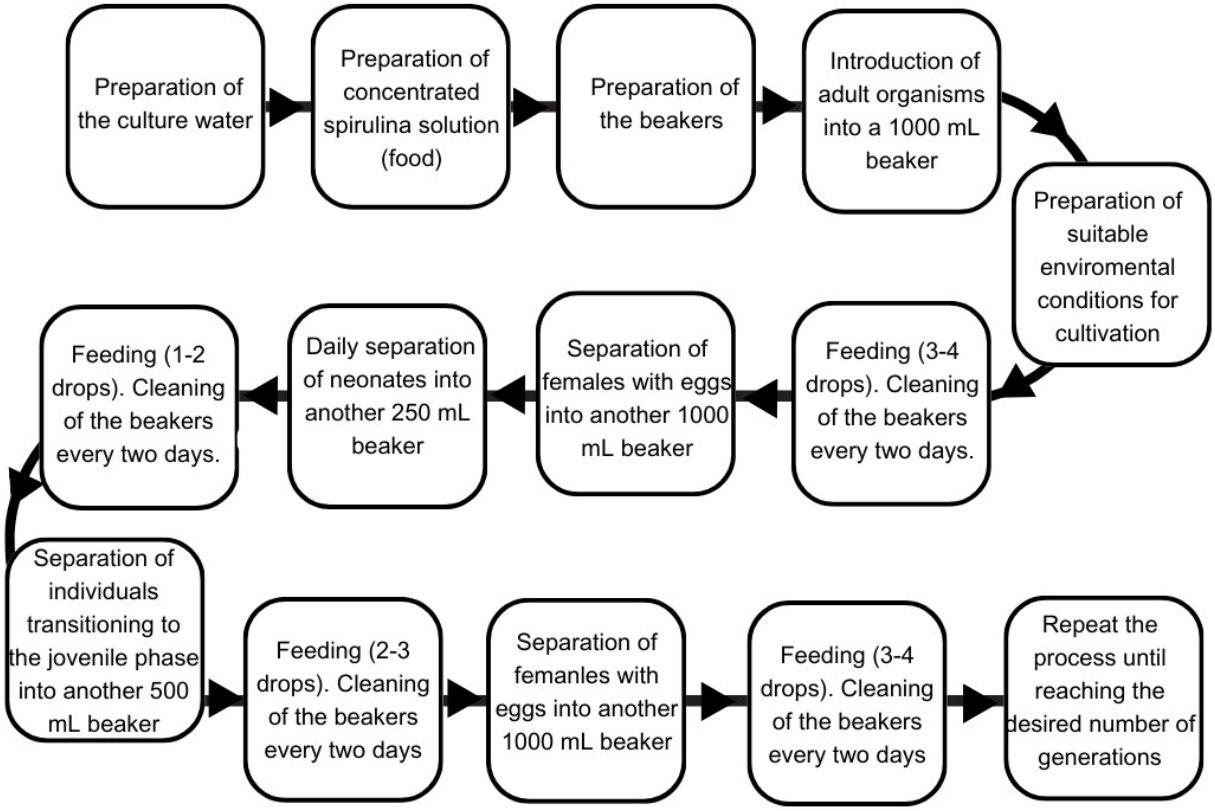
Chemical compounds	Measurement (g)
$CaCl_2 * 2H_2O$	
Calcium chloride dihydrate	19,587
$MgSO_4 * 7H_2O$	
Magnesium sulfate heptahydrate	0,822
$NaHCO_3$	
Sodium bicarbonate	0,648
KCl	
Potassium chloride	0,058

Note: this solution will be used to prepare the culture water.
Source: Díaz, Pica & Sombrero, 2008, and ISO 6341.

4.3. Proposal for breeding and reproduction protocol for Daphnia sp

Based on the experimental process conducted over six months, an effective and low-cost breeding and reproduction protocol for *Daphnia sp.* has been developed. Figure 1 presents a summary of this protocol, followed by a detailed explanation of its most important parts.

Figure 1: Summary of the Daphnia sp. cultivation protocol.



Note: never use the oxygenator with the individuals in the beaker or in the presence of food in the water.

To prepare the culture water, reconstituted hard water was prepared by mixing the salts in Table 2. Approximately 20 mL (3 Pasteur pipette measures) of this solution should be added per liter of distilled water used for the beakers (for example, 20 mL for 1000 mL beakers, 10 mL for 500 mL beakers, 5 mL for 250 mL beakers). To prepare the concentrated spirulina solution, approximately 5 g of commercial spirulina should be mixed with 250 mL of distilled water to obtain a concentrated food solution. It should be ensured that the solution is well mixed. For the preparation of the beakers, each beaker should be labeled with the generation number and the phase in which the individuals are (for example, G1 for first generation, A for adult, J for juvenile, and N for neonate).

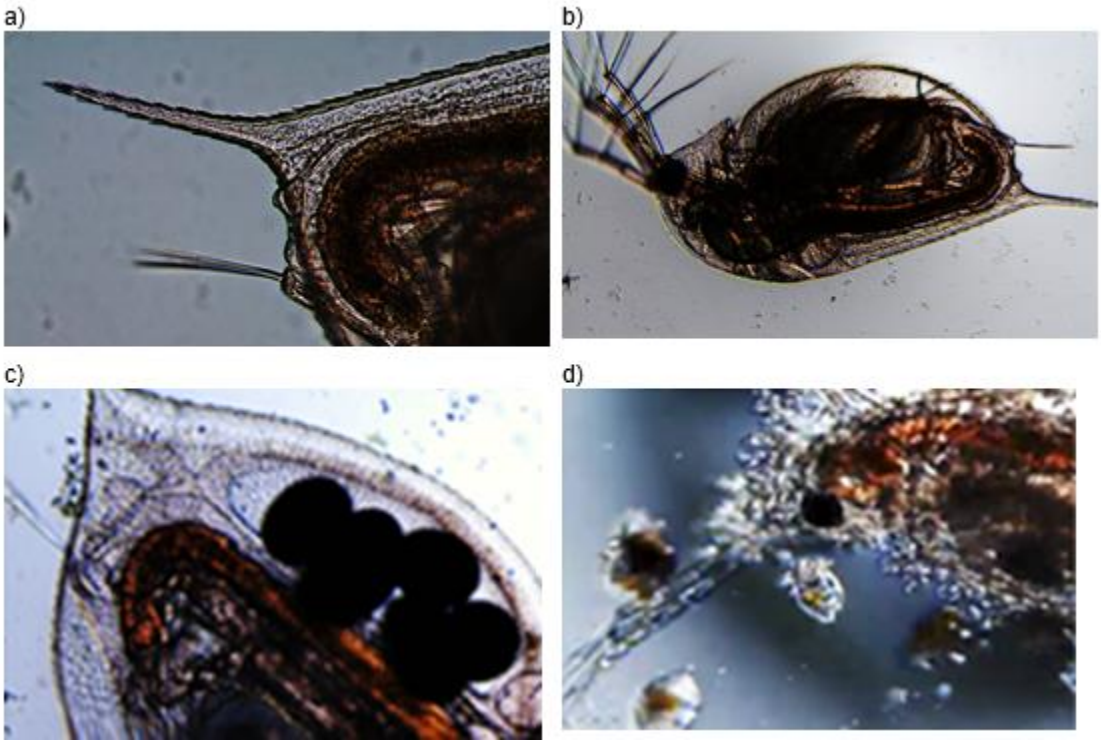
During the introduction of the local macroinvertebrates, a 1000 mL beaker should be prepared with 500 mL of distilled water, 10 mL of reconstituted hard water, and 500 mL of *Daphnia sp.* collection water. Subsequently, it should be aerated for 3 minutes. To transfer the adult Daphnia sp. individuals to the beakers, a Pasteur pipette should be used, so as not to harm the individuals. The beaker should then be covered with Parafilm, leaving a small opening for ventilation. Then, the beakers should be placed in a location with diffuse natural light and constant temperature (between 15-20ºC). During maintenance, molts and corpses should be removed from the bottom of the beaker daily, and the water should be changed every two days to maintain suitable conditions in the breeding systems. During the first week, the water change should be prepared with half of the water used in the old beaker, the other half with clean distilled water, and the corresponding amount of reconstituted hard water solution. Four drops of concentrated spirulina solution should be added each time the water

is changed, and three drops if the water is not. When the water is not changed, aeration should be done by generating bubbles inside the beaker with a Pasteur pipette, never using the aerator with the individuals inside the beaker.

The separation of females with eggs begins in the first week. First, females with eggs should be identified and separated (Figure 2) into separate beakers to have better control over reproduction. For the control of neonates (first generation and subsequent) and their transfer to another beaker, daily observations of the beakers should be made against a light source to identify and separate the present neonates. For this, the transfer location should be prepared, in this case, a 250 mL beaker of distilled water with 5 mL of reconstituted hard water, and the medium should be aerated for one minute before the transfer. After transferring the neonates to the beaker, the beaker should be covered with Parafilm, leaving a small space for ventilation. Each time the water is changed (every two days), two drops of concentrated food should be added, and one drop if the water is not changed. During the first generation's growth and development, individuals' development in the 250 mL beaker should be observed. Prepare a 500 mL beaker with distilled water and add 10 mL of reconstituted hard water solution, aerating it for two minutes. After this, individuals should be transferred to the 500 mL beaker as they reach the juvenile stage. Three drops of concentrated food should be added each time the water is changed, and 2 drops if the water is not changed.

Subsequently, the evolution of the population in the 500 mL beaker should be observed. Females with eggs found in this beaker should be selected and introduced into a separate 1000 mL beaker, prepared as indicated previously but only with distilled water and 20 mL of reconstituted hard water. From this point on, the process should be repeated to obtain additional generations until the required number of generations is achieved. The following figure shows different notable aspects of Daphnia sp.

Figure 2: Different notable aspects of Daphnia sp.



Note: a) Tail of Daphnia sp., b) Adult Daphnia sp., c) Female Daphnia sp. with eggs, d) neonates around an adult specimen.

To analyze the process, a daily record of the number of neonates and each time the beakers are cleaned, the number of adult and juvenile individuals, and the presence of females with eggs should be made. Any changes in the behavior of the individuals in each beaker should be recorded. It is possible that slightly different conditions may be required depending on the location where the cultivation is carried out. During the analysis of the growth and development of the individuals, factors such as temperature, light intensity, and photoperiod should be considered. The effectiveness of the cultivation and reproduction protocol in terms of survival, reproduction, and life cycle of *Daphnia sp.* should be evaluated. The results should also be compared with other studies, and independent conclusions should be established.

5.Discussions

The results obtained in this study on the cultivation of *Daphnia sp.* reveal the importance of considering specific environmental conditions when developing cultivation protocols. The comparison of methodologies used in different cities in Latin America, as shown in Table 1, highlights significant differences in terms of temperature, type of containers, and cleaning frequency (de la Lanza, Hernández, & Carbajal, 2000; Monterrubio Palma et al., 2022; Núñez & Hurtado, 2005). These variations can influence the development and reproduction of *Daphnia sp.* (Shaw et al., 2006), underscoring the need to adapt protocols according to specific local conditions.

The success of the proposed protocol is observed in the survival and reproduction of the local macroinvertebrates, reaching up to a seventh generation with a stable number of individuals. Adapting specific cultivation protocols to local conditions, as done in this study for the Andean highlands, can enhance result reproducibility and enable more precise comparisons between studies conducted in different geographical locations. The geographic variability in *Daphnia sp.* cultivation, as evidenced by the comparison of protocols, underscores the need to consider these differences when designing experiments and generalizing an adaptable protocol.

During the development of the protocol for the cultivation and reproduction of *Daphnia sp.* for the study area conditions, several aspects affecting the success of cultivation have been identified, such as maintaining the cleanliness of the culture water and avoiding overfeeding. Excessive spirulina concentration can be detrimental and lead to a higher mortality rate in individuals. Developing a specific standardized protocol for these conditions can facilitate water quality monitoring and assessment, thus contributing to the sustainable management of water resources.

Aquarium oxygenators during cultivation can also lead to the death of individuals, which is why it should only be used when water changes are made, before introducing the organisms. Oxygenating the water when the beakers are not cleaned involves producing bubbles at the bottom with a Pasteur pipette. The results of this study suggest that temperature is the factor that most influences the life cycle of *Daphnia sp.*, as an increase in temperature in the culture medium accelerated the life cycle. This is consistent with the study by Hebert and Ward (1972).

It is also very important to separate females with eggs to ensure successful asexual reproduction and to have well-differentiated generations. It has been determined through experimentation that organisms develop better in smaller containers, such as beakers, than in 3-liter fish tanks. These favors reducing the space required for the cultivation and reproduction systems of the studied macroinvertebrate.

Daphnia sp. is viable as a bioindicator due to its characteristics as established in studies by Colbourne, Hebert & Taylor, 1997. This macroinvertebrate species is sensitive to

environmental changes, such as water quality or the presence of contaminants. It is present in a wide variety of habitats. It reproduces rapidly, allowing for short-term assessments. It is easy to collect and sample in the field.

It is important to highlight some potential limitations of the study. Firstly, it should be noted that variability in environmental conditions, both within La Paz, Bolivia and in other Latin American cities, could have led to certain biases in the comparison of protocols. Although the protocol was adapted to specific local conditions, factors such as altitude, ambient temperature, and resource availability can influence results unexpectedly. Another limitation may be the lack of standardization in the collection and handling of organisms used in the study. Despite following established procedures from the scientific literature, there are variations in how organisms are collected, handled, and maintained. Additionally, the study focused on cultivating *Daphnia sp.* and its application as a water quality bioindicator in the Katari Basin. However, other species of aquatic macroinvertebrates could also be used as bioindicators and have not been considered.

6.Conclusions

This research presents a comparison of different methodologies for the cultivation and reproduction of *Daphnia sp.* applied in regions of Latin America, allowing for the identification of possible improvements or adaptations to propose a protocol that fits the conditions of the study area. Considering this, a protocol was developed with detailed information on optimal cultivation conditions, feeding methods, and management practices that have proven to be effective. The proposed protocol can be used as the basis for the integration of the local macroinvertebrate as a comprehensive biological tool to strengthen the water quality monitoring program in the Katari Basin.

This research provides valuable information for designing and implementing cultivation and reproduction protocols for *Daphnia sp.* in regions with similar climatic conditions to the Andean highlands. This information has implications for developing effective and cost-efficient strategies for monitoring and assessing water quality, as well as for future research in this area. Additionally, this work contributes data on the life cycle, reproduction, and adaptation of *Daphnia sp.* to different climatic conditions, which may be relevant for research related to aquatic ecology and the conservation of aquatic ecosystems.

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