The challenge of reducing the fecal indicator bacteria load and preventing spawning during depuration of *Perna perna* mussels

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Abstract – *Perna perna* mussels commonly spawn during depuration under water temperatures above 18°C. A previous study conducted under experimental conditions (15L aquariums) has suggested reducing the temperature to decrease the odds of spawning. This study monitored the concentrations of *Escherichia coli* in mussels during three 48-hour and two 72-hour depuration cycles conducted in a small (600L) commercial depuration unit, under temperatures of 14°C and 17°C. The protocol prevented mussels from spawning, as this behavior was observed only in a few mussels in one out of the four depuration cycles. The reduction of fecal indicator bacteria loads, in turn, was unsatisfactory in all depuration cycles, with at least 67% of the samples from cycles 1, 3, 4, and 5 presenting *E. coli* concentrations above the end product testing limit (230MPN $100g^{-1}$) after 48 hours of depuration. Moreover, none of the sample cycles showed bacterial concentrations below that limit, even after 72 hours of depuration.

Index terms: Mollusks; Sanitary control; Post-harvest treatment.

O desafio de reduzir a carga de bactérias indicadoras fecais e evitar a desova durante a depuração de mexilhões *Perna perna*

Resumo – Os mexilhões *Perna perna* comumente desovam durante a depuração em temperaturas acima de 18°C e um estudo em escala experimental (aquários de 15L) sugere reduzir a temperatura da água para diminuir as chances de desova. O presente estudo monitorou os níveis de *Escherichia coli* em mexilhões durante três ciclos de depuração de 48 horas e dois de 72 horas, realizados em uma pequena unidade de depuração comercial (600L), em temperaturas de 14°C e 17°C. O protocolo evitou a desova dos mexilhões, uma vez que este comportamento foi observado apenas em alguns mexilhões em um dos quatro ciclos de depuração. A redução das cargas de bactérias indicadoras fecais, por sua vez, foi insatisfatória em todos os ciclos de depuração, com pelo menos 67% das amostras dos ciclos 1, 3, 4 e 5 apresentando níveis de *E. coli* acima de 230MPN $100g^{-1}$ (limite para comercialização para consumo humano) após 48 horas de depuração e nenhum ciclo com todas as amostras abaixo desse limite, mesmo após 72 horas de depuração.

Termos para indexação: Moluscos; Controle sanitário; Tratamento pós-colheita.

Introduction

Filter-feeding bivalve mollusks accumulate microorganisms, including human pathogenic bacteria and viruses when grown in sewage-polluted waters, posing a health risk when consumed raw or lightly cooked (Lees, 2000; Butt *et al.* 2004). Depuration is a post-harvest treatment applied to reduce microbiological contaminants from commercially-harvested mollusks and consists of placing mollusks harvested from moderately polluted waters in tanks with clean seawater for a period of time (commonly at least 42 hours). This allows mollusks to cleanse or purge themselves of microbiological contamination while continuing their normal filter-feeding and digestive processes (Rees *et al.*, 2010). This procedure can be applied to any species of mollusks traded worldwide, including mussels.

The South American rock mussel, *Perna perna*, is an important aquaculture resource in several countries and Brazil largely contributes to the production volume of this species, with a production of 7,000 tonnes in 2022 (available at: www.infoagro.sc.gov.br). Microbiological results from research studies (Souza *et al.*, 2022) and from the Santa Catarina state mollusk monitoring program (available at: www.cidasc.sc.gov.br) indicate that mollusks from most farming areas should undergo a post-harvest treatment to reduce

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microbiological contamination and ensure that the mollusks are safe to consumers.

However, *P. perna* depuration is still incipient in Santa Catarina due to difficulties inherent to the depuration of this species and the weak law enforcement by sanitary authorities. Temperature variations and physical stress can trigger spawning in bivalve mollusks (Seed and Suchanek, 1992) and mussels such as *P. perna* are prone to undesirable spawning during depuration. Previous depuration studies at temperatures above 18°C resulted in widespread mussel spawning (Suplicy, 1999). A recent study (Suplicy *et al.*, 2024) evidenced the high frequency of spawning during *P. perna* depuration, in which 21 out of 22 depuration assays resulted in spawning. Spawning reduces depuration efficiency since gametes in the water column make the water turbid, which, in turn, reduces the efficiency of the UV disinfection systems (Lees *et al.*, 2010). Furthermore, spawning reduces the meat yield and increases the physiological stress of the animals, which can affect their pumping activity and impair the elimination of microbiological contaminants (Power and Collins, 1989).

Suplicy *et al.* (2024) showed that, at experimental scale (15L-aquariums), conditioning the depuration water to temperatures 5°C lower than those in the harvesting area reduces the spawning frequency by more than 50%, and this chance drops to 8.2% when the temperature difference reaches 10°C. However, the authors did not investigate the implications of temperature modulation during depuration in the reduction of microbial loads and recommended further studies to "*show how reducing water temperature during depuration influences its efficiency in terms of pathogen reduction*." Following this recommendation, we investigated the impact of reducing the water temperature in the tanks on the efficiency of *P. perna* depuration in terms of reduction of *E. coli* (a species of bacterium that occurs in sewage-polluted waters and indicates the potential presence of pathogens in the mollusks), aiming to prevent the mussels from spawning. In the trials, we used commercial scale depuration units to ensure that the experimental conditions matched those in industrial premises.

Material and methods

Experimental setup and sampling

The study monitored the concentrations of the bacteria *Escherichia coli* in mussels during three 48-hour and two 72-hour depuration cycles conducted from February to October 2023 in a small-scale depuration unit. The unit was composed of a 600L depuration tank and a recirculation system containing a water pump (1hp and water flux adjusted at 2.000L hour^{-1}), a chiller (1hp), and an ultraviolet disinfector (50WATTS lamp) (Fig. 1). The water temperature in the first four depuration cycles was maintained around 14°C, being increased to 17°C in the fifth experiment.

The mussels used in this study were obtained from a known sewage impacted aquaculture zone. The mussel density adopted was 90kg per batch, with animals accommodated in nine mesh trays containing 10kg of mussels each. Every 12 hours, water parameters were monitored and three groups with six mussels each were carefully collected from the upper trays, avoiding water disturbance that could resuspend feces or pseudofeces sedimented during depuration. The water parameters monitored included salinity and pH, obtained using a HI9829 multiparameter probe (Hanna, USA), and NH_3 concentration, using a photocolorimeter (Alfakit, AT100P, Brazil). The water temperature was measured every hour using a temperature logger (HOBO Pendant MX Water Temperature Data Logger, Onset, USA) deployed inside the tank. By the end of each depuration cycle, after draining the water from the tank, three additional mussel samples were collected from the trays positioned close to the bottom of the tank.

The mussel samples were immediately packaged in plastic bags, placed in styrofoam boxes and transported to the laboratory for microbiological analysis within 5 hours. Concentrations of *Escherichia coli* in mussel flesh were estimated using the Most Probable Number (MPN) technique (ISO 16649-3 2015). The Condition Index (CI) of 24 randomly selected mussels was estimated before and after each depuration cycle. For this, the mussels were hand-picked from the trays and dried for 24 hours in an oven at 60°C and the weight of their dry flesh was divided by their total dry weight.

Data analysis

The *E. coli* and CI results did not meet the normality and homoscedasticity assumptions; thus, the analysis was performed using non-parametric methods. The CI of mussels used in the different depuration cycles was compared using the Kruskal-Wallis H test followed by the Dunn's test. The comparison of CI before and after depuration and the comparison of the final *E. coli* levels in samples collected from the upper and lower trays were both performed using the Mann-Whitney U test.

In total, two E. coli limits (230 and 700MPN 100g−1) were used as benchmarks in the data analyses since they are the legal limits established in the Codex Alimentarius (Fao and Who,

2008) for live or raw mollusks that says that the legal limits are that in five (5) 100g samples of the edible parts none may contain more than 700 E. coli and not more than one (1) of five (5) samples may contain between 230 and 700 E. coli, or equivalent as decided by the competent authority having jurisdiction.

Results and discussion

Regarding water parameters, little variations on pH and salinity were recorded during the study. The maximum ammonia level was 0.35mg L^{-1} (Table 1). The water temperatures adopted prevented mussels from spawning, as this behavior was observed in only a few mussels and only in cycle 3 out of the four monitored depuration cycles.

The mussel CIs were significantly

Figure 1. Small-scale depuration unit used in the study. The unit was composed of a 600L depuration tank and a recirculation system containing a water pump, a chiller, and an ultraviolet sterilizer

Figura 1. Unidade de depuração de pequena escala utilizada no estudo. A unidade era composta por um tanque de depuração de 600L e um sistema de recirculação contendo bomba d'água, chiller e esterilizador ultravioleta

Table 1. Physical-chemical parameters of the seawater during the different depuration cycles

Tabela 1. Parâmetros físico-químicos da água do mar durante os diferentes ciclos de depuração

Depuration	Date	Temperature (°C)	pH	Salinity (ppt)	NH_3 (mg l^{-1})
1	02/13/2023	14.49 ± 0.44	7.05 ± 0.15	35	0.35 ± 0.02
2	04/03/2023	14.23 ± 0.38	7.08 ± 0.19	35	0.33 ± 0.11
3	05/16/2023	13.86 ± 0.32	7.29 ± 0.35	34	0.17 ± 0.02
4	05/23/2023	14.00 ± 0.26	7.54 ± 0.27	35	0.71 ± 0.06
5	08/02/2023	17.62 ± 1.00	7.44 ± 0.43	35	0.27 ± 0.03

different between the depuration cycles (KW chi-squared=91.0, p-value<$2.2e^{-16}$), with the highest indexes recorded in cycle 2 and the lowest in cycle 5 (Fig. 2). Analysis of all data combined, as well as of data from each depuration cycle separately, revealed virtually no difference of CIs before and after depuration, indicating that the animals did not lose weight during the process (Fig. 3). The exception was depuration cycle 5, which was conducted with the highest water temperature and showed lower CI after depuration compared to the initial values (W=431, p-value=0.003).

The reduction of fecal indicator bacteria concentrations, in turn, was unsatisfactory in all depuration cycles (Fig. 4). After 48 hours, at least 66.6% (two out of three) of the samples from cycles 1, 3, 4, and 5 presented *E. coli* levels above 230MPN $100g^{-1}$. The cycle 2, which held the lowest initial *E. coli* levels, showed the best results after 48 hours; however, it still presented one sample recording an *E. coli* level higher than 700MPN $100g^{-1}$. Prolonging the depuration period to 72 hours in cycles 4 and 5 did not help reduce *E. coli* concentrations. By the end of the 72 hours cycle, one sample was still above 230MPN $100g^{-1}$ in cycle 4 and all samples were above this limit in cycle 5. The final *E. coli* levels in mussel samples obtained from the upper or lower layers of trays did not differ significantly (W=15.5, p-value=1).

The efficiency of depuration is influenced by the system design and by different parameters and practices (e.g. shellfish load, water flux and physicochemical parameters, physical shocks) during the process (Souza *et al.*, 2021). The depuration system used in this study is similar to those used by the industry around the globe (Seafish, 2018) and its efficiency has been proven for oysters *Crassostrea gigas* in a study also conducted in Santa Catarina state (Bobermim, 2013). The employed depuration practices mostly corroborate the best recommendations, and the monitored parameters were within the limits recommended for mussels by the technical literature (Souza *et al.*, 2021; Seafish, 2018). The registered ammonium concentrations

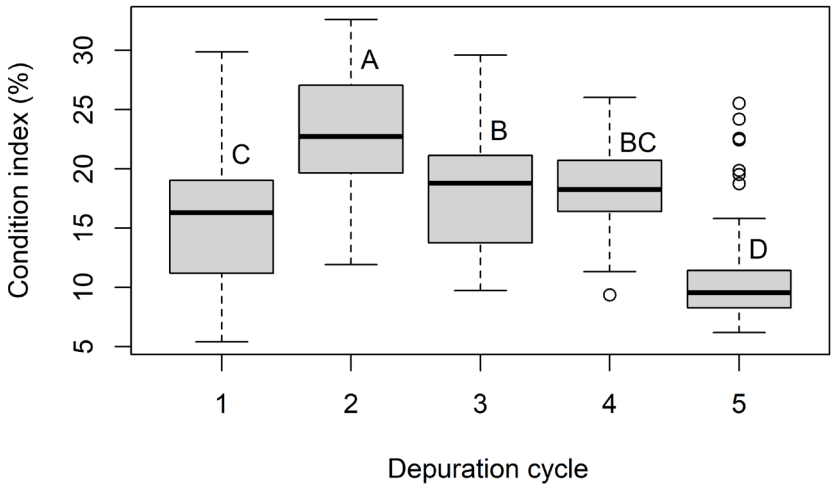

Figure 2. Condition index of mussels before the five depuration cycles monitored. The letters indicate homogeneous groups according to the Dunn's Test

Figura 2. Índice de condição dos mexilhões antes dos cinco ciclos de depuração monitorados. As letras indicam grupos homogêneos segundo o Teste de Dunn

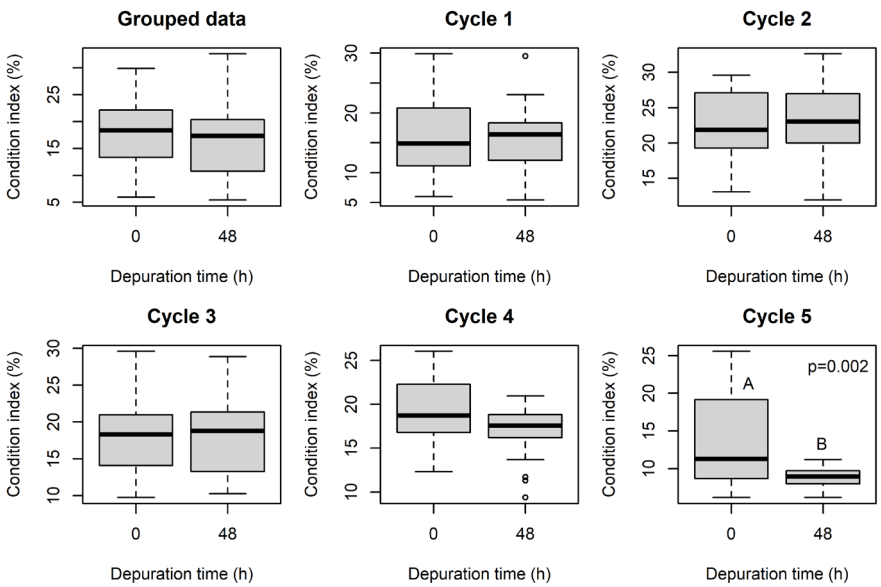

Figure 3. Condition index of mussels before and after 48 hours of depuration in different depuration cycles

Figura 3. Índice de condição dos mexilhões antes e após 48 horas de depuração, em diferentes ciclos de depuração

were all below the tolerance limit of 15.5mg/L^{-1} (48h LC 50) for mussels of the genus *Perna* (Reddy e Menon, 1979). Therefore, considering that the depuration conditions were all ordinary and that previous studies employing similar conditions succeeded in reducing the microbial load during depuration of *P. perna* under temperatures above 18°C (Suplicy, 1999; Guimarães Filho *et al.*, 2022), the poor efficiency of the process observed in the present study is probably related to the colder water used.

Water temperatures ranging from 5°C to 15°C are recommended to depurate two of the most produced species in the world, namely the Mediterranean mussel *Mytilus galloprovincialis* and the blue mussel *Mytilus edulis*, to ensure adequate mollusk filtration and prevent spawning (Lee *et al.*, 2008). This study shows that such temperatures are too low for *P. perna*, which can be expected since this species has different water temperature requirements to grow and reproduce from those of *M. galloprovincialis*. In a monitoring study in Plettenberg Bay, South Africa, Zardi *et al.* (2007) evaluated the reproductive behavior of these coexisting species and observed that *M. galloprovincialis* spawning events always occurred at temperatures ranging from 16.4 to 19.5°C, whereas *P. perna* spawned at the highest and lowest temperatures recorded in the 18 months of the survey (~14.5°C and ~24.2°C, respectively).

Further than impairing an efficient reduction of microbial loads, the water temperatures used in this study might have affected the mussel's post depuration survival. This aspect was not specifically addressed by the study. However, the mussels used in the trials were returned to the marine farm after depuration and the farm manager reported large mortalities. Further studies are needed to check whether the observed mortality is related to the temperatures adopted during depuration or if it is common occurrence for mussels submitted to depuration in general.

Based on the results, we can state that reducing the fecal indicator bacteria load and preventing *P. perna* mussels from spawning during depuration remains a challenge. In Brazil, these mussels are traditionally sold and consumed cooked (Furlan *et al.*, 2007). However, to date, there is no specific protocol that ensures the effectiveness of heat treatment for minimizing the microbiological risks of *P. perna* mussels. Moreover, different studies provide evidence that the light cooking practices (steaming, searing) usually adopted by final consumers and restaurants do not necessarily provide the temperature/time combination required for the efficient inactivation of pathogens in bivalves (Souza *et al.*, 2022). We highlight that there is still a high demand for live mussels in local markets or specific niches, such as haute cuisine restaurants. Therefore, future studies could try different approaches to allow microbial load reduction without

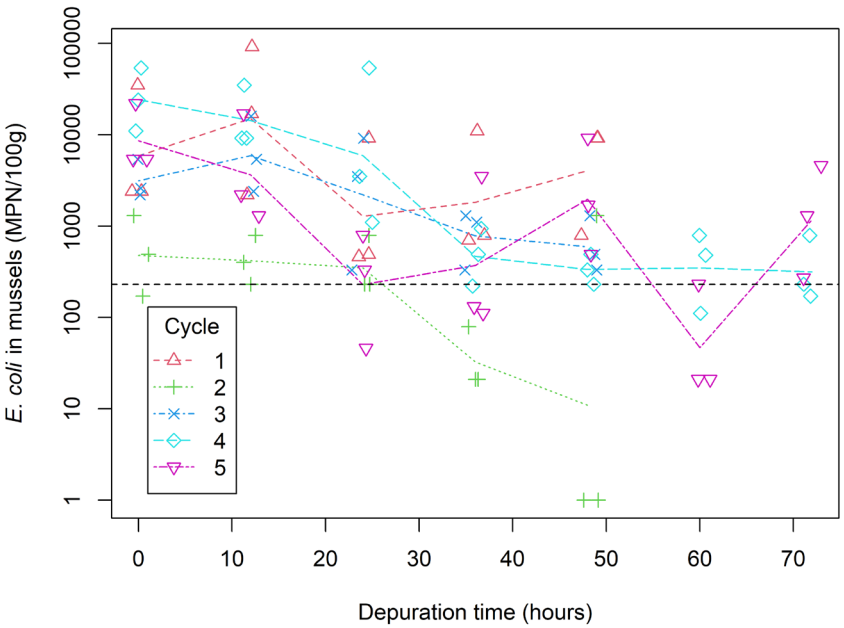

Figure 4. Evolution of Escherichia coli levels in mussels during the five depuration cycles monitored. Symbols indicate raw values and lines indicate the summary results in terms of geometric mean. The black horizontal dashed line indicates the legal limit of 230MPN $100g^{-1}$

Figura 4. Evolução dos níveis de Escherichia coli nos mexilhões durante os cinco ciclos de depuração monitorizados. Os símbolos indicam valores brutos e as linhas indicam os resultados resumidos em termos de média geométrica. A linha tracejada horizontal preta indica o limite legal de 230MPN $100g^{-1}$

mussel spawning during depuration. However, it is also urgent to validate a protocol that ensures the effectiveness of heat treatment for minimizing the microbiological risks of *P. perna* mussels produced in Brazil.

Conclusion

This research confirms that maintaining water temperature from 14°C to 17°C is an effective strategy to prevent mussels from spawning during depuration on a commercial scale, as previously observed on an experimental scale.

Depuration is not capable of efficiently reducing the fecal bacteria load. Even with extended depuration periods of up to 72 hours, *E. coli* levels were not reduced to within internationally established legal limits (Fao and Who, 2008).

We recommend that future studies try different approaches to prevent *P. perna* spawning during depuration. Moreover, we also suggest validating heat treatment protocols, as well as relocating mussels to approved areas to ensure the safety of *P. perna* mussels cultivated in Brazil.

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