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ENHANCING SHELF-LIFE OF LIVE CLAMS VIA MODIFIED ATMOSPHERE COMBINED WITH PHYSICAL CONFINEMENT THROUGH PACKAGING

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Dissertation presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Biotechnology and Innovation.

by
Cintia Borghetti Goes

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Abstract

Clams are the most expensive seafood products because they have a low yield compared to other bivalve's molluscs. *Venerupis corrugata* (pullet carpet shell) is a premium European native clams' species due to high gastronomic, nutritional and economic value, found and appreciated in Portugal. This species is highly perishable. It is typically sold alive within three or four days under refrigeration. The shelf-life period is defined based on the time the clams are alive. Significant risk of loss and waste exists during this period and efforts to extend its shelf-life are relevant. This study aimed to evaluate the shelf-life and physiological quality of live *V. corrugata* clams stored at 3 ± 1 °C in different packaging systems evaluating the effect of high-oxygen modified atmosphere packaging (MAP) versus ambient air, and of physical confinement in net bags versus loose storage. Survival percentage, gases concentration, volatile organic compounds (VOCs), pH, glycogen content, and biogenic amines were monitored throughout storage (7 days). Results showed that survival and physiological quality were strongly influenced by the combined effects of oxygen availability and confinement. High-oxygen MAP delayed mortality by sustaining aerobic metabolism for longer, thereby reducing the respiration quotient. Physical confinement was critical in maintaining intravalvular liquid and preserving clam viability. Nevertheless, the modelled survivability extension was limited to only 1 day under optimal MAP conditions and confinement, highlighting the intrinsic sensitivity of this species.

Keywords: bivalve molluscs; tightness packaging; shelf-life; MAP; confinement.

Resumo

As amêijoas são os produtos do mar mais caros porque têm um rendimento baixo em comparação com outros moluscos bivalves. *Venerupis corrugata* é uma espécie de amêijoa nativa europeia considerada premium devido ao seu elevado valor gastronómico, nutricional e económico, encontrada e apreciada em Portugal. Esta espécie é altamente perecível. Normalmente é vendida viva entre 3 ou 4 dias sob refrigeração. O período de vida útil é definido com base no tempo em que as amêijoas estão vivas. Existe um risco significativo de perdas durante este período e os esforços para prolongar o seu prazo de validade são relevantes. Este estudo teve como objetivo avaliar o prazo de validade e a qualidade fisiológica de amêijoas *V. corrugata* vivas armazenadas a 3 ± 1 °C em diferentes sistemas de embalagem, avaliando o efeito de embalagens com atmosfera modificada e alto teor de oxigénio versus ar ambiente, e do confinamento físico em sacos de rede versus armazenamento solto. A percentagem de sobrevivência, a concentração de gases, os compostos orgânicos voláteis, o pH, o teor de glicogénio e as aminas biogénicas foram monitorados durante todo o armazenamento (7 dias). Os resultados mostraram que a sobrevivência e a qualidade fisiológica das amêijoas foram fortemente influenciadas pelo efeito combinado da disponibilidade de oxigénio e do confinamento. A atmosfera modificada com elevado teor de oxigénio retardou a mortalidade ao manter o metabolismo aeróbio por mais tempo, reduzindo assim o quociente respiratório. O confinamento físico foi fundamental para manter o líquido intravalvular e preservar a viabilidade da amêijoa. No entanto, a extensão de sobrevivência modelada foi limitada a apenas 1 dia sob condições ideais de MAP e confinamento, destacando a sensibilidade intrínseca desta espécie.

Palavras-chave: moluscos bivalves; embalagem apertada; prazo de validade; embalagem em atmosfera modificada, confinamento.

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List of abbreviations

ANOVA	Analysis of variance
BA	Biogenic amine
CAS	Chemical abstracts service
CODEX STAN	Codex Alimentarius Standards
CO_2	Carbon dioxide
DMA	Dimethylamine
DSP	Lipophilic diarrhetic shellfish poisoning
EFSA	European Food Safety Authority
EVOH	Ethylene vinyl alcohol
EPA	Eicosapentaenoic acid
FAO	Food and Agriculture Organization
GC-MS	Gas chromatography – Mass spectrometry
HCl	Hydrochloric acid
HPLC	High-performance liquid chromatography
INE	Instituto Nacional de Estatística
IPMA	Portuguese Institute for Sea and Atmosphere
LOD	Limit of detection
LOQ	Limit of quantification
MAP	Modified atmosphere packaging
NaCl	Sodium chloride
NH_3	Ammonia
O_2	Oxygen
OPA	Ortho-phthalaldehyde
PA	Polyamide
PAHS	Polycyclic aromatic hydrocarbons
PE	Polyethylene
PET	Polyethylene terephthalate
PLA	Polylactic acid
PO	Polyolefins
PP	Polypropylene
PUFA	Polyunsaturated fatty acid
RH	Relative humidity

RQ	Respiratory quotient
SPME	Solid phase microextraction
TCA	Trichloroacetic acid
TMA	Trimethylamine
TVB-N	Total volatile basic nitrogen content
VOC	Volatile organic compound
VP	Vacuum packaging

1. Introduction

1.1. Live clams – from sea to fork

1.1.1. The clam as food

Venerupis corrugata (Gmelin, 1791) (pullet carpet shell), also popularly known as "*ameijoa macha*" in Portugal and "*almeja babosa*" in Spain and formerly referred to as *Venerupis pullastra* in the literature, is a bivalve mollusc species classified in the family Veneridae (Figure 1.1). Its shell is typically thin and elongated, with concentric lines pronounced posteriorly on its surface. This species differs from other similar clams in several respects, such as its united siphons, which run along almost the entire length of the animal except at the terminal zone, and the secretion of mucus, which provides protection against desiccation and pathogens while also assisting locomotion.

Through the siphons, seawater flows, in and out, enabling the clam to collect food in the form of organic matter and phytoplankton, while also facilitating gaseous exchange for respiration process (FAO, 2009). Ecologically, clams differ from other bivalves such as mussels and oysters. Clams inhabit sandy or muddy substrates in shallow waters, whereas mussels and oysters are permanently attached to rocks and can form large reefs. These contrasting life strategies mean that, while clams are relatively mobile within sediments and cannot survive for long outside water, mussels and oysters are fixed and rely on strong attachment or cementation to withstand waves and desiccation in the intertidal zone (Gosling, 2003).

(A) (B)

Figure 1.1. *Venerupis corrugata clam: (A) live specimen outside water and (B) detail of the edible meat.*

The pullet carpet clam is often found in the coastal areas of the north-eastern Atlantic Ocean, including the North Sea and the Mediterranean Sea. It can be produced through aquaculture or harvest at sea. The maximum shell length is approximately 5 cm, while in Portugal the minimum legal size for capture and sale is 3.8 cm (Ministério da Agricultura, do Desenvolvimento Rural e das Pescas, 2011).

Clams are a highly sustainable source of animal protein due to their low trophic level. *V. corrugata* is popular with consumers in Portugal, Spain, France and Italy for its flavour, and like other clams, it offers nutritional benefits. It is rich in vitamins (particularly B12) and essential minerals (such as iodine, selenium and calcium), while being low in fat and a good source of omega-3 fatty acids, which are well-known for their health benefits (Elvevoll et al., 2023).

1.1.2. Food processing of live clam

The clam's life product as food begins once they are removed from the water. However, for harvesting to take place, the Portuguese Institute for Sea and Atmosphere (IPMA) - which monitors water quality in production areas and consequently the bivalve molluscs (as they are filter feeders) - must classify the areas and grant authorisation. The classification of production determines whether bivalves are suitable for direct sale or requires additional processing before commercialisation. Clams from Zone A may be sold directly, those from Zone B must undergo a depuration (purification) period, while clams from Zone C must undergo prolong depuration or industrial transformation (European Parliament & Council of the European Union, 2004a and 2004b).

European regulations establish limits for *Escherichia coli*, marine toxins, as well as metals and polycyclic aromatic hydrocarbons (PAHs) and production zones are classified accordingly.

Depuration is a purification process commercially applied for 24 to 48 hours at water temperatures between 10 and 15 °C. It facilitates the removal of sand inside the shells and microorganisms from the digestive tract and gills (Silvestre et al., 2021). The microbial load of bivalve mollusc's is usually the cause of spoilage once clams die, and microbial activity can also cause chemical changes leading to off-odours and off-flavours in food (Sivertsvik et al., 2002). A comparative study on the impact of the depuration in *Ruditapes decussatus* clams showed that the process delayed the growth of mesophilic and psychotropic microorganisms by three days and the postponed rejection in sensory evaluation. (Mota, 2013).

Once collected, the clams must be transported carefully to avoid shells breakage. After purification, fresh clams are packaged in bags weighing between 500 g and 2 kg, then stored

under refrigeration until the point of sale, or processed further for freezing or canning. Shell damage usually results in mortality, directly associated with improper handling during post-harvest processes. A regulatory requirement states that, once packaged and shipped, bivalves cannot be re-immersed in or sprayed with water (European Parliament & Council of the European Union, 2004a). This makes it essential to maintain shells integrity and closure until sale.

This species is highly perishable, surviving alive for only around 3-4 days under refrigeration (after depuration process and outside of the water). According to the Codex Alimentarius Standard (CXS 292-2008) for live and raw bivalve molluscs, "Bivalves must be alive when sold", with a maximum tolerance of 5% damage or dead clams by count (FAO & WHO, 2008). Other commercial standards have also been proposed, such as a minimum viability threshold of 80% (Bernárdez & Pastoriza, 2011; Ratnawati et al., 2023).

1.1.3. Clam quality parameters

As food fresh product, clams must remain alive, intact, and present a good appearance and odour. Some authors have described methodologies for the sensory evaluation of bivalve molluscs (Gonçalves et al., 2009; Bi et al., 2023). These often involve applying physical stimuli to test whether the clam, as a self-defence mechanism, closes its shell and resists opening. When the clam dies, the adductor muscle can no longer close the shell, which remains open (Bernárdez & Pastoriza, 2011). This exposes the intravalvular fluid, causing the meat to dry out (Fratini et al., 2013). The number of open clams, together with the release of the intravalvular fluid (exudate) and lack of response to manual stimulation, are reliable indicators of loss of quality.

The metabolic state of live bivalves directly influences the sensory properties of their edible meat (Fu et al., 2024). Once removed from water, clams lose access to oxygen and food, leading to stress and reliance on stored energy reserves, mainly glycogen. Glycogen levels in the adductor muscle contribute to organoleptic attributes and are associated to post-harvest stress and survivability (Gonçalves et al., 2009; Bernárdez & Pastoriza, 2011; Anacleto et al., 2014b; Bi et al., 2023). Storage and transport conditions significantly affect glycogen retention. In *Ruditapes philippinarum*, refrigerated (4 °C) and semi-dry transport preserved higher levels of glycogen compared with warmer or more humid conditions (Bi et al., 2023). Species differences have also been reported: *V. corrugata* shows higher glycogen content than *R. philippinarum* under comparable conditions (Anacleto et al., 2013a). Bernárdez and Pastoriza (2011) further observed no detectable glycogen consumption in *Mytilus galloprovincialis* after

10 days of storage at different oxygen concentrations (20, 75 and 85%), suggesting that glycogen consumption depends on the duration of stress exposure. However, a pH decrease was detected. Several authors have consistently reported a decrease in pH during storage, reflecting a shift to anaerobic metabolism and acid production (Pastoriza et al., 2004; Gonçalves et al., 2009; Bernárdez & Pastoriza, 2011; Ratnawati et al., 2023). These metabolic changes negatively affect sensory parameters, reducing clam quality.

In regulatory terms, specific requirements for bivalve mollusc quality are limited compared with fish (European Parliament & Council of the European Union, 2004a and 2004b; European Commission, 2005). Therefore, limits for some parameters are usually adopted as a reference, such as total volatile basic nitrogen (TVB-N), with a freshness threshold of 30 mg of nitrogen / 100 g of flesh (European Commission, 2005). This measure quantifies nitrogenous compounds produced during protein degradation, such as ammonia (NH_3), trimethylamine (TMA) and dimethylamine (DMA). Biogenic amines (BA), including histamine, tyramine, putrescine, and cadaverine formed by amino acid decarboxylation, are also recognized as spoilage indicators. While regulatory European limits exist primarily for histamine in fishery products (mean value $\leq$ 100 mg/kg or max. value $<$ 200 mg/kg), their presence provides valuable insights into product quality and microbial activity (Visciano et al., 2020; Arulkumar et al., 2023).

As filter-feeding animals, the clams have a natural microflora, even after being purified. European regulation (European Parliament & Council of the European Union, 2004a) set limits for indicator microorganisms (less than 300 faecal coliforms or less than 230 *E. coli* per 100 g of flesh and intravalvular liquid) and pathogens (absence of *Salmonella spp.* in 25 g of flesh) for a production area of bivalves suitable for direct human consumption (Zone A). During refrigerated storage, microbial counts increase and may signal tissue deterioration in weaker clam (Bernárdez & Pastoriza, 2011; Pastoriza & Bernárdez, 2012). Some authors suggest that bacteria proliferation in molluscs is facilitated when their defence system is compromised by stress factors such as anoxia (de Zwaan et al., 2002).

Shellfish quality and safety is strongly connected with sensorial aroma (flavour), as volatile organic compounds (VOCs) directly influence consumer perception of seafood freshness (Fratini et al., 2012). Both fresh flavour and off-flavour notes such as aldehydes, alcohols, ketones and acids can arise by microbial activity, enzymatic or auto-oxidative reactions, even before visible tissue degradation occurs (Zhang et al., 2010; Fratini et al., 2012).

From a consumer perspective, quality criteria are crucial for market acceptance. In Portugal, clams are generally consumed lightly cooked, and strong culinary traditions have led to highly

demanding consumers expectations. At the point of purchase, Portuguese consumers prioritise smell, shell size and appearance. The preference is overwhelmingly for live clams, particularly in summer, which are bought in supermarkets, cooked at home or eaten in restaurants (Anacleto et al., 2014a).

1.1.4. Market overview

V. corrugata species is a strong candidate as a new sustainable source of protein and for aquaculture production. Considered a premium species, it is around three times more expensive per kilogram than other invasive clam species (Cruz et al., 2020). Due to its relatively low yield compared with other bivalves, it is regarded as one of the most expensive seafood products.

Despite the global clam market already being worth over EUR 6.3 billion (FAO, 2018), there are a few gaps in the value chain that need to be overcome to improve industry standards in the shellfish sector and support the production, packaging and supply. Currently, there is a lack of large-scale production and supply capacity. Therefore, increasing its shelf-life is of major interest for commercial value and economic benefits. The Food and Agriculture organization (FAO) has highlighted the need for innovation to reduce the loss and waste of aquatic foods (FAO, 2024). In 2018, the global production of molluscs in aquaculture, primarily bivalves, reached 17.7 million tonnes, equivalent to 2.6 kg per person. Clams accounted for approximately 38% of this total (FAOSTAT, 2021).

Among bivalves, clams are the most important species consumed in Portugal, particularly *V. corrugata* and *R. philippinarum* species (Anacleto et al., 2014a). Portuguese aquaculture production of molluscs increased by 17.7% between 2022 and 2023, representing 57.3% of total aquaculture production. Clams reached 5,820 tonnes, corresponding to 48.7% of all molluscs (INE, 2025). High consumption levels are accompanied by significant losses across the supply chain. Between 2022 and 2023, clam losses totalled around 1,300 kg in the warehouses of a specific Portuguese retailer. Meanwhile, around 82,000 kg of clams were rejected at the retail stage. Overall, this represented 21% of supply chain losses, including the retail phase.

In the warehouse, the main causes of loss were lack of vitality (62%) and packaging defects (4%), with the remaining 34% due to transport-related issues, clam size, labelling problems and quality control concerns (e.g. broken shells). In retail, much higher losses were caused by lack of vitality (around 93%), with only 1% attributed to packaging problems. Standard practice shows that these products remain alive for around three days once they reach the retail outlet.

Therefore, effective strategies are required to extend the shelf-life of clams and thereby reduce both losses and waste (Carneiro et al., 2025).

1.2. Factors affecting survivability and quality of clams

The scientific literature includes shelf-life studies for bivalve molluscs (Hauzoukim & Mohanty, 2020; Odeyemi et al., 2023), mainly mussels (Bernárdez & Pastoriza, 2011; Ratnawati et al., 2023) and oysters (Rodezno et al., 2023; Tian & Liu, 2023). However, there is limited information available for clams (Gonçalves et al., 2009; Goes et al., 2025), particularly for this species.

The survival, and consequently the quality, of live clams is influenced by multiple factors. Beyond the need to avoid shell damage, clam longevity is related to conditions, such as temperature and the ability to maintain the shell closed, thereby preventing the loss of intravalvular fluids (Pastoriza & Bernárdez, 2012; Pastoriza et al., 2004).

Khripounoff et al. (2017) reported that the respiration rate of bivalves is affected by several factors, including size, species, environmental temperature, food availability, stress, and ecological conditions. Different gas concentrations in modified-atmosphere packaging have been applied to extend the shelf-life of mussels (Bernárdez & Pastoriza, 2011; Ratnawati et al., 2023) and clams (Goes et al., 2025), by maintaining or slowing down the respiration process.

1.2.1. Temperature

Temperature must be carefully controlled from depuration to packaging, distribution, retail display and finally by the consumers. Inadequate modes of transportation have associated with low survival rates and severe quality loss in fresh aquatic products (Zhang et al., 2019). Anacleto (2013a; 2013b) studied the impact of depuration and two different temperatures of transport (4 and 22 °C) in physiological and microbiological responses of *V. corrugata* and *R. philippinarum* clams. Significant differences were observed between species; depuration had a positive effect on outcomes, but the temperature differences had a strong influence on mortality: transport at 4 °C resulted in higher survival rates than 22 °C. Similarly, as previously mentioned, Bi et al. (2023) also found higher survival results when *R. philippinarum* was transported at 4 °C than at 15 °C. According to Anacleto et al. (2014b), clams closer to 4 °C enter a dormant or semi-dormant state, characterised by weak metabolic activities, which helps maintain vitality for extended periods. Conversely, high transport temperatures under

anhydrous conditions can reduce the immunity of the shellfish, leading to bacteria accumulation and, consequently increased mortality (Anacleto et al., 2013b).

The survival rate and sensory attributes of live clams *V. corrugata* packed on plastic net bags (1 kg) were evaluated over six days of storage at four different temperatures: 3 ± 1 °C, 5 ± 2 °C, 8 ± 1 °C and 12 ± 2 °C (Goes et al., 2025). Intermediate temperatures (5 °C and 8 °C), best maintained the threshold of 95% survival, in line with the CODEX guideline. Storage at 12 °C gave the worst results, while lower temperatures (3 °C and 5 °C) were associated with lower mortality after the threshold period. In addition, clams stored at 8 °C and 12 °C developed unacceptable odours two days earlier than those stored at 3 °C and 5 °C, underlining the importance of maintaining clams at low temperatures.

1.2.2. Gas composition

Modified atmosphere packaging (MAP), which uses active gases, and vacuum packaging (VP), which excludes gases, are well-established technologies for shellfish and fish products. They help to reduce respiratory rate and metabolic activity, inhibit microbial growth during storage, and extend shelf-life (Sivertsvik et al., 2002; Bernárdez & Pastoriza, 2011; Pastoriza & Bernárdez, 2012; DeWitt &Oliveira, 2016; Odeyemi et al., 2023; Carneiro et al., 2025). The key parameters influencing the effectiveness of MAP in bivalves are the composition of the gas mixture, the packaging system (material and geometry) and the temperature control. These determine the ratio between the gas and product, the barrier to gas permeation, and the ability to maintain the desired gas composition during storage. For example, Pastoriza & Bernárdez (2012) applied MAP in live mussels and reported a shelf-life of 5 -15 days in MAP depending on pre-packaging treatments, the gas mixture used, and the refrigeration temperature during storage, to keep the bivalves in a lethargic state.

Carbon dioxide is widely used in MAP due to its antimicrobial effects. Dissolved CO_2 increases lag phase and a slows microbial growth during the logarithmic phase (Hauzoukim & Mohanty, 2020). Some studies with mussels (*M. galloprovincialis*) focused on the effect of different gas mixtures in MAP to extend the survival percentage (Pastoriza et al., 2004). They observed that the mortality of mussels increased with the presence of CO_2 gas in the initial gas composition. While low CO_2 levels are recommended, mixtures with high-oxygen concentration (> 50%), have also been shown to extend survival compared to storage in air. For instance, Ratnawati et al. (2023) showed a shelf-life of 7-9 days for blue mussels (*M. edulis*) in MAP with 60% O_2: 40% CO_2 stored at 4 °C, with mortality remaining below 20%. This highlights that different

bivalve's species respond differently to gas mixture, reinforcing the need for species-specific studies of commercial interest.

A high-oxygen modified atmosphere a (75 - 85% O_2) extended the shelf-life of Mediterranean mussels (*M. galloprovincialis*) by up to 8 days, when stored at ≤ 2 °C (Bernárdez & Pastoriza, 2011). The effect of a gas mixture with a high concentration of O_2 in MAP (70% O_2: 30% N_2) was studied in live clams (*R. decussatus*) stored at 6.0 ± 0.7 °C and noted the maintenance of sensory attributes, such as the characteristic sweet taste resulting in a shelf-life of six days (Gonçalves et al., 2009).

A comparative MAP study was conducted on live *V. corrugata* packed in plastic trays and stored for six days at 3 ± 1 °C with two gas mixtures (70% O_2: 30% CO_2 and 70% O_2: 30% N_2). The results showed a negative effect of CO_2, with lower survival in samples exposed to this gas. The study also compared two higher oxygen levels (70% and 90% O_2) but found no improvement. The shelf-life increased by only 1–2 days compared to the four days typically achieved under commercially conditions for this species.

1.2.3. Confinement

The appearance and taste of the edible meat depend on retaining the intravalvular liquid inside the shells (Gonçalves et al., 2009). The natural opening and closing of the shells in bivalves are related to feeding, respiration and excretion. After the clams are removed from water, with the absence of food and water, bivalves tend to open the valves to access oxygen (Ratnawati et al., 2023).

Clam confinement depends entirely on the packaging system. To keep the clams closed, avoid loss of intravalvular liquid and mechanical damage to the shells, it is essential that they are packed tightly (Goes et al., 2025). This packaging parameter is neither mentioned nor measured in the literature on bivalve shelf-life studies. In industry, mesh bags are generally used to group the product into suitable selling weights. This packaging and closure system can provide adequate confinement of the shells to keep them closed (Carneiro et al., 2025). However, further research is required to assess the impact of confinement on clam shelf-life, and to enable the industry to optimise the packaging system.

Vacuum is typically applied to many food products in hermetically sealed packaging to confine them in gas-free packaging. However, modified atmosphere packaging (MAP) requires free space containing the gas mixture at the appropriate concentration, which makes it difficult to simultaneously confine the product. A comparison of different pressure of gas mixture in MAP

of *V. corrugata* clams was conducted by Goes et al. (2025). The live clams were packaged in plastic trays and vacuum (50 mbar) was applied before injecting the gas mixture (70% O_2: 30% N_2) at two different pressures (120 and 150 mbar). The clam's survival was monitored for 0, 3 and 6 days under refrigerated storage (3 ± 1 °C). The results showed that samples with the higher gas mixture pressure (150 mbar) presented higher survival over the six days study period.

1.3. Packaging

1.3.1. Net bags

Fresh alive clams are sold in labelled net plastic bags, also known as "mesh bags" or "netting bags" closed by a plastic or metal clip (Figure 1.2).

Figure 1.2. *Clams in net plastic bag.*

Nets bags are lightweight, versatile and easy to handle, but have also some disadvantages such as not offering protection to the shell from handling and not holding dripping fluids, which can affect hygiene (Pastoriza & Bernárdez, 2012).

The most used nets are made from plastic, such as polypropylene (PP), polyethylene (PE) and polyamide (PA) and can be produced by filament knitting and woven (Figure 1.3A) or extrusion (Figure 1.3B) with different patterns and sizes.

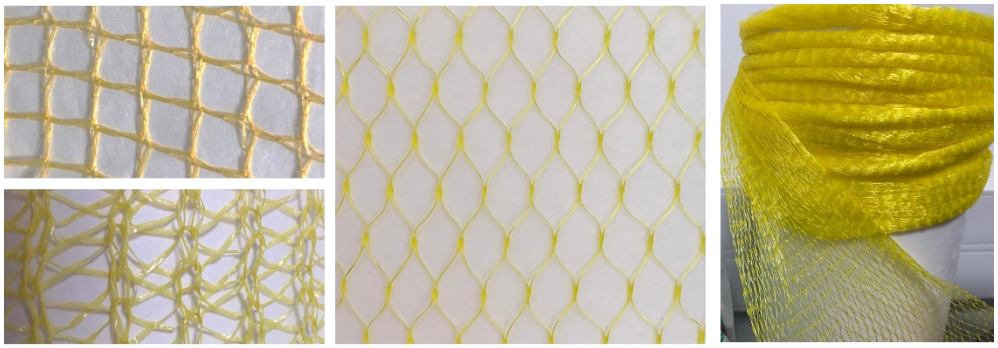


Figure 1.3. *Plastic net: (A) Knitted and woven net (B) extruded net and (C) sleeve of extruded net.*

For plastics, these types of nets are subjected to a thermal process to ensure that the knots are sufficiently fixed for the intended use. Mechanical properties are one of the most important characteristics of the net in particular the tensile resistance as it is important for keeping the bag tightness around the clams (Carneiro et al., 2025). The nets are produced as a continuous sleeve possibility to package the product in different sizes of the bag (Figure 1.3C).

New sustainable materials for packaging seafood are in constant development. Nets in biobased fibres, such as cotton, starch and cellulose-based or plastics nets claimed to be biodegradable may also be found commercially available. For renewable nature and compostability characteristics, starch and cellulose-based fibres are attractive as environmentally friendly net bag alternatives. Materials derived from other sources like corn, wheat, and wood pulp, offer inherent biodegradability and strength under dry conditions. However, the performance of these materials under the humid conditions, in particular in marine environmental, usually observed in clams chain needs to be ensured (Carneiro et al., 2025). Company like Packnatur® (Packnatur GmbH, 2025) are already utilising bio-based fibres for net production, signalling a growing trend towards sustainable alternatives in this industry. Lyocell®, another product developed using cellulose, with high tensile strength, has applications in the textile industry and holds promise for broader use (Lenzing AG, 2025).

1.3.2. Modified Atmosphere packaging – trays and bags

MAP is not widely used for live clams on the market. However, it is already applied for live mussels. MAP requires packaging with high barrier to gases under humid conditions, good mechanical resistance and sealability. Traditional materials able to provide the required barrier consist of multilayer materials combining polyolefins (PO) ethylene-vinyl alcohol (EVOH)

and/or PA. However, these complex structures have limited recyclability. Therefore, the benefit of shelf-life extension should compensate the lower circularity of the system.

Ratnawati et al. (2023) studied live blue mussels (*Mytilus edulis*) stored under high-oxygen MAP at 4 °C for 16 days using multilayer trays (PP/EVOH/PP) with a cover film (PA/EVOH/PA/PE/PP). Similarly, Gonçalves et al. (2009) used PA/PE barrier films to study the effect of high-oxygen storage in live clams *R. decussatus* for 6 days under refrigeration (6.1 ± 0.7 °C). In these cases, barrier properties and hermetic seal of packaging are critical. Even multilayered plastics exhibit gas permeability over making product shelf-life a key factor in selecting the packaging material. For live clams, which continue to respire after packaging, careful material selection and monitoring of gas concentrations over time are essential.

MAP operations using pre-formed trays typically involve vacuum drawing to remove the initial atmosphere following the injection of the atmosphere with the selected gas composition and sealing. For large volume packaging lines, form-fill-seal systems are used where the trays are formed in the machine before filling with the product. In these processes, a continuous flow of gas mixture replaces the atmosphere surrounding the product before sealing. Trays are typically produced using polyethylene terephthalate (PET) or PP the main structural material combined with the barrier materials (EVOH and PA) if needed.

With advances in super-clean recycling processes and positive evaluations by the European Food Safety Authority (EFSA), PET trays with the incorporation of recycled resin are common in the market for different applications particularly for fresh fish. Polylactic acid (PLA) trays are also available for low-contact time applications. However, PLA alone does not provide sufficient gas barrier properties for MAP or other products that need to avoid oxidation.

2. Objectives

Given the ecological and economic importance of *V. corrugata*, extending its post-harvest shelf-life is essential for promoting production, minimizing waste, and expanding market access. This study aimed to evaluate the effect of two parameters that packaging can control on the shelf-life and physiological quality of live *V. corrugata* clams stored at 3 ± 1 °C. The packaging systems were compared by examining clams in:

(i) high-oxygen modified atmosphere (MAP) versus ambient air and,
(ii) physical confinement in commercial net bags versus loose storage.

Survival percentage, pH, glycogen content, VOCS and biogenic amines were monitored over time.

3. Materials and methods

3.1. Clam samples and experimental set-up

Specimens of *V. corrugata* (shell length: 3.8 – 4.4 cm; body weight: 14.3 ± 1.9 g) were sourced from a commercial aquaculture facility in Ria de Aveiro (RIAV), Portugal, in January 2025. After 24 – 48 hours of depuration in a local installation (Falcamar – Comércio de Mariscos Lda.), the clams were refrigerated at 3 ± 1 °C and transported to the research facility for analysis. Empty and/or damaged shells were discarded; the remaining live clams were weighed, counted and divided into groups of 24 clams (average weight: 323.5 ± 1.9 g). Half of the samples were packed in plastic PE net bags commercially used by Falcamar and sealed using a plastic clamp, thus replicating supplier conditions.

Each batch was randomly assigned to one of four experimental conditions:

- **C1:** Loose clams stored under air;
- **C2:** Loose clams stored under MAP (96% O_2);
- **C3:** Clams packed in net bags under air;
- **C4:** Clams packed in net bags under MAP (96% O_2).

The four experimental conditions were tested using a sealed jar system designed for controlled storage and gas monitoring. This experimental setup allowed precise control of environmental variables to simulate and monitor each condition effectively (Figure 3.1).

Figure 3.1. *Jars sample with loose and confined clams in plastic net bag.*

All the jars were closed with a hermetic metal lid. The jars used of the MAP condition and for headspace monitoring had two inlets to gas addition and a rubber septum for headspace sampling (Figure 3.1). Eight jars were prepared for each group/condition: five to assess clam survival and three for measuring changes in the gas composition (non-destructive sampling) over time. For the MAP conditions, two additional jars were used as gas composition control (without clams). The jar system was tested, and the loss of oxygen was lower than 2% after one week. For creating the modified atmosphere, oxygen gas (100% O_2, Gasin, Air Products Group - Spain) was inserted in the hermetically closed jars to generate a modified atmosphere with 96 ± 1% O_2 concentration. All samples were stored in a refrigerated incubator at 3 ± 1 °C (MIR253, Sanyo) and evaluated in six storage time sampling points (0, 3, 4, 5, 6 and 7 days).

3.2. Clam's quality monitoring

3.2.1. Survivability, opened clams, exudation

Clam survivability was assessed by opening one of the five jars of each condition and assessing all the clams in that jar. In the last sampling day (7^{th}), the three jars used to measure gas concentration over time were also evaluated. Opened clams and sensorial aspects were registered and clams were classified as alive or dead based on valve closure upon tactile response. The percentage of live clams was calculated by observing the state of the valves, as well as by stimulating the clams (Bi et al., 2023; Wang et al., 2024). Essentially, a clam was considered alive if the shell valve closed or open during the evaluation, or if the body moved either before or after stimulation. The survival percentage was calculated according to Equation 3.2.1:

$$Live\ clams\ (\%) = \frac{No.\ live\ individuals}{No.\ total\ clams\ evaluated}\ x\ 100 \qquad (3.2.1)$$

The exudated liquid collected in the jar was weighed and expressed in grams.

For each condition and group, the clams were prepared for chemical analysis as follows. A subsample of 10 whole clams (n=10) was separated, and the adductor muscles were extracted and stored at -80 °C for glycogen quantification. Death and alive clams were distinguished. The remaining edible meat of the same 10 clams was extracted for analysis of pH and biogenic amines. For identification of volatile compounds via gas chromatography mass spectrometry (GC–MS), three whole clams were stored separately. These samples were collected and stored at -20 °C for simultaneous analysis.

3.2.2. Gas composition and estimation of respiration rate

The gas composition (% CO_2 and % O_2) inside the jars was measured over time with a headspace gas analyzer (CheckMate 3®, Dansensor, MOCON Europe A/S, Denmark).

The oxygen consumption and carbon dioxide production rate were calculated through equation 3.2.2.1 for each sampling point:

$$Rate\left(mg_{gas}\ g_{clams}{}^{-1}day^{-1}\right) = \frac{P\ V_{hs}\left|\Delta\%_{gas}\right|\ M_{gas} \times 1000}{R\ T\ m_{clams}\ \Delta t} \quad (3.2.2.1)$$

where P = pressure (atm), V_{hs} = headspace volume (L), $\Delta\%_{gas}$ = variation in gas concentration (percentage), M_{gas}= molar mass (O_2 = 32.0 g mol^{-1}; CO_2 = 44.0 g mol^{-1}), R = 0.0821 L atm mol^{-1} K^{-1}, T = temperature (K), m_{clams} = edible meat of clams (g), and Δt = time difference (days).

The respiratory quotient (RQ) was calculated as the ratio of CO_2 production rate to O_2 consumption rate according to equation 3.2.2.2:

$$RQ = \frac{CO_2 rate}{O_2 rate} \quad (3.2.2.2)$$

The estimative method was based on Ho et al. (1997) with minor modifications. Changes in the gases were assumed to be caused exclusively by the clams, with the microflora being ignored. To calculate the volume of headspace and the mass of the clams without shells, previous results for average clam density (1.48 ± 0.03 g/mL) and edible meat percentage (49.83 ± 1.49%) were considered. Both data were obtained from fresh clams of the same species and size.

3.2.3. Volatile organic compounds

The clam's meat was prepared for analysis in accordance with Zhang et al. (2010). Whole clams' meat was mixed in a 1:1 ratio with NaCl (0.06 g mL^{-1}), following homogenisation in a T25 ULTRA-TURRAX® (IKA, Germany) and spliting into aliquots for analysis. The analytical method was in accordance with Wang et al. (2024), with certain adaptations. GC–MS analysis is performed in a system Bruker® EVOQ 456 TQ GC/MS, with the following operating conditions: vector gas Helium at 1 mL min^{-1}; injection temperature 250 °C and column DBWAX, 30 m × 250 µm × 0.25 µm. Temperature was isothermal at 40 °C for 3 min, ramped to 90 °C at a rate of 5 °C/min, then ramped to 230 °C at a rate of 8 °C/min, and held at 230 °C for 10 min, in splitless time (1 mL min^{-1}). The mass spectrometer is operated in the electron impact mode with the electron energy set at 70 eV. The ion source temperature was set

at 230 °C, and mass range was set at: 20–350 amu (m/z). Samples were extracted using a SPME fibre of 50/30 mm divinylbenzene/carboxen/ polydimethylsiloxane (DVB/CAR/PDMS) at room temperature, with 5 min pre-incubation period and 40 min extraction period, with constant stirring at 250 rpm.

The identification of the volatile substances was carried out by comparing the retention indexes and mass spectra data with those from the NIST Tandem Mass Digital Library (Version 2.3, build May 4, 2017). Semi-quantitative analysis was carried out using an internal standard (deuterated dodecane, CAS N_0. 16416-30-1, concentration of 5.32 mg L^{-1}) from Sigma-Aldrich (Germany), prepared beforehand and used at room temperature.

3.2.4. Glycogen quantification

Glycogen was quantified in the adductor muscles of clams, in triplicate, applying a colorimetric detection method using anthrone-sulfuric acid and following the protocol of a commercial CheKine^{TM} Micro Glycogen Assay Kit (Abbkine Scientific Co., Ltd; Georgia, USA). Briefly, 0.1 g of tissue was mixed with extraction buffer, boiled for 20 min (covered to prevent water loss), diluted in deionized water and centrifuged (8000 g for 10 min). The samples were incubated at 95 °C for 10 min after the chromogenic reagent was added. The absorbance was measured at 620 nm with a Microplate reader Agilent BioTek Synergy H1 (Agilent Technologies, Santa Clara, CA, USA). A calibration curve was prepared using a glucose standard. The kit detection range and sensitivity are 0.003125-0.25 mg mL^{-1} and 0.003125 mg mL^{-1} respectively. The results were expressed as mg per g wet weight (WW).

3.2.5. pH

The pH was measured, in duplicate, at 20 ± 2 °C using a pH meter (Crison GLP 22+, Barcelona, Spain) and a combined pH electrode (Crison 50 14T) in a sample of 10 clams edible meat mixed in a T25 ULTRA-TURRAX® homogenizer (IKA, Germany).

3.2.6. Biogenic amines

The flesh of 10 clams' individuals was pooled to obtain a homogeneous and representative composite sample. Briefly, a 10 g portion of the composite was weighed into a 50 mL test tube and mixed with 35 mL of 10% (w/v) trichloroacetic acid (TCA) using a T25 ULTRA-TURRAX® homogenizer (IKA, Germany). The homogenate was then diluted to a final volume of 50 mL with ultrapure water and filtered through a 0.45μm membrane filter. Biogenic amines

were quantified by high-performance liquid chromatography (HPLC) after derivatization with *ortho*-phthalaldehyde (OPA), based on the method described by Komprda et al. (2004). The analysis was performed using a Beckman HPLC system (Beckman Coulter, USA) equipped with a fluorescence detector (excitation wavelength: 350 nm; emission wavelength: 445 nm). A Chromolith® Performance RP-18 column (100 mm × 3 mm; Merck, Germany) was used for the separation. The following amines were determined: histamine*, tyramine*, isoamylamine*, ethylamine*, putrescine**, cadaverine**, 2-phenylethylamine** and methylamine**. The method has limits of detection and quantification (LOD/LOQ): * < 0.6 / 2.0 and **<0.9 / 3.0.

3.2.7. Statistical analysis

One-way and two-way analysis of variance (ANOVA) was performed to compare results between conditions and to compare conditions x time, respectively. A statistically significant result was considered when p-value was lower than 0.05. Statistical analysis was performed using IBM SPSS STATISTICS, 29.0.2.0 Package (IBM Corporation, New York, USA, 2024).

The experimental data for the survival percentages were fitted to a Boltzmann sigmoidal function according to equation 3.2.7:

$$y = \beta + \frac{(\alpha - \beta)}{\left(1 + \exp\left(\frac{x - \varphi}{\tau}\right)\right)} \qquad (3.2.7)$$

where y is the % live clams and x is the time (days). The model parameters, α (initial value), β (final value), φ (centre) and τ (time constant), were estimated by non-linear regression analysis using the Levenberg-Marquardt algorithm to minimise the sum of the squares of the differences between the predicted and experimental values. The precision of the parameters was evaluated by their 95% confidence intervals, and the regression quality assessed by residuals analysis and the coefficient of determination R^2.

Principal Component Analysis (PCA) was performed using survival percentage, % open shells, exudate (g), gases concentration (% O_2 and % CO_2), pH, glycogen content and VOCs in Origin Pro (Version 2024 by OriginLab, USA).

4. Results and discussion

4.4.1. Survivability, open clams, exudation

The results are presented in Figure 4.1 showing a decrease in percentage of live clams (the survival percentage) over the 7 days of refrigerated storage period, as expected.

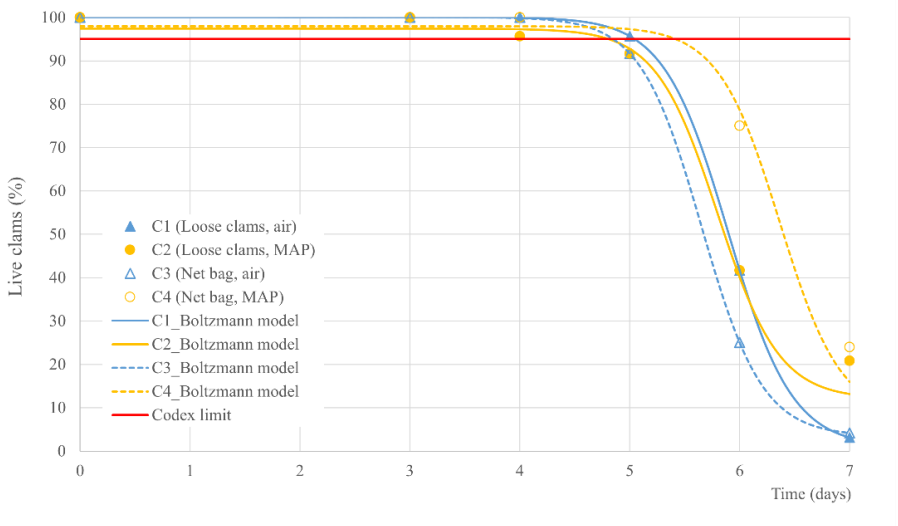


Figure 4.1. *Survival percentage (%) of clams stored at conditions (C1 - C4) over time in scatter markers. Boltzmann model for each condition represented in lines. Red line represents the CODEX limit of 5% of non-live clams.*

The 5% mortality threshold established by the Codex Alimentarius proved to be a stringent benchmark. Based on experimental data, the time to reach 95% live clams was as follows: C1 (loose clams, air) – 5.0 days; C4 and C3 (net bag, MAP and air) – 4.6 days; and C2 (loose clams, MAP) – 4.2 days. Beyond day 5, condition C4 (net bag, MAP) began to outperform the others in maintaining clam viability, showing a significantly higher percentage of live clams compared to conditions C1 (loose, air) and C3 (net bag, air). Condition C2, under MAP, shows an intermediate improvement, although no significant difference from the other conditions. On day 7, the percentage of live clams in C4 was 24.3 ± 12.0%; in C2, 21.0 ± 10.6%; in C3, 4.3 ± 6.1% and in C1, 3.3 ± 6.5%.

Table 4.1 presents the model parameters obtained for each packaging condition. This model should be seen as indicative only because of the low number of sampling points compared with the number of the parameters of the fitting equation. Additionally, the sampling period does not allow to define with precision the parameter β (final value) for all experimental conditions. According to the model (Figure 4.1), there is an increase in time of survivability within the

Codex limit when packaging confinement and modified high-oxygen atmosphere (C4) is applied, as compared to storage of loose clams in air (C1). However, the difference in time was less than one day in this study. Once the death process begins, the rate of decline in the number of alive clams appears to be similar among the conditions tested, as denoted by the slope of the model between days 5 and 7.

Table 4.1. *Parameters obtained from Boltzmann sigmoidal model fitting of survival curves under conditions (C1 - C4) overtime.*

Parameters	**C1**	**C2**	**C3**	**C4**
α	100.05 ± 0.05	98.85 ± 1.27	100.08 ± 0.08	98.00 ± 4.58
β	0.94 ± 0.11	19.60 ± 2.46	3.41 ± 0.16	7.50 ± 132.2
φ	5.90 ± 0.00	5.71 ± 0.05	5.65 ± 0.00	6.37 ± 1.90
τ	0.29 ± 0.00	0.31 ± 0.04	0.28 ± 0.00	0.28 ± 1.07
Reduced Chi-sqr	0.01	4.55	0.20	60.47
R^2	1.00	1.00	1.00	0.97
Adj R^2	1.00	1.00	1.00	0.93

Shell closure in bivalves is controlled by the adductor muscle, which contracts to keep the valves shut. Upon death, muscle relaxation prevents valve closure, resulting in shell opening and exposure of the intravalvular fluid, ultimately leading to flesh desiccation (Fratini et al., 2013). However, in experiments conducted in closed system such as the jar, no drying of the flesh could be observed. Although the clams remained succulent in sensory appearance, a loss of vitality was noticed from day 3 onwards, with the shells opening and exudate being released (Figure 4.2). Up day 5, no signs of spoilage were detected, but after that, an unpleasant odour was easily detected when the jars were opened. On the 6th day, the clams under condition C2 had a strong bad odour; those under conditions C1 and C3 had a bad odour less intense; and those under condition C4 were without spoilage notes, appearing in this sample condition only on day 7.

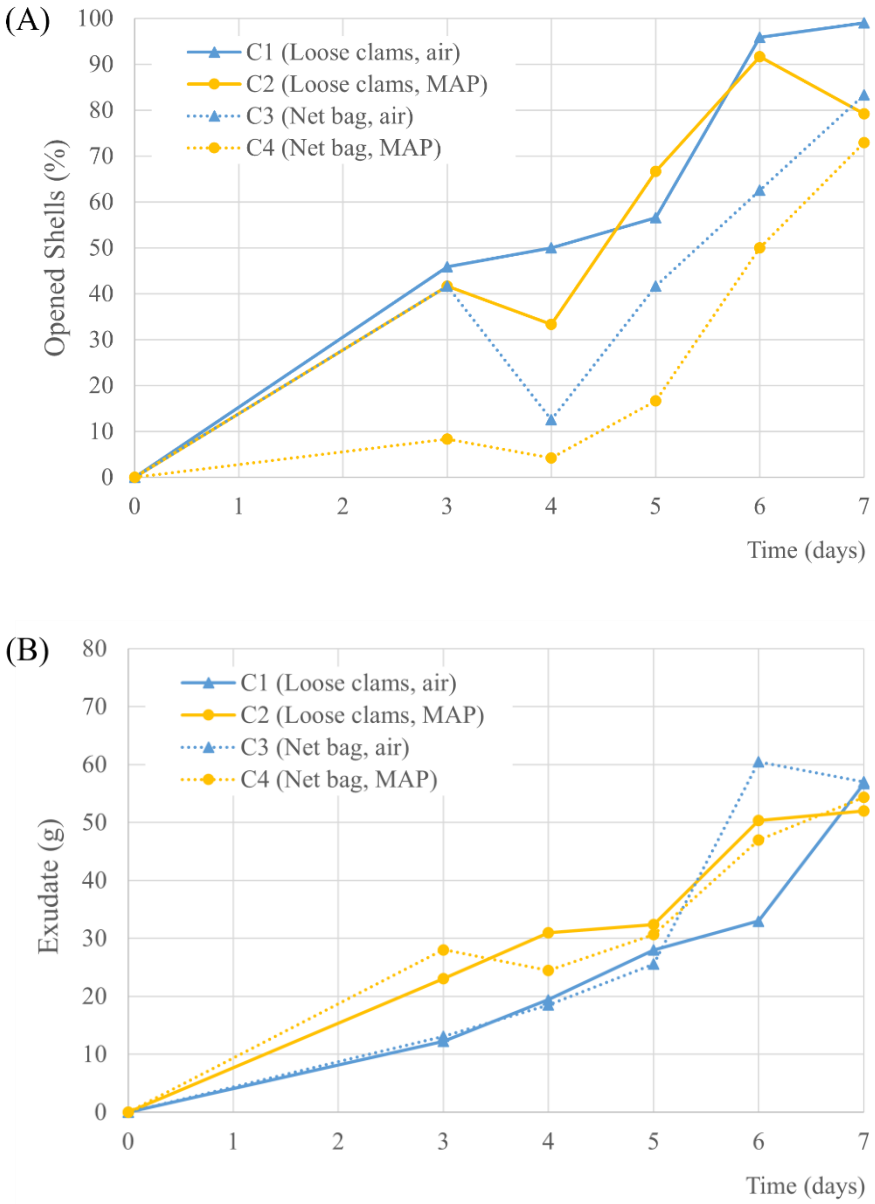


Figure 4.2. *Percentage of opened clam shells (A) and mass of exudate (B) in samples stored at different conditions (C1 - C4).*

A progressive increase in number of open shells (Figure 4.2A) and amount of exudate release (Figure 4.2B) was observed over time across all storage conditions. Figure 4.2A shows a trend for the clams confined in net bags (C3, C4) with high number of closed shells reflecting the effect of the confinement process. Until days 4 – 5, when a high number of clams were alive in all conditions, some clams were naturally opened at the time of evaluation possibly as result of their search for nutrients and oxygen. However, after this period, the proportion of opened clams showed to increase steadily, suggesting a relationship with the rising number of dead clams shown in Figure 4.1. On the day 7, the % opened clams were higher for the samples

under air condition with C1 (99.0 ± 2.1%), C3 (83.3 ± 5.9%) compared to MAP conditions C2 (79.2 ± 0.0%) and C4 (72.9 ± 8.7%).

Figure 4.2B shows the amount of exudate released by the clams. The values increased over time for all conditions, and there was a tendency for lower values in the air sample conditions (C1, C3). Although with no statistically significant difference between the conditions tested, the results show a tendency for samples with MAP to have higher amount of exudate compared to air. The results may be affected by a possible difference in the amount of initial liquid inside the clams (including water) that could not be controlled. The subsequent loss of this liquid would account as exudate in the conditions of test. It is suggested in the literature that the continuous aerobic metabolic process in clams with MAP, with or without the use of glycogen, produces metabolic water that is retained in a closed environment. After the fifth day, with an increase in dead clams mainly in C1 - C3, an increase in the exudate rate of clams in these conditions was noted. In addition to the loss of intravalvular fluid, the death process releases intracellular fluid and haemolymph (Bejaoui et al., 2020).

4.4.2. Changes in gases concentration and respiration rate

The results for the concentration of gases (O_2 and CO_2) in the headspace, for all conditions tested overtime, are presented in Figure 4.3.

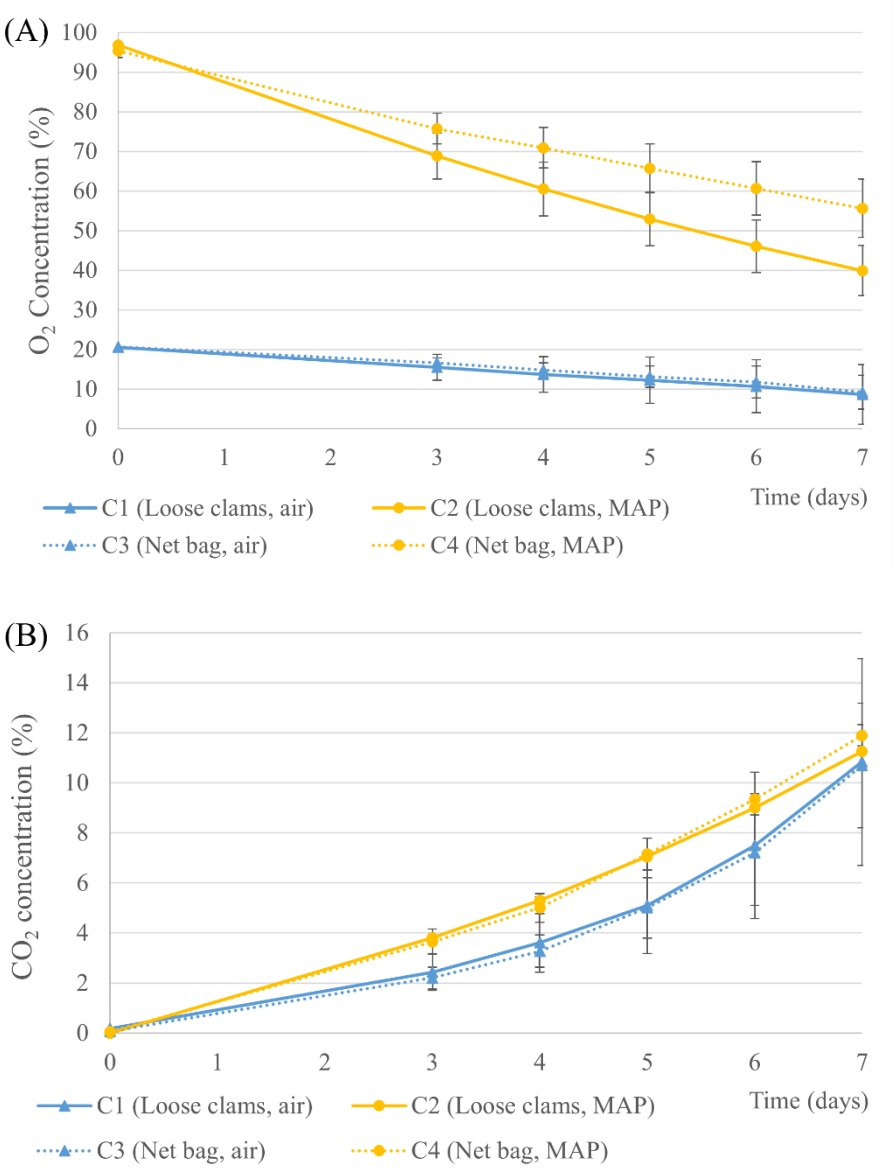


Figure 4.3. *Gas concentration (average ± standard deviation) in headspace: (A) percentage oxygen and (B) percentage carbon dioxide of each condition (C1 - C4) over time.*

Figure 4.3A illustrates the progressive decline in O_2 concentration across all conditions throughout the storage period. The conditions under air (C1, C3) started with 20.5 ± 0.0% and 20.7 ± 0.1% respectively and, after 7 days they were 4.6 ± 3.6% and 6.9 ± 2.2% of oxygen respectively. The conditions under MAP (C2, C4) initiated with 96.9 ± 0.1% and 95.4 ± 1.6% and, in the 7^{th,} day of evaluation were 39.9 ± 6.4% and 55.6 ± 7.4% respectively. Although no statistically significant differences were observed between conditions C1 and C3 or between C2 and C4 because of replicates variability, two consistent trends emerged: the higher rate of O_2 consumption under MAP conditions than in air and the slower decrease in O_2 levels in confined clams compared to loosen ones. This trend was particularly pronounced in the MAP groups after day 4 of storage.

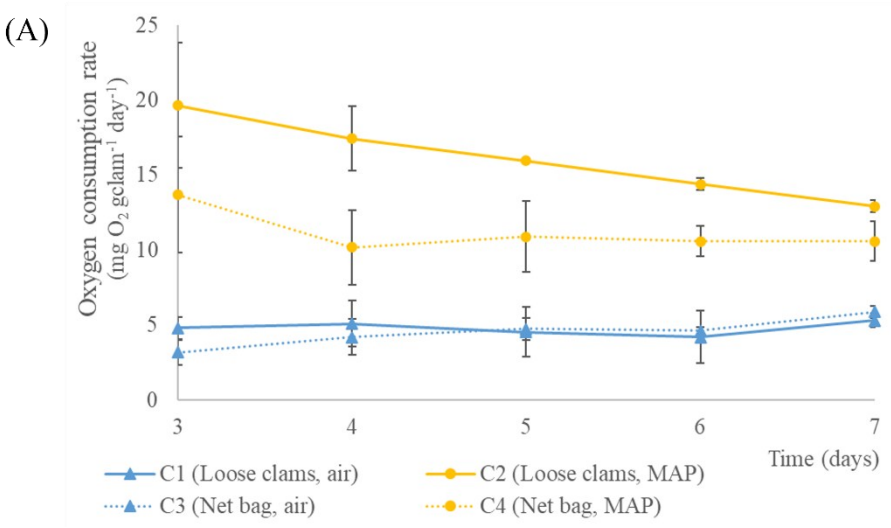
The O_2 consumption rates were calculated for the periods between sampling point times (Table 4.2). The conditions under MAP (C2, C4) showed a O_2 consumption rate four times higher than the samples under air (C1, C3).

Table 4.2. *Oxygen consumption rate, carbon dioxide production rate and respiration quotient calculated for each condition (C1 - C4) over time.*

	Day	C1	C2	C3	C4
Oxygen consumption rate (mg O_2 g_{clam}^{-1} day^{-1})	*Initial (0-3)*	4.80 ± 0.75	19.66 ± 4.17	3.19 ± 0.83	13.70 ± 3.84
	4	5.08 ± 1.54	17.45 ± 2.13	4.21 ± 1.17	10.17 ± 2.51
	5	4.55 ± 1.68	15.98 ± 0.05	4.74 ± 0.72	10.91 ± 2.36
	6	4.19 ± 1.74	14.40 ± 0.40	4.62 ± 0.26	10.59 ± 1.03
	7	5.31 ± 0.47	12.93 ± 0.41	5.88 ± 0.39	10.59 ± 1.32
Carbon dioxide production rate (mg CO_2 g_{clam}^{-1} day^{-1})	*Initial (0-3)*	2.55 ± 0.21	3.66 ± 0.01	2.17 ± 0.47	3.46 ± 0.55
	4	4.05 ± 0.82	4.34 ± 0.40	3.33 ± 0.60	3.89 ± 0.21
	5	5.49 ± 1.23	5.06 ± 0.22	5.94 ± 1.00	6.20 ± 0.21
	6	8.38 ± 2.05	5.64 ± 1.04	7.96 ± 0.58	6.35 ± 1.22
	7	11.70 ± 0.63	6.50 ± 0.63	10.86 ± 0.26	7.36 ± 1.83
Respiration quotient	*Initial (0-3)*	0.54 ± 0.04	0.19 ± 0.04	0.68 ± 0.03	0.27 ± 0.12
	4	0.81 ± 0.08	0.25 ± 0.05	0.80 ± 0.08	0.40 ± 0.12
	5	1.24 ± 0.19	0.32 ± 0.01	1.25 ± 0.02	0.58 ± 0.15
	6	2.08 ± 0.37	0.39 ± 0.08	1.72 ± 0.03	0.60 ± 0.06
	7	2.21 ± 0.08	0.50 ± 0.06	1.85 ± 0.08	0.69 ± 0.09

Data are average ± standard deviation (n=2).

After the 4th day, the rates at conditions C1, C3 and C4 apparently stabilized and the rate at condition C2 decrease until the 7th day. The oxygen rate for each condition is plotted in Figure 4.4A.



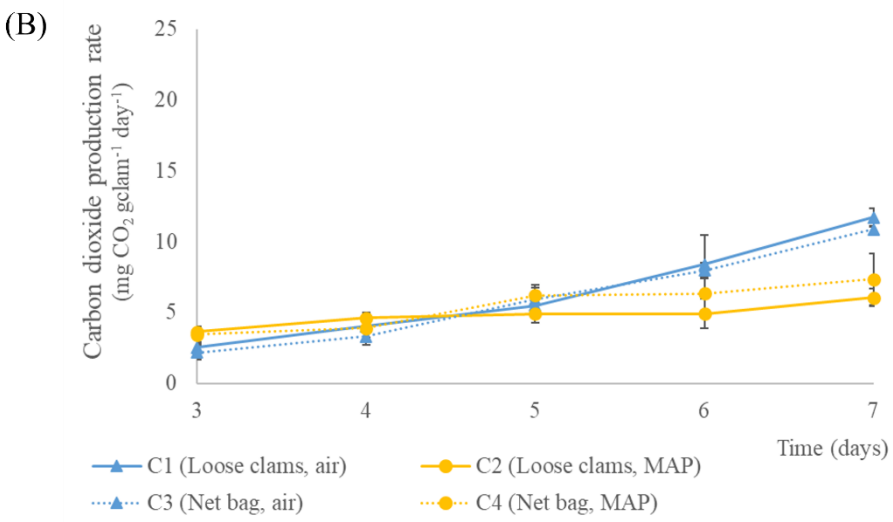


Figure 4.4. *(A) Oxygen consumption rate and (B) Carbon dioxide production rate for each condition (C1 - C4) over time. Average ± standard deviation. (n=2)*

The carbon dioxide concentration increased over time for all conditions (Figure 4.3B), and no statistical differences were found among the samples. The results showed a trend towards higher CO_2 production in samples under MAP conditions (C2, C4) until day 5-6, with a reversal of the scenario from this point onwards. The CO_2 concentration after 7 days was C1 (13.1 ± 1.8%), C3 (12.1 ± 1.2%), C4 (11.9 ± 0.4%) and C2 (11.3 ± 0.5%). The carbon dioxide production rate increased over time for all samples (Figure 4.4B). Initially, the samples under air (C1, C3) produces less carbon dioxide than the MAP samples (C2, C4), but after days 5-6, the rates increased. This behaviour aligns with the increase in the number of dead clams in conditions C1 and C3 (Figure 4.1).

Although the decrease in O_2 levels and increase in CO_2 levels throughout the storage period suggest that this is a result of bivalve respiration (Gonçalves et al., 2009), the higher availability of O_2 in modified atmosphere packaging (MAP) conditions may improve oxygen diffusion in the clam tissues and in the intravalvular liquid, allowing aerobic respiration to continue for longer. This may also favour the auto-oxidation of lipids, proteins and pigments, which are reactions that consume oxygen without producing CO_2. Additionally, microbial activity may also consume oxygen and store some of the carbon as biomass.

The respiratory quotient (Q) is an indicator of a ratio between the carbon dioxide evolution rate to oxygen consumption rate overtime is presented in Figure 4.5.

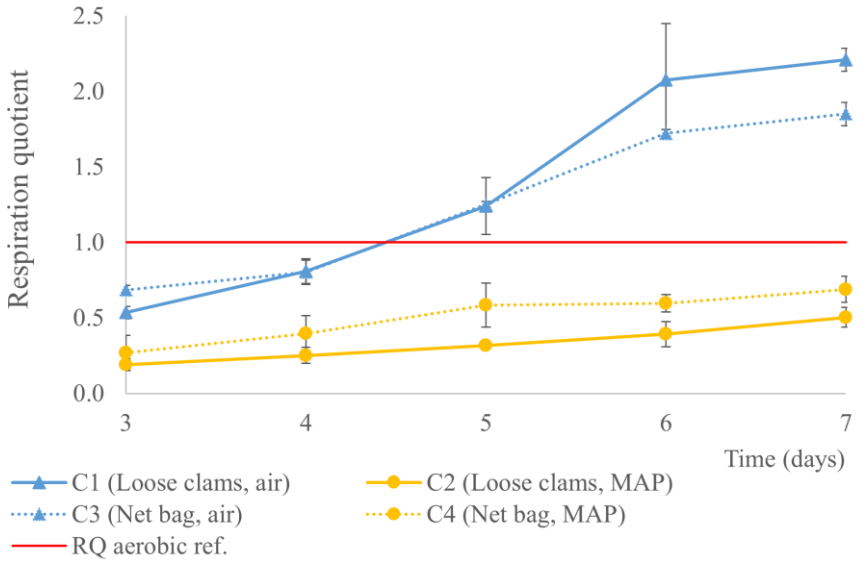


Figure 4.5. *Respiratory quotient (RQ) for V. corrugata clams stored at different conditions (C1 - C4) over time. Data are average ± standard deviation (n=2).*

RQ values increased over time for all conditions tested (Table 4.2). However, for samples under MAP, values remained below 1, with a tendency to be higher in C4 than in C2 (Figure 4.5). For samples under air, values started between 0.5-0.7, but between days 4 and 5 they crossed the reference line for metabolism based on aerobic oxidation of carbohydrates and continued to increase until day 7. Information on the respiratory quotient of bivalve molluscs under air or MAP is lacking in the literature, particularly for the *V. corrugata* clam species. Ho et al. (1997) found RQ values ranging from 1.4 to 1.9 for *M. mercenaria* (hard clams) when individuals were exposed to different MAP conditions (6% O_2: 16% CO_2; 11% O_2: 10% CO_2; and 16% O_2: 4% CO_2). A study conducted by Khripounoff et al. (2017) with submerged *Christineconcha regab* clams and *Bathymodiolus azoricus* mussels obtained RQ values ranging from 0.8 to 1.5. Similar oxygen consumption between mussels and clams was noted, but clams showed higher carbon dioxide production attributed to anaerobic metabolism.

4.4.3. Identification of volatile compounds via GC-MS

To monitor the progression of spoilage and aroma compound formation, volatile organic compounds (VOCs) were analysed by GC - MS. Table 4.3 shows the VOC profile of the clams stored at 3 ± 1 °C under conditions C1 - C4 for 0, 4 and 6 days. Four groups of chemical compounds were detected in all conditions and sample points, i.e. alcohols, aldehydes, ketones and carboxylic acids, with a varying number of compounds in each group. A plot showing the VOC profile for each condition and time is presented in Appendix (Figure A1).

Among the detected VOCs, alcohols were predominant (87 – 92% of total volatiles), followed by aldehydes (4 – 9%), ketones (2 – 4%) and carboxylic acids (<2%). The predominant alcohol in all conditions tested was 1,5-octadien-3-ol. Notwithstanding the high intensity, there was no significant differences in the concentration among the conditions tested for the period analysed. This compound is characterized by a fresh mushroom or moss-like odour (Kawai, 1996) and is commonly found in oysters such as *Ostrea edulis* (Fratini et al., 2012; van Houcke et al., 2016) and *Crassostrea gigas* (van Houcke et al., 2016), as well as in *Cerastoderma edule* (Fratini et al., 2012), *Pollicipes cornucopia* and in *V. corrugata* (Fratini et al., 2012).

1-penten-3-ol, is known to result from the lipoxygenases on PUFAn-3 (Hu & Pan, 2000; German et al., 1991), was one of the few volatiles in which a significant difference was observed between the conditions tested (Table 3). It has been detected in various bivalves, including *M. galloprovinciallis*, *V. corrugata* (Fratini et al., 2012), and oysters (Fratini et al., 2013, van Houcke et al., 2016) and is associated with a grassy odour (Hu & Pan, 2000). Results indicate a much higher concentration for this alcohol at day 4, compared to the other sampling points, for conditions C1, C2, and C4 (Figure 4.6). Both 1-penten-3-ol and 1-octen-3-ol have also been identified as volatile organic compounds produced by seafood spoilage bacteria (Odeyemi et al., 2018). 1-Octen-3-ol has been cited as a marker of lipid oxidation in horse mackerel muscle (Iglesias & Medina, 2008) and was also detected in *E. ensis* (Fratini et al., 2012) and it is associated with a plant-like odour (Hu & Pan, 2000). Additionally, 1-pentanol, which is associated with a desirable apple-like aroma (Hu & Pan, 2000), was detected in *V. corrugata, M. galloprovinciallis* (Fratini et al., 2012) and oysters (Fratini et al., 2013). Both 1-penten-3-ol and 1-pentanol were significantly more abundant in *V. corrugata* compared to other shellfish species (Fratini et al., 2012). Both these compounds showed significant differences between time and results indicate a significant interaction condition x time (Table 4.3).

Table 4.3. *Volatile organic compounds (VOCs) profile of V. corrugata clams under different conditions (C1 – C4) of gas composition and confinement during storage at 3 ± 1 °C. Values are expressed as chromatographic peak /I.S. area.*

VOCs	**C1**			**C2**		**C3**		**C4**		**Condition**	**Time**	**Condition x Time**
Days of storage:	0[a]	4[a]	6[b]	4[a]	6[b]	4[a]	6[ab]	4[a]	6[ab]		*p value*	
Alcohols												
1-Penten-3-ol	0.37±0.30	2.49±0.83	0.37±0.05	0.42±0.17	0.31±0.11	0.97±0.44	1.02±0.55	1.74±0.27	0.34±0.16	0.044*	<0.001*	0.010*
1-Pentanol	0.02±0.02	0.08±0.03	0.03±0.00	0.02±0.01	0.04±0.02	0.04±0.02	0.16±0.09	0.04±0.01	0.02±0.01	0.061	0.048*	0.030*
1,5-Octadien-3-ol	3.87±3.07	7.47±2.28	4.70±0.57	2.48±0.97	3.90±1.59	6.07±2.90	8.63±4.82	5.70±0.66	3.19±2.06	0.325	0.479	0.555
1-Octen-3-ol	0.28±0.22	0.67±0.20	0.28±0.04	0.22±0.08	0.27±0.11	0.41±0.19	0.68±0.39	0.53±0.05	0.25±0.17	0.400	0.249	0.281
Aldehydes												
2,4-Heptadienal	0.00±0.00	0.02±0.01	0.01±0.00	0.00±0.00	0.01±0.00	0.02±0.01	0.01±0.01	0.01±0.00	0.00±0.00	0.125	0.042*	0.347
Lilac aldehyde D	0.36±0.38	0.69±0.14	0.38±0.10	0.19±0.12	0.37±0.19	0.47±0.23	1.42±1.04	0.35±0.10	0.44±0.32	0.286	0.324	0.348
Ketones												
2-Nonanone	0.04±0.04	0.10±0.04	0.05±0.00	0.05±0.03	0.04±0.02	0.14±0.08	0.10±0.07	0.08±0.02	0.04±0.02	0.239	0.109	0.770
3,5-Octadien-2-one	0.04±0.03	0.28±0.07	0.07±0.01	0.04±0.00	0.07±0.02	0.27±0.11	0.20±0.09	0.16±0.02	0.07±0.03	<0.001*	<0.001*	<0.001*
2-Undecanone	0.00±0.00	0.01±0.00	0.01±0.00	0.00±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	1.000	0.391	0.889
Carboxylic acids												
Octanoic acid	0.01±0.01	0.02±0.01	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.01	0.03±0.03	0.02±0.02	0.02±0.01	0.489	0.801	0.443
Nonanoic acid	0.03±0.02	0.04±0.01	0.04±0.00	0.01±0.00	0.03±0.01	0.14±0.06	0.06±0.04	0.04±0.03	0.04±0.03	0.026*	0.312	0.089*

Data are average ± standard deviation (n=2).
Superscript letter in day means a = live clams, b = dead clams, ab = relative proportion value of live and dead clams.
**Means significant difference (p<0.05)*

Aldehydes are the second largest group (4 – 9%) of VOCs detected in the samples under the analytical conditions used. Lilac aldehyde was well represented in this group, but with fluctuations in values over time and no significant differences among conditions (Table 4.3). It is characterized by a floral lilac odour (Fratini et al., 2012; van Houcke et al., 2016; Acree & Arn, 2008) was detected in *V. corrugata, O. edulis* (Fratini et al., 2012, Fratini et al., 2013; van Houcke et al., 2016), *C. gigas* (Fratini et al., 2013, van Houcke et al., 2016), *E. ensis* and *P. cornucopia* species (Fratini et al., 2012). In *C. ariakensis* fresh oysters this compound also dominated the aldehyde group (Fu et al., 2024). 2,4-Heptadienal was identified as the main product of a polyunsaturated omega-3 fatty acid - eicosapentaenoic acid (EPA) - degradation through lipid autoxidation (Kawai, 1996) and described as having an extremely rancid odour (Hu & Pan, 2000). Other authors have described this compound as having a mushroom, moss or green odour (Fratini et al., 2012). This compound had low representation among VOCs, but a significant difference was noted over time (Table 4.3).

The most representative ketone (2 - 4% in total) was 3,5 octadien-2-one. It showed a large increase in values on day 4 and a decrease on day 6 for conditions C1, C3 and C4. This volatile compound contributes to the fatty fruity odour (Fratini et al., 2012; Hu & Pan, 2000; Kawai, 1996) and has been detected in *C. gigas* (Fratini et al., 2013; van Houcke et al., 2016), *O. edulis* (van Houcke et al., 2016), *E. ensis* and *P. cornucopia* (Fratini et al., 2012). The other ketone 2-nonanone was detected in *V. corrugata*, *E. ensis*, *P. cornucopia* (Fratini et al., 2012) as well as in *C. gigas* (Fratini et al., 2013). The values found for 2-nonanone showed the same behaviour of rising on the fourth day and falling on the sixth day for all conditions tested. 2-Undecanone, ketone with lower representation, showed slight variations over time between conditions. This compound has been found in the *E. ensis* and *P. cornucopia* species (Fratini et al., 2012).

Two carboxylic acids (0 - 2%) were identified in all samples under the analytical experimental conditions applied. No reference was found for these compounds in bivalve molluscs, but they were reported as off-odours in fish species in aquaculture (Noguera et al., 2024). Nonanoic acid (pelargonic acid), the most representative within this group, showed significant different values among conditions tested (Table 3), with C3 condition (net bag, air) standing out. This compound was found in fish collagen peptides and characterised as having a fatty, musty odour (Mahmoud & Buettner, 2017). Octanoic acid (caprylic acid), although there were no statistical differences among the conditions tested, it also showed the highest values for C3 on days 4 and 6. It is a saturated fatty acid found in Atlantic salmon, aquafeed and rainbow trout and is

characterized as fruity-acidic, irritating, musty, coriander-like, fresh and roasty odour (Wang et al., 2023).

The volatile compounds with statistically significant differences among conditions C1 - C4 are showed in Figure 4.6.

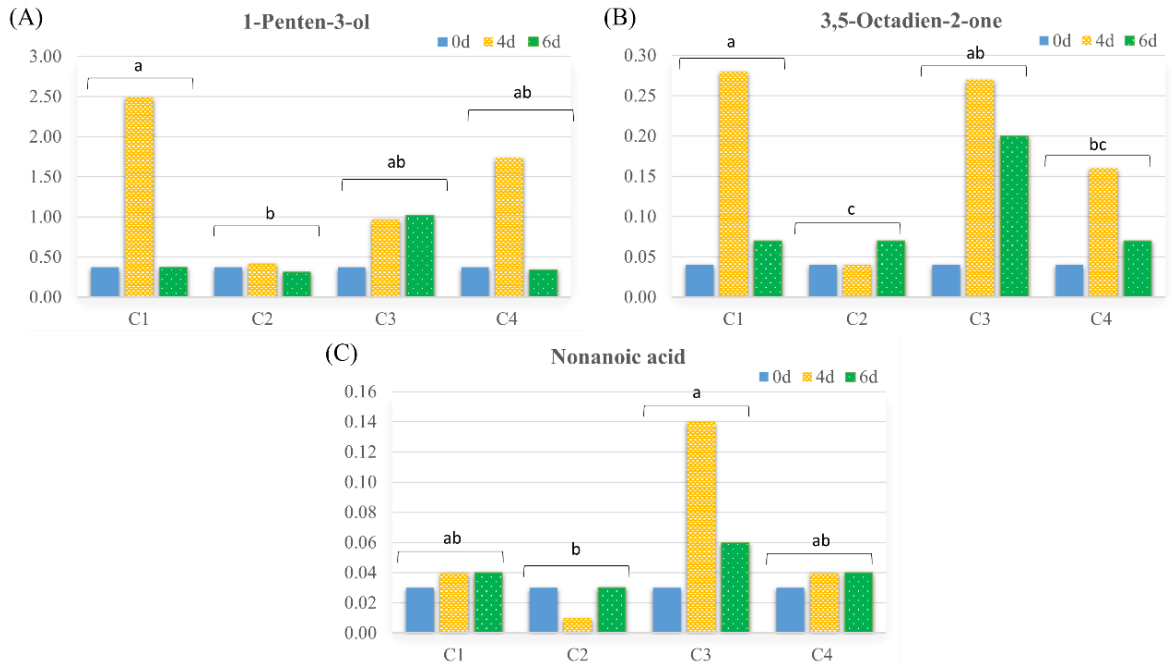


Figure 4.6. *Changes in concentration of volatile compounds: (A)1-penten-3-ol, (B) 3,5-ocadien-2-one, and (C) nonanoic acid, in V. corrugata clams stored at different conditions (C1 - C4) over time. The values are expressed chromatographic peak /I.S. area. Different letters indicate statistical differences among conditions ($p<0.05$).*

According to previous results, the largest number of clams died earlier under air conditions. Considering this, the formation and modification of volatiles should also occur at slightly different times. Figure 4.6 shows higher values for the compounds evaluated in samples under air conditions (C1, C3) compared with MAP (C2, C4), in addition to a tendency for most compounds to decrease after the death of the clam. Further studies are necessary to understand the evolution of these compounds over time and what impact they have, particularly on the consumer´s experience with clams in a closed modified atmosphere packaging.

4.4.4. Glycogen quantification and pH

Glycogen is a principal energy reserve in bivalves and serves as a key physiological marker of stress response and survival under refrigerated storage (Anacleto et al., 2013a; Liu et al., 2020). In addition, glycogen also contributes to organoleptic properties. Therefore, the measure of its content is relevant (Gonçalves et al., 2009). The content of glycogen and pH values over time

are reported in Figure 4.7. The full line represents the values obtained in clams alive, while the dotted line represents the values obtained for dead clams (storage times of 6 and 7 days).

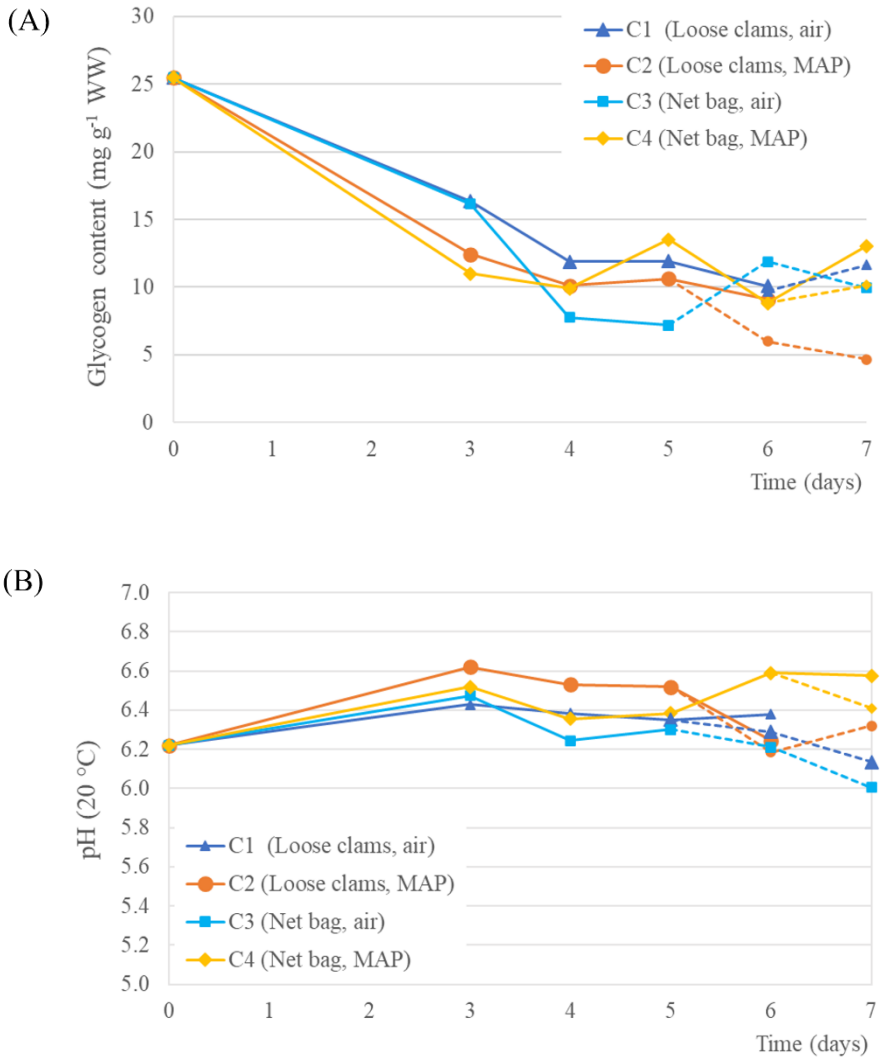


Figure 4.7. *(A) Glycogen content (mg g^{-1} WW) and (B) pH of the clams stored at different conditions (C1 - C4) over time. Full line: results for live clams and dotted line: results for dead clams. Average ± standard deviation.*

The initial glycogen content of live clams was 25.5 ± 0.6 mg g^{-1} WW (Figure 4.7A). Anacleto et al. (2013) reported values of about 17 mg g^{-1} WW for the same species. The values of glycogen decreased over time particularly between days 0 and 3, with a more severe drop observed in samples under MAP conditions (C2, C4) than in samples under air conditions (C1, C3). Between day 3 and day 7, the values oscillated and, on day 7, it was observed to be significantly lower in value for C2 condition (4.7 ± 0.04 mg g^{-1} WW) in relation to the other conditions tested C1 and C4 (11.6 ± 0.2 mg g^{-1} WW), C3 (9.9 ± 0.2 mg g^{-1} WW). The individual results for glycogen are plotted in Appendix (Figure A2).

Anacleto et al. (2013) demonstrated that *V. corrugata* maintained the metabolic reserves when kept at 4 °C in contrast to clams transported at 22 °C. Furthermore, when compared to *R. philippinarum,* the native clam showed higher glycogen content than the exotic clam. Bi et al. (2023) studied the species *R. phillippinarum* under different temperatures and modes of transport and found a decrease in glycogen content over time, which was explained by the consequence of environmental stress caused by a rapid change in temperature. Under stressful conditions, clams need to consume more polysaccharides, which leads to a decrease in glycogen content to maintain needs. A correlation has also been suggested with the formation of lactic acid explained by the degradation of glycogen via glycolysis, also mentioned by Chen et al., 2021.

On days 6 and 7 of storage, the glycogen content was quantified in both live and dead clams. On day 6, live clams in condition C1 showed 10.1 ± 0.1 mg g^{-1} WW while dead clams showed 9.7 ± 0.4 mg g^{-1} WW. Live clams in condition C2 showed 9.1 ± 0.3 mg g^{-1} WW, while dead clams showed 6.0 ± 0.0 mg g^{-1} WW. On day 7, for condition C4, live clams presented 13.0 ± 0.1 mg g^{-1} WW and dead clams presented 10.1 ± 0.3 mg g^{-1} WW. The results showed a tendency for lower glycogen values in dead clams than in live clams (Figure A2).

At the beginning of the experiment, the pH of live clams was 6.22 ± 0.01 (Figure 4.7B). This value showed a slight increase by day 3 across all conditions. For samples stored in air (C1, C3), a gradual decrease in pH was observed over time, reaching 6.14 ± 0.01 and 6.01 ± 0.01 on day 7, respectively. In contrast, samples stored under modified atmosphere packaging (MAP) conditions (C2, C4) exhibited a more stable pH trend, with values of 6.49 ± 0.05 and 6.32 ± 0.00 on day 7. These findings align with Gonçalves et al. (2009), who studied live *R. decussatus* clams conditioned in MAP (70% O_2: 30% N_2) compared to clams stored in air within net bags at 6.1 ± 0.7 °C for 6 days. Their results showed that clams stored in air had lower pH values than those in high-oxygen conditions. The authors attributed this to the higher oxygen availability allowing the clams to maintain predominantly aerobic metabolism, whereas oxygen-restricted conditions induced anaerobic metabolism, leading to acid production and a consequent pH decline.

4.4.5. Biogenic amines

Biogenic amines were evaluated, and results are shown in Figure 4.8. The amount of 2-phenylethylamine, isoamylamine and cadaverine showed values below the LOD in the samples evaluated. The results showed that the BAs formation is very low until day 5. Tyramine, an

aromatic monoamine originated from tyrosine decarboxylation, was detected only on day 6 and increased the values in day 7 for all conditions. The condition C3 (net bag, air) had the higher value 17.8 mg/kg and the condition C4 the lower value 6.6 mg/kg. In the literature, for crustacean and mollusc species, the highest amount of tyramine recorded was 29.0 mg/kg in sea squirt samples, with an average value of 15.2 mg/kg. It is suggested that the maximum average value for fresh fish, cephalopods, crustaceans and molluscs should not exceed 100 mg/kg, as it has been reported that tyramine can be toxic if consumed at 100 - 800 mg/kg food (Arulkumar et al., 2023).

Putrescine, an aliphatic diamine originated from ornithine, was also detected on day 7 in conditions C1 and C3 with 3 mg/kg. Although not acutely toxic, putrescine can potentiate the effects of histamine and other BAS, as it favors its adsorption and interfere with the detoxication system (Visciano et al., 2020). In seafood, highest amounts of putrescine were registered for baby octopus' sample (190 mg/kg), and the highest mean value was Japanese mystery snail with 134.1 mg/kg (Arulkumar et al., 2023).

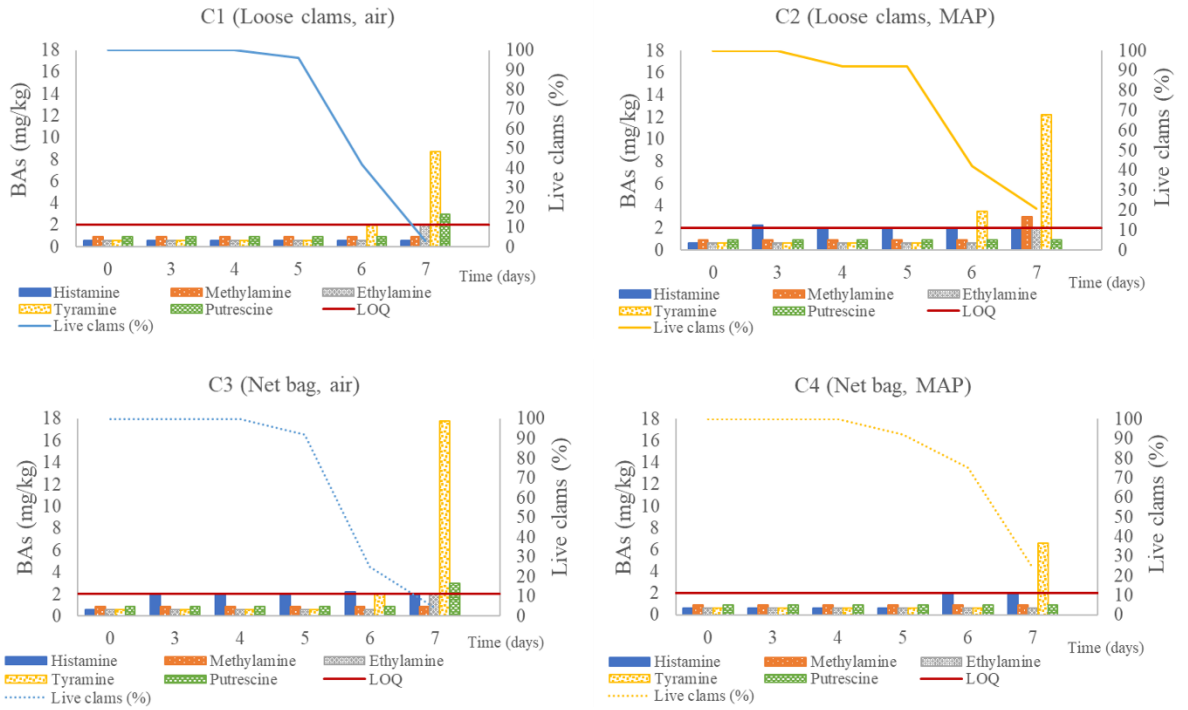


Figure 4.8. *Biogenic amines (mg/kg) and % live clams stored at different conditions (C1 - C4) overtime. Limit of Quantification (LOQ): 2.0 mg/kg. (n=10)*

Many authors refer the relation between BAs formation and microorganisms, temperature, NaCl, pH, oxygen availability and other factors (Visciano et al., 2020; Arulkumar et al., 2023). At low pH, microorganisms are more likely to produce decarboxylase as a protective mechanism against acidity. Conversely, conditions that lead to a reduced redox potential,

enhance histamine formation. Some bacteria, such as *Pseudomonas*, produce low quantities of BAs when NaCl concentrations are 4 - 5%, even though decarboxylation reactions are still occurring (Visciano et al., 2020). In this study, microorganisms´ counts were not evaluated but in Figure 4.8 it is possible to note that the higher values of BAs were found in condition C3 (net bag, air) with lower pH (6.01 ± 0.01) on day 7. MAP with different concentrations of gases may provide the optimal conditions for effectively retarding both microbiological and chemical processes, although in the present study this was not targeted, but rather the effect on keeping the clams alive. High O_2 concentrations in MAP (% CO_2/O_2 at 60/40 and 40/60) had an inhibiting effect on the production of BAs, including tyramine and putrescine, in chilled hake (*Merluccius merluccius L.*) stored for 33 days at 2 ± 1 °C (Ruiz-Capillas & Moral, 2011). In our study, the condition C4 (net bag, MAP) had the lowest level of tyramine, however C2 (loose clams, MAP) had almost the double of the condition C4 and, for putrescine, C4 and C2 were below the limit of quantification.

4.4.6. Multivariate analysis of storage and packaging conditions

Principal component analysis (PCA) was applied to all data (all replicates) of survival percentage (% live clams), number of open shells, exudate released by the clams, head space gas concentrations (% O_2 and % CO_2), pH and glycogen content (Figure 4.9). The first two components explained 80.97% of the variance (PC1: 60.00%, PC2: 20.97%).

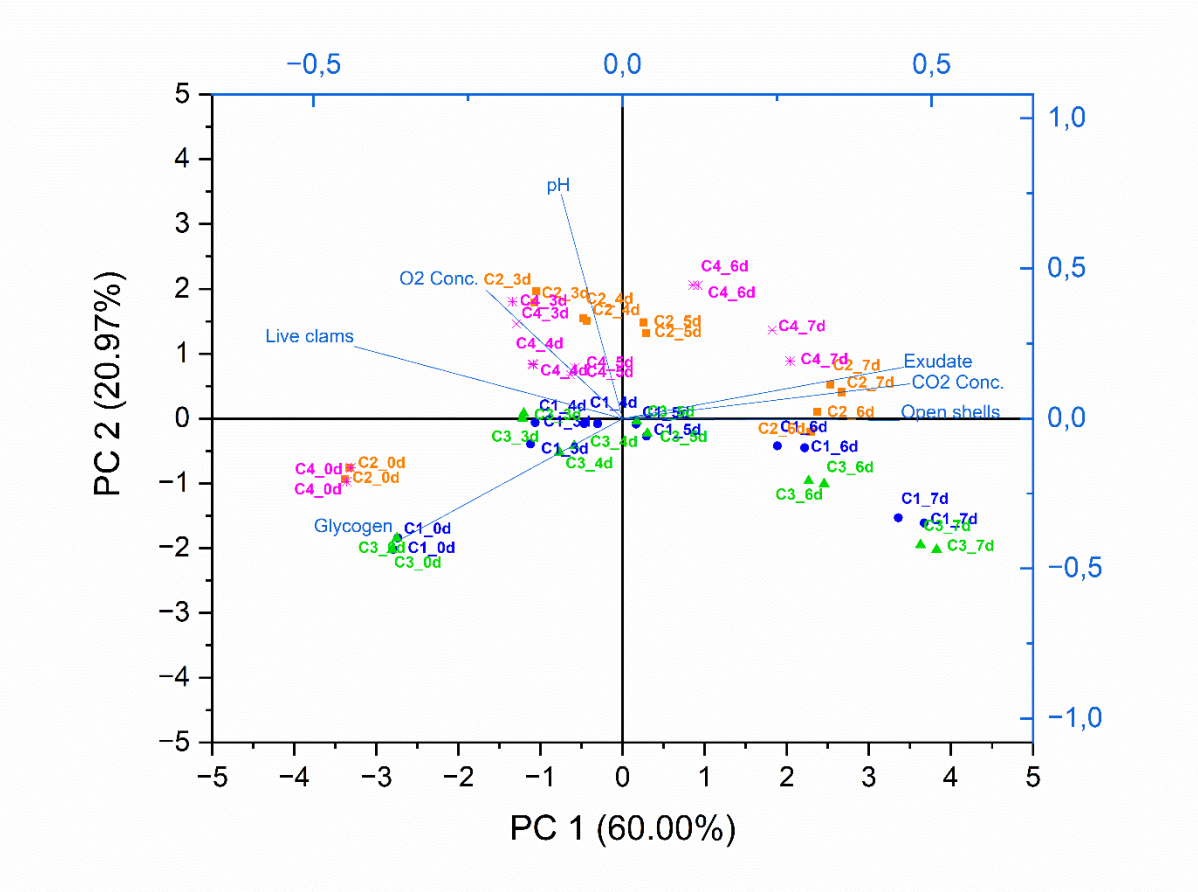


Figure 4.9. *Score plot of PC1 versus PC2 for parameters of V. corrugata samples stored at conditions C1 - C4 overtime (0, 3, 4, 5, 6 and 7 days). Data points are shown as blue circles (C1), orange squares (C2), green triangles (C3) and pink stars (C4).*

PC1 represented the main spoilage gradient, with positive loadings for % CO_2, amount of exudate and number of open shells, and negative loadings for % live clams. Movement towards positive PC1 values thus indicated progressive deterioration. Fresh samples (C1_0d, C2_0d, C3_0d, C4_0d) clustered at the negative side of PC1 with low variation between replicates and conditions, as expected before storage. Late air-stored samples (C1_7d, C3_7d) rest in the far positive side, reflecting advanced spoilage, related to the low number of live clams, high number of open shells and high % CO_2 produced in head space, aligned with Figure 4.1, Figure 4.2A and Figure 4.3B, respectively.

PC2 captured variability associated with changes in pH, where higher PC2 scores (e.g., C4_6d, C2_3d) reflected higher pH, and lower scores (e.g., C3_7d) corresponded to pH decline aligned with Figure 4.7B. Glycogen content and % O_2 shared contributions to both components: their loading vectors were oriented partly along PC1 (spoilage gradient) and partly along PC2 (pH-related variability), indicating that these parameters influenced both factors. Samples stored under air (C1, C3) tended to align with negative PC2 scores, while MAP-stored samples (C2,

C4) shifted toward positive PC2 values, reflecting the influence of MAP on pH and O_2 dynamics.

MAP-stored clams (C2, C4) also show attenuated spoilage progression along PC1 compared with air (C1, C3). For example, C2_7d scored at PC1 ≈ 2.8 while C1_7d reached ≈ 3.6–3.8. Packaging format further modulated outcomes: under MAP, net bag clams (C4) showed higher pH and O_2 than loose clams (C2) at later storage times.

Zhang et al. (2010) previously demonstrated that PCA effectively depicts transitions from fresh to deteriorated states in seafood through volatile. In the present study, VOCs were excluded from PCA because the other parameters already explained > 80% of the variance and clearly distinguished spoilage progression across conditions. While VOCs could provide additional biochemical detail insight, the selected parameters sufficiently captured the deterioration trends.

Overall, PCA revealed clear distinct differences between storage conditions. Air-stored clams deteriorated most rapidly, whereas MAP delayed spoilage. The effect of the confinement using net bag under MAP (condition C4) showing the greatest preservation.

5. General conclusions

This study evaluated the evolution of specific physiological characteristics of live *V. corrugata* clams during the shelf-life, highlighting the impact of shells confinement through packaging and modified atmosphere.

The analysis of survival rate, number of open clams, and exudate released confirmed the effectiveness of confinement. This extension of shelf-life was directly associated with the lower oxygen consumption rate. The effect of MAP is confirmed and associated with the CO_2 release and glycogen depletion.

The increase in clams' mortality was closely linked to the higher number of opened clams, higher exudate release, originated both from the metabolic water during life and from intravalvular fluid and tissue breakdown after death, together with pH decline and biogenic amine formation.

The effectiveness of packaging in high-oxygen modified atmosphere alone, i.e., without confinement, seems to be not relevant, while the two factors synergically result in increased preservation. Shell confinement was crucial to preserve intravalvular liquid and ensure the beneficial effect of oxygen availability. Without confinement, liquid losses compromised preservation.

Overall, the intrinsic variability of this highly valued species, combined with the sensitivity, represents a major challenge for extending shelf-life. Ensuring that the clams reach the consumers fresh and alive is essential to safeguard product quality and minimize loss.

6. Limitations and future work

Some limitations of the work need to be recognised. Additionally, during the study, different ideas emerged for future work that could complement this research and benefit the shelf-life of the clams.

One main aspect is the sampling times which could be increased for a more precise definition of the time for 5% CODEX threshold and for 100% dead clams and survivability modelling.

The pre-processing stage, prior to packaging and controlled storage, was not analysed in this study and could be examined in more detail in following work. For example, the purification process could be examined. The number of hours of purification treatment, the level of oxygenation of the purification water, or even the extent to which this water could be retained by the clams, may influence the extension of shelf-life, since they breathe through water.

As for the packaging process and the packaging itself, the impact of different confinement pressures and volumes of free space, contact with exudates and light exposure during shelf-life could be studied. The use of active packaging containing fast CO_2 absorbers, oxygen releasers or exudate absorbers could also be investigated.

In terms of testing methodologies, improvements could be made by measuring the dissolved oxygen in the intravalvular liquid to verify that it is being consumed exclusively by respiration. New technologies could also be used to improve the comparison between odours, such as the electronic nose.

Appendixes

Appendix A.1. Volatile organic compounds profile in *V. corrugata* clams' samples.

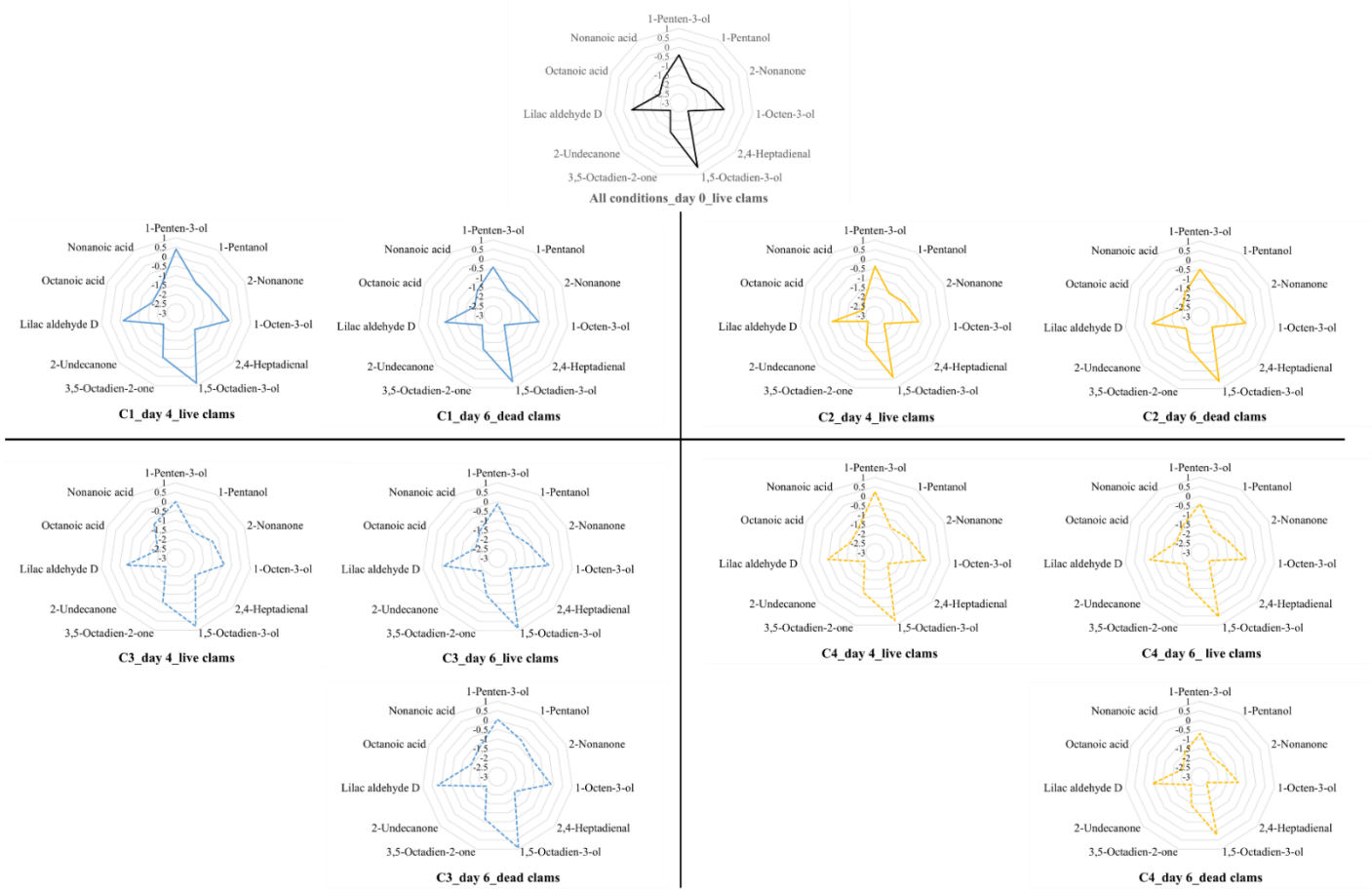


Figure A.1. *Volatile organic compounds profile in V. corrugata clams at different conditions (C1 - C4) and refrigeration (3 ± 1 °C) for 0, 4 and 6 days. The average values are expressed as chromatographic peak /I.S. area.*

Appendix A.2. Glycogen content along the time.

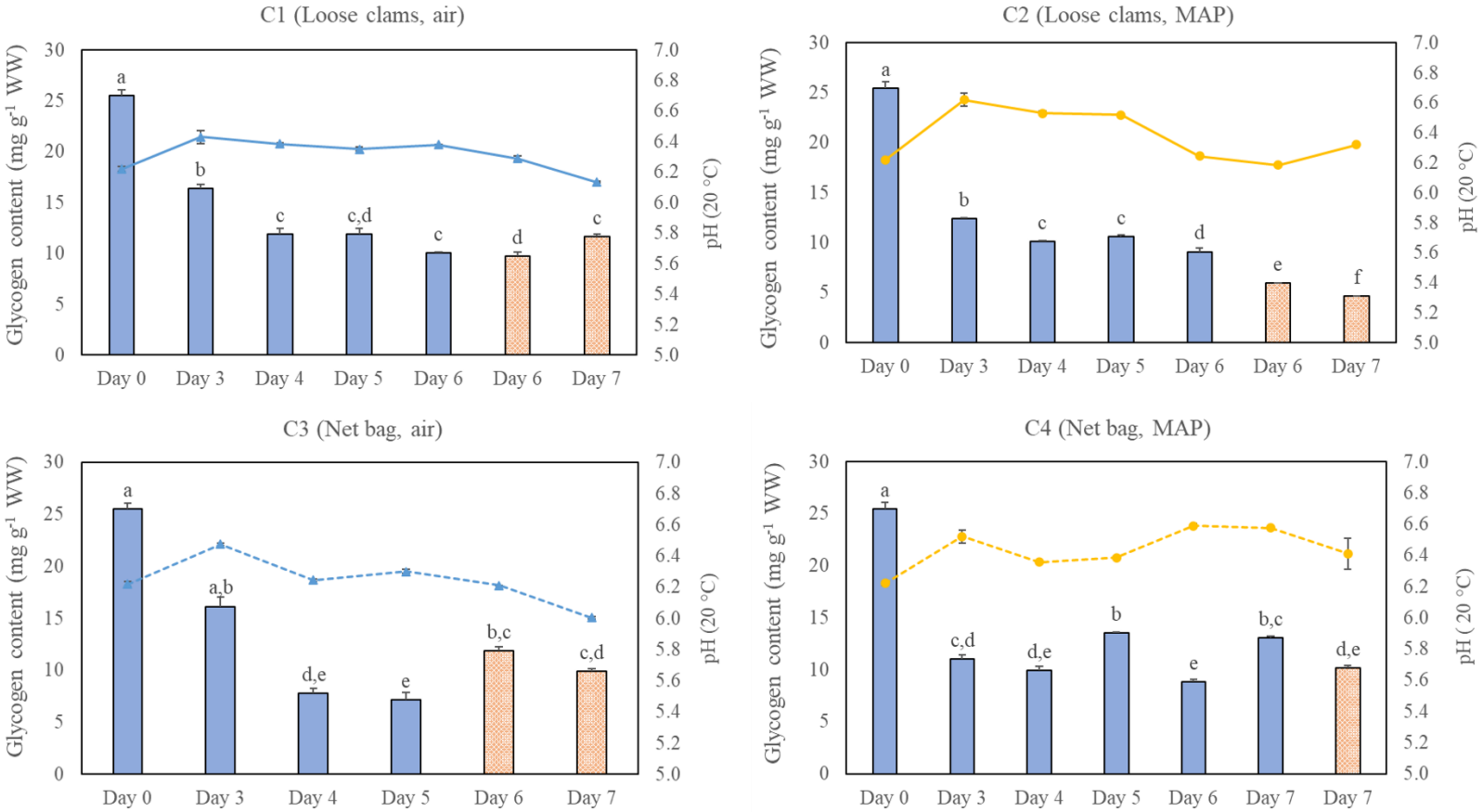


Figure A.2. *Glycogen content (mg g^{-1} WW) and pH of the clams stored at different conditions (C1 - C4) over time. Within each plot site, different letters on the bars denote significant differences between days of storage ($p < 0.05$, n=10). Legend: blue solid-filled bar means that analysis was done with live clams and orange-dotted bars with dead clams.*

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