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Multifactorial analysis and food safety issues of edible marine gastropods intended for human consumption

(Analisi multifattoriale e di sicurezza alimentare di gasteropodi marini destinati al consumo umano)

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Index

Abstract

According to the European Legislation, marine gastropods placed unprocessed on the market must comply with the same requirements established for live bivalve mollusks. *Tritia mutabilis* and *Bolinus brandaris* are scavengers and carnivorous, respectively, edible marine gastropods. It is known that gastropods have an open circulatory system and could accumulate bacteria in their flesh, but little is known in the literature about their food safety for consumers.

The present thesis aims to evaluate a method to assess the viability; estimate the bacterial and viral (Hepatitis A and Norovirus) contamination; describe how some parameters change during a week in refrigerated condition and after 24 hours of immersion; estimate indole-producing bacteria and biogenic amines; evaluate the presence of saxitoxin and tetrodotoxin.

The method to assess the viability using sea salt is easy to apply. Marine gastropods did not accumulate fecal contaminants, but vibrios due to their feeding. The *Vibrio* spp. load was 5.44 ± 0.70 $\log_{10}$ CFU g^{-1} for *T. mutabilis* and 6.52 $\log_{10}$ CFU g^{-1} for *B. brandaris* and is notable because even higher than the average *Vibrio* spp. load registered on *Ruditapes philippinarum* belonging to the same sea area (4.69 $\log_{10}$ CFU g^{-1}). Moreover, the bacteria did not be eliminated during re-immersions, in contrast to Bivalvia. For what to concern the evaluation during a week in refrigerated condition and after 24 hours of immersion, non-re-immersed gastropods exceeded the acceptable mortality (10%) after three days in refrigerated conditions, but the *Vibrio* spp. load did not show a significant increase within three days. The TVC was already high from the beginning (more than 5 $\log_{10}$ CFU g^{-1}) and its major part consisted of SSOs, which could be explained by gastropods' feed, such as the *Pseudomonas* spp. load and the abundance of IPB. The BAs amount partially could be explained in the same way, but it was also correlated with viability and had a statistically significant difference within a week on refrigerated conditions, principally because putrescine, tyramine, spermidine, and cadaverine rise in non-re-immersed samples, while remained constant in re-immersed samples. It also should be noted that the BAs amount was higher on average than the recommendation of literature (50 mg kg^{-1}). Moreover, re-immersed batches showed acceptable viability even after 3 days, and the *Vibrio* spp. load, TVC, SSOs, and biogenic amines remained almost constant (except for a decrease of spermine) within a week contrary to non-re-immersed samples. Finally, *T. mutabilis* and *B. brandaris* did not accumulate NoVs and TTX. We obtained only one positivity of the HAV sample and traces of STX (not at levels toxic to humans).

Our results contribute to identifying food-borne hazards for *T. mutabilis* and *B. brandaris* that may be related to the accumulation of biogenic amines and indole-producing bacteria due to carrion feeding. Moreover, suggests that the usage of sea salt is easy and successful to assess the viability and the BAI index could be used as a safety target. Moreover, the post-harvest treatment of re-immersion could extend the shelf-life and keep constant the microbiological and toxicological parameters.

Section 1 – Introduction

1. Gastropoda

Gastropoda belongs to the phylum Mollusca and is the wider and more varied class, including almost 70.000-80.000 species living in freshwater, marine, and terrestrial ecosystem [Bouchet et al., 2005; Hickman et al., 2012; Lobo-de-Cuhna, 2019]. Gastropods range in size from less than 1 mm to almost 1 m and can be grazers, browsers, suspension-feeders, carnivores, predators, scavengers, detritivores, and parasites [Hickman et al., 2012; Lobo-de-Cuhna, 2019; Manzoni, 2010; Santhanam, 2019]. The class Gastropoda is divided into three subclasses: Pulmonata, Prosobranchia, and Opisthobranchia [Smolowitz, 2012]. *Tritia mutabilis* and *Bolinus brandaris* belong to the second subclass.

1.1 Torsion

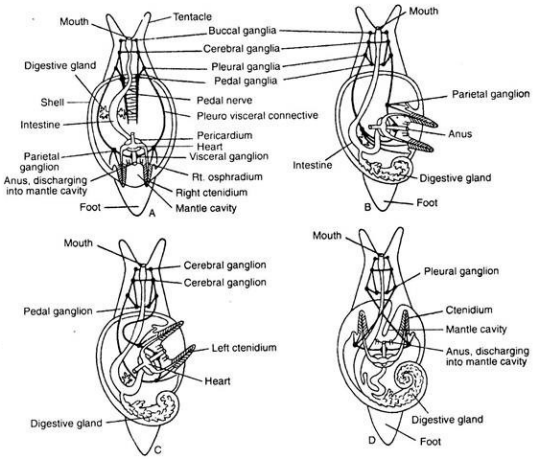

Figure 1.1: Representation of Gastropods' torsion. A. Hypothetical ancestor with symmetrical structure. B. Displacement of the mantle cavity to the right side. C. 90° torsion. D. Complete torsion [w1].

Gastropods are distinguished from other mollusks by the 180° torsion of visceral mass during the larval stage [Lobo-de-Cuhna, 2019; Manzoni, 2010; Santhanam, 2019]. In fact, during the veliger stage, a symmetrical larva is converted into an asymmetrical adult due to different growth rates and contractions of muscles, connecting the shell and the foot [Hickman et al., 2012; Kurita and Wada, 2011]. In

the beginning, larvae have a straight alimentary canal, with an anterior mouth and a posterior anus, and a mantle cavity; in addiction, ctenidia and nephridiopores are situated posteriorly [Hickman et al., 2012; w1]. Afterward, two rotations are followed: a first one of 90°, which is usually shorter, and a second for reaching 180° [Hickman et al., 2012]. Finally, the mantle cavity, anus, ctenidia, and nephridiopores are placed anteriorly, nerve cords are twisted in the form of 8 and all the left side of visceral mass becomes topographically right and vice versa [Falniowski, 1993; Ghiselin, 1966; Hickman et al., 2012; Lobo-de-Cuhna, 2019; w1].

1.2 Shell

Shells have usually three layers. The periostracum, the outermost layer, is thin and proteinaceous and its function is to protect gastropods from boring organisms and abrasions. The central layer is the prismatic one and it is made of solid calcium carbonate precipitate into conchiolin. The mother-of-pearl layer is the innermost and is composed of calcium carbonate's laminae immersed into a proteic matrix [Fleury et al., 2008; Grégoire, 1972; Hickman et al., 2012; Webster and Palmer, 2018; Santhanam, 2019].

The protoconch is an embryonic stage of the gastropod shell and forms the apex of the spire, which is constituted by all the whorls (a whorl is a complete coil), except the body one, that is larger than the rest joined together. Whorls are connecting with a continuous line, which is called the suture. The main opening of the shell is known as the aperture which is surrounded by the inner and outer lip and can have two notches: an anterior is called the siphonal canal, and a posterior is the anal canal. The operculum is a hard structure that is attached to the dorsal part of the foot and is used to close the aperture when gastropods withdraw into their shells. Inside there is a central pillar named columella [Manzoni, 2010; Sangeeta, 2017; Santhanam, 2019].

1.3 General anatomy and physiology

The soft parts of gastropods may be separated into the foot, head, visceral mass, and mantle [Smolowitz, 2012].

The foot of Gastropods is provided with secretory epithelium and cilia to secrete mucus and pedal muscles to allow the animal locomotion [Brown and Lydeard, 2010]. The foot muscle is smooth muscle, and its cells may occur singly or be arranged in muscle blocks and commonly have numerous mitochondria to allow

pedal escape movements that are presumably energetically expensive [Chaparro et al., 1998; Trueman and Hodgson, 1990].

In the subclass Prosobranchia, the mantle cavity contains the head, anus, gill, and penis or oviduct opening and ovipositor [Brown and Lydeard, 2010; Smith, 2016]. The gill contains only one ctenidium with leaf-like triangular plates, that commonly have four ciliary fields: lateral (which provides ventilation) and frontal, abfrontal, terminal (which together handle fine particular matter) [Brown and Lydeard, 2010; Lindberg and Ponder, 2001; Rodriguez et al., 2019; Smolowitz, 2012].

Anteriorly the ctenidium, all prosobranchs have a gland termed as osphradium, whose function is chemoreception [Beninger et al., 1995; Brown and Noble, 1960; Smolowitz, 2012]. In Neogastropoda it is a large and bipectinate gill-like organ with a simple chemosensory structure (a primary sensory neuron within the sensory epithelium) [Emery, 1992; Lindberg and Ponder, 2001]. Caenogastropods possess eyes at the base of their tentacles, which also have the function of chemoreception and the same chemosensory pattern of osphradium [Brown and Lydeard, 2010; Copeland, 1918; Emery, 1992].

The mantle covers the visceral mass, underlays the shell (which it secretes), and as a prolongation termed siphon, that has skin chemoreceptors [Brown and Lydeard, 2010; Copeland, 1918; Emery, 1992]. Siphon commonly possess ciliated sensory cells, which are sensitive to contact chemical stimuli, and, on its tip, it has photoreceptors, which probably are an adaptive value in animal periods of inactivity during which gastropod lie buried in the sand with only the siphon above the surface [Crisp, 1971].

The digestive tract can be divided into buccal mass, esophagus, stomach, and intestine.

The muscular buccal mass is usually on a proboscis, which can be completely o partially retracted, and consists of a mouth and a pharynx [Smolowitz, 2012]. The lateral walls of the pharynx are lined by the odontophore, which contains single pair of cartilages and is almost lost in Neogastropoda [Katsuno and Sasaki, 2008]. The radula, which covers the odontophore, is a ribbon-shaped structure bearing chitinized teeth and can be protruded from the buccal cavity when gastropod eats [Brown and Lydeard, 2010; Paoulayan and Remigio, 1992/1993]. The salivary glands are usually parallel to the esophagus and secrete mucus fluid and compounds (cysteine-rich

glycoprotein in some Nassariidae and Muricidae) into the buccal cavity to facilitate the intake of food [Gawad et al., 2018; Ponte and Modica, 2017]. Two types of salivary glands are known: tubular (accessory) and acinous (primary) glands; neogastropods are characterized by the possession of tubular salivary glands in addition to the single pair of acinous glands [Andrews, 1991]. The secretory epithelium of primary glands includes two cell types: superficial ciliated cells secreting mucus and basal cells with apocrine secretion, while accessory salivary glands have a columnar secretory epithelium surrounded by a richly innervated sub-epithelial muscular coat [Ponte and Modica, 2017].

The esophagus is a thin-walled tube connecting the buccal mass to the stomach and is characterized by a wide bend due to the gastropod torsion [Paoulayan and Remigio, 1992/1993]. In *Tritia* spp. the posterior part of the esophagus does not constitute a crop and in *Tritia reticulata* it consists of two regions: the anterior part is whitish and flaccid, while the posterior part is plump with an opaque creamy orange color [Payne and Crisp, 1989]. The esophagus contains mucous, ciliated, and secretory cells [Harris et al., 1998]. In Neogastropoda it is also present an absorptive and secretory gland of Leiblein with a valve (termed the valve of Leiblein), that is a pear-shaped structure that consists in a glandular chamber containing a cone-shaped valve [Lobo-da-Cunha, 2019].

The stomach is a maroon sac-like organ partly embedded in a digestive gland and plays a role both in digestion and in waste rejection [Harris et al., 1998; Lobo-da-Cunha, 2019; Paoulayan and Remigio, 1992/1993]. Its epithelium contains ciliate and unciliated columnar cells alternated with secretory cells and overlying a muscular lawyer [Harris et al., 1998; Lobo-da-Cunha, 2019]. The digestive gland is a greenish acinous structure being formed by blind-ending tubules (termed digestive diverticula) linked by a system of branch ducts [Lobo-da-Cunha, 2019; McLean, 1971; Paoulayan and Remigio, 1992/1993]. It primarily consists of basophilic and digestive cells, that have the function of secrete enzymes for extracellular digestion and digest food, respectively [Arrighetti et al., 2015; Lobo-da-Cunha, 2019].

The intestine makes several loops and ends into the mantle cavity behind the head [Brough and White, 1990; Paoulayan and Remigio, 1992/1993]. It commonly changes color throughout its length switching from beige to almost black and then light-brown/whitish color [Harris et al., 1998]. The intestine wall included an epithelium (mainly composed of tall ciliated columnar absorptive cells which also

may possess microvilli), connective tissue, and one or two muscular layers [Loboda-Cunha, 2019; Pfeiffer, 1992; Zaitseva and Filimonova, 2006].

The kidney is the major organ of excretion and, in aquatic gastropods, consists of one nephridium (divided into two lobes and formed by trabeculae lined by columnar cells), a nephridial lumen, and a nephridiopore, which opens into the mantle cavity [Paoulayan and Remigio, 1992/1993; Smolowitz, 2012]. The urine of aquatic gastropods commonly contains ammonia because it is extremely soluble in the surrounding water [Brown and Lydeard, 2010].

The circulatory system is open, and the heart consists of a single auricle and a single ventricle surrounding the pericardium [Bocxlaer and Strong, 2016; Paoulayan and Remigio, 1992/1993; Smolowitz, 2012]. The hemolymph is distributed from the ventricle to all body by the anterior and posterior aortae that further divide in open arterial sinuses (with an incomplete epithelium) and then, from veins, the hemolymph returns to the heart via the kidney and gill [Voltzow, 1985].

The nervous system is formed by aggregates of paired ganglia including cerebral, buccal, pedal, visceral, pleural, supraintestinal, and subintestinal ganglia [Chase, 2002; Smolowitz, 2012; Voronezhskaya and Croll, 2016]. The cortex of gastropod ganglia is composed of several layers of the somata of primarily unipolar neurons, which project their neurites into the neuropil (the central region of a ganglion) [Voronezhskaya and Croll, 2016].

Most prosobranchs have internal fertilization and are gonochoristic with females being commonly larger [Brown and Lydeard, 2010; Mallet et al., 2021; Smolowitz, 2012]. The coloration of mature gonads is in the specific section of *T. mutabilis* and *B. brandaris*. The microscopic stages are commonly divided in: (0) immature: gonads have little detailed internal structure and show groups of primordial germ cells dispersed in a large and loose matrix of connective tissue; (1) pre-active: few separated follicles and tubules are embedded in connective tissue and the cytoplasm comprises few spermatocytes and pre-vitellogenic oocytes in males and females respectively; (2) active: the connective tissue is reduced and the follicles become more packed; in males diverse spermatogenic stages are visible (spermatids, spermatocytes and a few spermatozoa) while in females vitellogenic oocytes appear at the periphery of follicles; (3) ripe: the connective tissue disappears and the follicles and tubules become highly compressed and more grouped; in males tubules are distended with spermatozoa particularly in the lumen (although spermatogenesis still

occurs near the wall), while in females the follicles are full of mature oocytes packed with yolk granules (although a few half-grown previtellogenic oocytes occurs near the wall); (4) partially spent: the lumen of follicles become emptier and surrounded by some connective tissue, but spermatozoa and ripe oocytes are still present in it; (5) spent: tubules and follicles are dispersed in loose network of thicker connective tissue and most of them are empty, even if a few may contain some mature oocytes and spermatozoa in the lumen [Barroso and Moreira, 1998; Elhasni et al., 2013; Ramón and Amor, 2002; Vasconcelos et al., 2012b; Wu et a., 2019].

In males, the sperms, formed into the testis, flow through the vas deferens (which is connected to the bilobated prostate and where the sperm is embedded in prostatic secretion), which leads to the penis [Abidli et al., 2009; Lobo-da-Cunha et al., 2018; Smith, 1980]. The prostate includes subepithelial glands with long ducts opening between the columnar ciliated epithelium, which continues through the vas deferens [Smith, 1980]. The vas deferens has abundant vacuolar cells beneath the ciliated epithelium and is encircled by a thick muscular wall. The penis is a muscular structure with a ciliated epithelium above densely packed vacuolar cells [Lobo-da-Cunha et al., 2018].

In females, the ovary is followed by the gonadial oviduct, which is divided into three sections (all embedded in a single mass of parenchymal tissue): the albumen gland/capsule gland complex (formed by the white opaque albumen gland and the creamish yellow capsule gland), the bursa copulatrix and the seminal receptacles; lastly, the oviduct is linked to a genital orifice, where the eggs are deposited [Catalán et al., 2006; Hershler and Davis, 1980; Rajalakshmi Bhanu et al., 1982]. The albumen gland synthesizes and secretes perivitelline fluid that provides nutrients to the embryos and consists of columnar secretory cells with short microvilli interspersed between ciliated labyrinthic cells, while the capsule gland stores a large amount of calcium that later forms the eggshell and includes groups of gland cells packed together with a thin layer of connective tissue [Cadierno et al., 2018; Catalán et al., 2006; Goldsmith et al., 1978; Rajalakshmi Bhanu et al., 1982]. The bursa copulatrix is an organ for the selection and digestion of excess or immotile sperm received during mating and internally is lined by a layer of columnar epithelium above several layers of visceral muscles interspersed with collagen bundles [Clelland et al., 2001; Kunigelis and Saleuddin, 1986]. The seminal receptacles is an organ for the temporary storage of sperm to allow gonads to release mature eggs and consists

of an internal single layer of epithelium and external muscular layers separated by a thick layer of collagen masses immersed in a fine granular matrix [Beninger et al., 2016; Giusti and Selmi, 1985].

Muricid gastropods possess the hypobranchial gland, which secretes not only mucus for trapping and cementing particulate matter sucked into the mantle cavity to its expulsion, but also biologically active compounds (including chromogens). It consists of several secretory cell types interspersed along with ciliated supportive cells [Naegel and Aguilar-Cruz, 2006; Westley et al., 2010].

2. *Tritia mutabilis* (Linnaeus, 1758)

2.1 Taxonomy

Subclass Caenogastropoda
Order Neogastropoda
Superfamily - Buccinoidea
Family - Nassariidae
Subfamily - Nassariinae
Genus - *Tritia*
Species - *Tritia mutabilis*

It was described by Linnaeus as *Buccinum mutabile*, since then the taxonomy has been changed multiple times; hence, there are several different names in the literature, such as *Buccinum foliosum*, *B. gibbum*, *B. jaspideum*, *B. rufulum*, *B. tessulatum*, *Nassa ebenacea*, *N. gibba*, *N. helvetica*, *N. mediterranea*, *N. mutabilis* (and its variations), *Nassarius mutabilis*, *Sphaeronassa adriatica*, *S. deformis*, *S. globulina* e *S. umbilicate* [Galindo et al., 2016; Molluscabase eds., 2020a; w2]. Even if it is unaccepted [Molluscabase eds., 2020b], the most common synonym is *Nassarius mutabilis*, enough to be the official scientific name in Italy [D.M. 19105/17].

Common English names are *mutable nassa* [Piroddi et al., 2015; Santhanam R., 2019; w2] or *changeable nassa* [Caprioli and Giansante, 2018; FAO FIES, 2019; Grati et al., 2010; Manzoni, 2010] and the Italian one is *lumachino* [D.M. 19105/17; Manzoni, 2010].

2.2 Description

The shell is ovate, smooth, and swollen. It is composed of seven whorls, of which the body whorl is larger than the rest joined together. In the spire, the three upper whorls are finely plaited. All the sutures are pretty evident and there are few, slightly-in-relief striae in the shell. The aperture is white, large, ovate, and has a short, deep canal. The outer lip is finely striated internally, and the inner is shining and partially covers the shell. The arcuated columella has a thick callosity and terminates at the base with a lightly projecting keel [Manzoni, 2010; Santhanam, 2019].

The external coloring of the shell is variable from fawn to reddish-brown with brownish or yellow flames and a band upon the upper edge of the whorls with white and violet lines. The internal is porcelain or chestnut-colored [Manzoni, 2010; Santhanam, 2019].

The maximum size is 38 mm [Manzoni, 2010] with a diameter of 23 mm [Fisher et al., 1987] and the common adult size is between 14 and 30 mm [Santhanam, 2019]. However, in the Adriatic Sea, the more frequently observed sizes ranged between 17 and 25 mm [Piccinetti and Piccinetti Manfrin, 1998].

2.3 Global distribution and habitat

T. mutabilis is a common nassarid in the Mediterranean Sea and the southern coast of Portugal until the Strait of Gibraltar [Fisher et al., 1987; Manzoni, 2010]. It is usually found in sandy or muddy bottoms from the tidal range to a depth of 15 meters [Minelli et al., 2006; Fisher et al., 1987; Santhanam, 2019]. *T. mutabilis* does not shift so far from an original place; Balducci (2011) notes an average displacement of 24 m/d with a maximum movement of 1 nmi from the placing point. Anyway, males are usually found in shallower waters than females, but, during the reproduction time, all of them migrate to deeper waters [Solustri et al., 2002].

2.4 Biology

This gastropod spends most of the day buried into the sand or mud [Caprioli and Giansante, 2018; Polidori et al., 2015; Santhanam, 2019]. It is a nocturnal animal, and it emerges only to seek food or in response to predators [Kohn, 1961; Polidori et al, 2015; Solustri et al., 2002].

Adults are scavengers, which means they normally feed on dead organisms [Polidori et al., 2015; Santhanam, 2019]; moreover, the genus *Nassarius* is famous to

gather over dead fish or crustaceans [Kohn, 1961]. Juveniles, instead, feed on a detritus-rich substrate [Polidori et al., 2015].

T. mutabilis has different predators, for example in the Tyrrhenian Sea *Xyrichtys novacula* (fish) [Cardinale et al., 1997] or in the Adriatic Sea *Liocarcinus vernalis* (crustacean), *Gobius niger* (fish), and *Naticarius stercusmuscarum* (gastropod) [Grati et al., 2010]. However, sea stars are the most known, because changeable nassas have a special escape route: they leap following a zigzag line [Feder, 1963; Kohn, 1961; Santhanam, 2019].

2.5 Reproduction

T. mutabilis was considered a proterandrous hermaphrodite, in fact, previous papers have found that there is a sex reversal at 20 mm (approximately) of shell length from male to female [Minelli et al., 2006; Polidori et al., 2015; Solustri et al., 2002] but recently, Mallet et al. (2021) contradicted this and affirmed that it is gonochoristic. The fertilization is internal [Caprioli and Giansante, 2018; Santhanam, 2019] and the reproductive period is from late winter to spring [Minelli et al., 2006; Polidori et al., 2015]. The spawning and the deposition are influenced by temperature and salinity: this is the reason why there are geographical and annual variations [Barroso and Moreira, 1998] and why the reproductive period correspond to a rise in the water temperature [Caprioli and Giansante, 2018].

Ripe gastropod's gonads are easily distinguished: males have an orange testis and a white vesicula seminalis expanded by mature sperm, while females have a large white capsule gland with red lips and a fawn ovary [Barroso and Moreira, 1998].

Females depose smooth and transparent egg capsules with 6-14 embryos per capsule [Caprioli and Giansante, 2018; Minelli et al., 2006] on hard substrates, such as stones, algae, or seagrasses [Polidori, 2015; Santhanam, 2019]. Each capsule is originally spherical, but the gastropod gives it an almond shape with its foot's movements [Zupo and Patti, 2009] and it also has complex lateral ridges and spines [Caprioli and Giansante, 2018]. This last peculiarity and the number of embryos distinguish the capsules of *T. mutabilis* from the ones of *T. reticulata* [Caprioli and Giansante, 2018; Zupo and Patti, 2009].

Veliger larvae hatch from benthic capsules and start their pelagic life, which will last for approximately two months to complete all the metamorphosis [Caprioli and Giansante, 2018; Polidori et al., 2015; Zupo and Patti, 2009], even if its duration

is influenced by temperature [Massé et al., 1977]. The life cycle is completed in 2-3 years [Caprioli and Giansante, 2018; Fiori, 2011; Gramitto, 2001].

2.6 Fishery

T. mutabilis is appreciated among the local population [Manzoni, 2010] and is an important resource in the Adriatic Sea for the small-scale fishery [Caprioli and Giansante, 2018; Fabi et al., 2006; Grati et al, 2010; Polidori et al., 2015]: between 2004 and 2011 the average of the gastropods' total catches was of 4.185 tonnes, that also corresponds to 7,6% of the average of the mollusks' total catches and 1,7% of the total catches' one [IREPA, 2012]. In addition, in Emilia-Romagna gastropods represented 8,4% of the total catches and 11,9% of the total revenues in the year 2011 [IREPA, 2012].

Fishing activity is conducted from the beginning of autumn to the end of spring, using cone-shaped basket traps (exclusively for this gastropod) baited with dead fish [Caprioli and Giansante, 2018; Minelli, 2006; Polidori, 2015]. The Italian legislation states that 20 mm is the minimum fishing size of *T. mutabilis* [D.M. 30/11/96]

The value of *T. mutabilis* was, on average, 4.03 €/kg between 2004 and 2011 [IREPA, 2012].

3. *Bolinus brandaris* (Linnaeus, 1758)

3.1 Taxonomy

Subclass Caenogastropoda
Order Neogastropoda
Superfamily - Muricoidea
Family - Muricidae
Subfamily - Muricinae
Genus - *Bolinus*
Species – *Bolinus brandaris*

In 1758 Linnaeus described it as *Murex brandaris*. However, in following years, due to its morphological variety, new synonyms were established, by changing

the genus (for example *Aranea cinera*, *Haustellum clavatum*, and *Purpura fuliginosa*), modifying species (for instance: *Murex clavisherculis*, *M. coronatus*, *M. girisus*, *M. monospinosus*, *M. moreanus*, *M. pseudobrandaris*, *M. subbrandaris*, *M. trifariaspinosus*, *M. tuberculatus*, and *M. tudiculoides*) or introducing a different variation of *M. brandaris*, like *aculeata*, *adunca*, *bicaudata*, *bifida*, *canaliaspinosa*, *cingulata*, *delicatula*, *ecaudata*, *fragilis*, *gigantea*, *intermedia*, *longispina*, *multicostata*, *nodosa*, *novemcostata*, *polii*, *spinotuberculata*, and *trifariaspinosa* [MolluscaBase eds., 2020c; Russo P., 2017]. Now, they are all unaccepted, but only the subspecies *Bolinus brandaris brandaris* remains [MolluscaBase eds., 2020d].

The common English name is *purple dye murex* [FAO-FIES, 2019; Santhanam, 2019] and the Italian one is *murice spinoso* [D.M. 19105/17; Manzoni, 2010].

3.2 Description

The shell is club-shaped with visible growth shutdowns, in which there are three laps of spines: one on whorls' upper part, another visible only in the middle of the last whorl, and the last (less marked) on the siphonal canal. Spines are more pronounced in juveniles, whereas the outer shell surface remains rough throughout muricid's life. The spire consists of low compact whorls, of which only the body whorl is swollen and shows a lengthy, straight siphonal canal. The aperture is large, brilliant, and ovate. The columella is callous, and its border is well-developed. The operculum is corneous and concentric [Fisher et al., 1987; Manzoni, 2010; Santhanam, 2019; w3].

The external shell color ranges from yellow to brownish, while the internal is reddish-brown [Manzoni, 2010; Santhanam, 2019].

Fisher et al. (1987) and Manzoni (2010) claim that the maximum shell size of *B. brandaris* is 92 mm with a diameter of 66 mm, however, Vasconcelos et al. (2016) have found specimens of 107.7 mm and Bañón et al. (2008) and Abidli et al. (2012) affirm also that the total shell length can be of 120 mm. Anyway, an adult's shell length is on average between 60 and 100 mm [Santhanam, 2019].

3.3 Global distribution and habitat

B. brandaris is one of the most common and abundant gastropod species of Mediterranean Sea and Atlantic Ocean from Portugal to northern Morocco [Manzoni,

2010; Ramón and Amor, 2002; Vasconcelos et al., 2008; Santhanam, 2019]. It inhabits sandy and muddy bottoms and occurs usually in the sub-littoral zone but can also be found up to 200 m [Abidli et al., 2012; Bañón et al., 2008; Martín et al., 1995; Santhanam, 2019; Vasconcelos et al., 2012a; Vasconcelos et al., 2016].

3.4 Biology

Muricidae are usually carnivorous, feeding on small worms, crustaceans, barnacles, shellfish, or other gastropods [Bertolino and Ferranti, 2018; Ramón and Amor, 2001; Mylona, 2015; Smith et al., 2019; Vasconcelos et al., 2012a]; in addition, they are famous for piercing mollusks' shell with their radula and making a characteristic hole on them [Bertolino and Ferranti, 2018; Mylona, 2015]. *B. brandaris* can also be a scavenger and a cannibal, feeding on smaller individuals [Bertolino and Ferranti, 2018; Mylona, 2015; Smith et al., 2019; Vasconcelos et al., 2012a].

It has been used for extracting the purple dye since the time of the Roman Empire [Abidli et al., 2012; Russo, 2017; Vasconcelos et al., 2012b]: in fact, its hypobranchial gland produces a colorless or yellowish pigment, that oxidizes when exposed to air or light, producing the characteristic coloring [Deshpande, 2002; Poli and Fabbri, 2012; Soufia et al., 2014]. Moreover, the same gland produces the murexine, a paralytic toxin for skeletal muscles in both prey and human [Poli and Fabbri, 2012; Soufia et al., 2014]: in fact, it has a neuromuscular blocking (acting by depolarization) and a nicotinic action (which raises blood pressure and produces local vasodilation) [Deshpande, 2002; Erspamer and Glässer, 1957; Rand and Stafford, 1967; Winbury, 1957]; both actions can cause death by secondary anoxia due to respiratory arrest [Erspamer and Glässer, 1957]. Despite this, its toxicity is not high, in fact, milligrams are necessary to cause lethal effects in adults patients intravenously [Erspamer and Glässer, 1957; Poli and Fabbri, 2012] and there was only a serious outbreak at the beginning of the 19th century, in which 43 persons were poisoned and 5 of them died after eating *B. brandaris* around the Gulf of Trieste [Deshpande, 2002; Halstead, 1965].

3.5 Reproduction

B. brandaris is a gonochoric species and has internal fertilization [Abidli et al., 2012; Vasconcelos et al., 2011; Vasconcelos et al., 2012b]. The reproduction

cycle is annual with a long period of spawning and a short resting phase between August and October [Ramón and Amor, 2002], but the peak of deposition and the length of the resting period are influenced by seawater temperature, photoperiod, and imposex, hence there are variations in different sites of Mediterranean Sea [Abidli et al., 2012; Elhasni et al., 2013; Santhanam, 2019; Vasconcelos et al., 2008; Vasconcelos et al., 2011]. In general, the main spawning periods occur in summer from May to July [Santhanam, 2019; Vasconcelos et al., 2008], however, Ramón and Amor (2002) have found two peaks, even if in April the number of immature females has been high, so it has been considered less important.

Females and males cannot be distinguished by external characters [Vasconcelos et al., 2011], and they usually have the same ripe size [Abidli et al., 2012; Vasconcelos et al., 2012]. Elhasni et al. (2013) have demonstrated that sexual maturation's size is slightly smaller in males (54,6 mm) than in females (56,4 mm), whereas, on the contrary, Ramón and Amor (2002) have shown that females have signs of gametogenic activity in a shorter shell length (26,87 mm, while males 29,17 mm). This can be explained because it has been found a slight asynchrony both in gonad development and gamete release due to seawater temperature and females' ability to store viable sperm after copulation [Abidli et al., 2012; Elhasni et al., 2013].

However, it is easy to distinguish between ripe females and males looking at the color of the gonad: indeed, females have a pinkish ovary and a white capsule gland, whereas males have a yellowish testis [Abidli et al., 2009; Ramón and Amor, 2002].

During spawning, females usually create aggregations and lay oothecae (namely capsules that enclose eggs), attached to each other, forming a protective mass fixed to solid substrata [Abidli et al., 2012; Elhasni et al., 2013; Ramón and Amor, 2002; Vasconcelos et al., 2011]. The growth of *B. brandaris* is influenced by currents, predation, and degree of pollution [Abidli et al., 2012], and it is normally considered a slow-growing muricid, with a typical growth of 1-2 mm in shell length per month, that can decelerate with increasing size/age [Vasconcelos et al., 2012a]. On the other hand, it seems to reach marketable size in approximately two years [Vasconcelos et al., 2012a].

3.6 Fishery

B. brandaris is a common artisanal fishery target in the Mediterranean Sea, especially along the coasts of Spain, Portugal, Italy, France, Greece, and occasionally Turkey and Tunisia [Abidli et al., 2012; Elhasni et al., 2013; Manzoni, 2010; Ramón and Amor, 2001; Ramón and Amor, 2002; Vasconcelos et al., 2008; Vasconcelos et al, 2012a; Vasconcelos et al., 2012b]. It is usually caught using trammel nets, basket traps, and dragged gears, of which "rastell" was specially designed of the muricid [Martín et al., 1995; Ramón and Amor, 2002]. In Portugal, it was used the "wallet-line" gear, but now it is illegal, due to a high amount of by-catch species [Vasconcelos et al., 2008]. *B. brandaris* is also the most common shellfish species caught ad by-catch by bottom trawlers [Elhasni et al., 2013].

This muricid is appreciated in western coasts of the Mediterranean Sea [Manzoni, 2010] with a high commercial value in Portugal and Spain, reaching 20-25 € kg^{-1} [Elhasni et al., 2013; Santhanam, 2019; Vasconcelos et al., 2008; Vasconcelos et al., 2012a]. However, in Tunisia, its consumption and value are increasing, because it is prohibited the collection of *Ruditapes decussatus* on account of biotoxins' presence [Abidli et al, 2012], and fisheries will be enhanced for exportation in foreign countries [Elhasni et al., 2013].

4. Legislation

Regulation (EC) No 853/2004 defines fishery products as "*all seawater or freshwater animals (except for live bivalve molluscs, live echinoderms, live tunicates and live marine gastropods, and all mammals, reptiles and frogs) whether wild or farmed and including all edible forms, parts and products of such animals*". Live marine gastropods intended for human consumption are included in products of animal origin as well as live bivalve mollusks, live echinoderms, and live tunicates [Reg. 853/2004/EC; Reg. 625/2017/EU].

Live marine gastropods are usually assimilated to live bivalve mollusks in terms of safety criteria for human consumption with exceptions: the purification is not applied to marine gastropods [Reg. 853/2004/EC], and, in addition, "*the classification of production and relaying areas is not required in relation to the harvesting of Pectinidae, marine gastropods and Holothuroidea, which are not filter feeders*" [Reg. 624/2019/EU]. The same concept is resumed in Regulation (EU) No

627/2019 and explained in Regulation (EU) No 558/2010: they seem to be incapable of concentrating fecal contaminants, such as *E. coli*.

According to Reg. 624/2019/EU reprising Reg. 853/2004/EC, marine gastropods must be placed on the market alive, specifically "*they must have organoleptic characteristics associated with freshness and viability, including shells free of dirt, an adequate response to percussion and normal amounts of intravalvular liquid*". Evidently, this applies only to bivalve mollusks, but the topic will be addressed in the specific chapter. Moreover, marine gastropods must not exceed particular limits of marine biotoxins [Reg. 853/2004/EC; Reg. 786/2013/EU; Reg. 624/2019/EU; Reg. 1374/2021/EU] and, here too, the subject will be deepened in the specific section.

Gastropods must follow two food safety criteria. Firstly, *Salmonella* must be absent in 25 g, following EN/ISO 6579 [Reg. 2073/2005/EC]. Secondly, as regards *E. coli*, gastropods are considered safe "*if all the five values observed are ≤ 230 MPN/100 g of flesh […] or if one of the five values observed is > 230 MPN/100 g of flesh […] but ≤ 700 MPN/100 g of flesh*", according to EN/ISO 16649-3 [Reg. 2285/2015/EU].

Like bivalve mollusks, marine gastropods must be accompanied by a registration document, that must contain the following information: the gatherer's identity and address; the date of harvesting; the location of the production area described in as precise detail as is practicable or by a code number; the health status of the production area; the shellfish species and quantity; and the destination of the batch [Reg. 853/2004/EC]. The document follows the batch between establishments.

Marine gastropods must be placed on the market for retail sale via a dispatch center and once they leave it, individual costumer-size packages must remain closed until presented for sale to a final consumer [Reg. 853/2004/EC]. The waterproof label must include: the species of bivalve mollusk (common name and scientific name); production method; the area where the product was caught or farmed; the date of packaging, comprising at least the day and the month; the country in which the establishment is located; the approval number of the establishment; the march CE EC, EF, EG, EK or EY; net weight; and the entry "these animals must be alive when sold" [Reg. 853/2004/EC; Reg. 1379/2013/EU].

5. Issues and challenges

In the last twenty years, *T. mutabilis* population and, consequently, catch rates have declined due to several aspects [Caprioli and Giansante, 2018; Grati et al., 2010; Fabi et al., 2006; Minelli et al., 2006; Polidori et al., 2015].

Firstly, females lay egg capsules on hard substrates, which are not frequent in the Adriatic Sea [Caprioli and Giansante, 2018]; consequently, they spawn on basket traps, but the eggs are not always discarded by fishermen and even if they are, they need to find another hard substrate to survive. In addition, encapsulated larvae are preyed on by crabs or hermit crabs, which represent a further threat [Caprioli and Giansante, 2018].

Secondly, *T. mutabilis* occupies the same ecological niche as *T. reticulata* [Minelli et al., 2006], which is usually discarded by fishers on account of its little commercial value. The fishing effort and the continuous re-entry of *T. reticulata* lead to the changeable nassa decrease and this antagonistic species increase [Fabi e Grati, 2004; Fabi et al., 2006]; hence, *T. mutabilis* is disadvantaged in the interspecific competition.

Thirdly, in the literature, it is well-know that prosobranch gastropods, e.g. *B. brandaris* [Abidli et al., 2009; Ramón and Amor, 2002; Vasconcelos et al., 2011], *T. reticulata* [Barreiro et al., 2001; Barroso et al., 1998; Minelli et al., 2006] and *T. mutabilis* [Lahbib et al., 2013], are afflicted by pseudohermaphroditism or imposex, which is defined as a "*superimposition of male sexual characters […] on normal female*" [Abidli et al., 2009]. This condition is the consequence of a sub-lethal exposition to pollutants and anti-fouling products, especially tributyltin (TBT) and triphenyltin (TPT), which have been used worldwide in boats and pipes [Abidli et al., 2009; Minelli et al., 2006; Vasconcelos et al., 2011]. Imposex leads to a reproduction decrease because it may cause female sterilization [Barreiro et al., 2001]; therefore, this is a further threat to the conservation of the stock.

Lastly, management measures (minimum landing size, maximum daily catch amount, and fishing season) are not enough to preserve this resource [Caprioli and Giansante, 2018; Polidori et al., 2015]. In fact, basket traps are not selective and undersized individuals are not discarded into original fishing grounds, but usually far from them (sometimes even inside fishing harbors), where water quality is poor and seabed substrate may be unsuitable: this could decrease the survival rate [Grati et al.,

2010]. In addition, fishermen are prone to retain and land marine gastropods, in event of low catches [Grati et al., 2010].

Furthermore, there are other issues in the context of legislation. First of all, according to D.M. 19105/17, *T. mutabilis* and *T. reticulata* have an identical commercial name and, as well, the netted dog whelk does not have an alpha 3 code. In this manner, a small number of specimens of *T. reticulata* may be found in the same packages of the changeable nassa.

Moreover, individual costumer-size packages must remain closed until the final customer [Reg. 853/2004/EC], but they could be opened and relabeled between dispatch centers. Nevertheless, the label reports only the packaging date [Reg. 853/2004/EC], so the final customer can only see the last date of packaging and cannot know the landing one. This could be a big problem if it is coupled with the fact that there is not an official method to verify the viability.

Finally, marine gastropods were defined as "generally not filter feeder animals" (Reg. 558/2010/EU), but in the new regulations, they are distinguished in filter feeder and not filter feeder [Reg. 624/2019/EU]. *T. mutabilis* is a scavenger [Polidori et al., 2015] and *B. brandaris* is carnivorous [Ramón and Amor, 2001], so it may be harvested in not-classified areas [Reg. 624/2019/EU]. It is known that gastropods have an open circulatory system and could accumulate bacteria in their flesh [Jones, 1983; Narain, 1976; Smolowitz, 2012]. However, *E. coli* has been found in *Rapana venosa* [Altug and Güler, 2002], which is a carnivorous gastropod. It is necessary to demonstrate that not filter feeder gastropods actually do not concentrate *E. coli*, even if they are fished in not-classified areas.

6. Purpose

The present thesis aims to illustrate a multifactorial analysis of edible marine gastropods, particularly *T. mutabilis* and *B. brandaris* since little is known about the food safety of sea snails. Considering the immensity of the project, we establish specific purposes, which correspond to different sections of the thesis, as follows:

- Evaluate a method to assess the viability, that should be cheap and easy to be replicated by official authorities and food business

operators, and we also concentrate on the smell, which may be another indicator of freshness.

- Estimate the bacterial contamination of marine gastropods to evaluate both legislation safety criteria (*Escherichia coli*) and pathogenic microbes (*Vibrio parahaemolyticus*, *V. vulnificus*, *V. cholerae*, and *V. alginolyticus*), that might also produce tetrodotoxin.
- Describe how some parameters change during a week in refrigerated condition and after 24 hours of immersion; in addition, we estimate indole-producing bacteria and biogenic amines, because of the association of these substances with carrions on which scavenger gastropods feed. Moreover, indole and biogenic amines are thermostable and may be found even after long cooking (which is the traditional way to consume marine gastropods).
- Focus on viral zoonosis (Hepatitis A and Norovirus) because sea snails might play an important role as carriers.
- Evaluate the presence of different toxins (saxitoxin and tetrodotoxin).

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Section 2 – Viability and smell

1. Introduction

Regulations No 853/2004/EC and No 624/2019/EU affirm that marine gastropods must be placed on the market viable with the peculiarities of freshness, but they define these characteristics as "*shells free of dirt, an adequate response to percussion and normal amounts of intravalvular liquid*". Obviously, it can only be referred to bivalves.

Palese and Palese (1991) claim that an alive marine gastropod has a glossy and bright body with a saltwater smell, whereas a dead individual has an opaque, slimy, and drooping body with an acrid and nasty smell. They also add that the body of fresh gastropods is well hydrated with clear dripping water and, as time passes, it dehydrates and the water becomes cloudy if it is present. In addition, they specify that *Tritia mutabilis* may change color during refrigeration: it may have a yellowish shade or a brown one if it is preserved for a considerable time, respectively, in a safe environment or an unhealthy and really moist one. Finally, they suggest stinging the gastropod's body with a pin to see if the specimen is alive because if it is viable, the body retreats quickly inside the shell. In fact, Orlandi and Perna (1968) claim that in *T. mutabilis* the viability is directly linked to the reaction to stimuli.

In literature, marine gastropods respond to different stimuli. Kohn (1961) affirm that dry gastropods react to immersion into seawater with the extension of the foot, head, and tentacles and, if the immersion persists, locomotion. It is appropriate to clarify that the water should be as close as possible to natural conditions or gastropods may stay close and only secrete mucus, while trying to recovery [Ho et al., 2019]. Moreover, *T. mutabilis* responds to NaCl stimulation with escape movements [Gäde et al., 1984], but it may also react to other salts, acids, and basis with escape movements or by retracting within shell [Kohn, 1961], hence this could be more dangerous for the official authorities and less effective.

Concerning the smell, Palese and Palese (1991) claim that gastropods' odor changes from saltwater to sour, passing through pungent. Similarly, Orlandi and Perna (1968) assert that the smell of *T. mutabilis* varies from saltwater to stale and, lastly, to fecaloid. Obviously, the legislation does not include the shelf-life of marine gastropods and their alterations, because they must be sold alive. Nevertheless, Regulation (EC) No 2406/96 establishes freshness ratings for fish, crustaceans,

cephalopods, and selachii and one of the scores is the smell. In most cases, the odor changes from seaweed to neutral and, lastly, to sour, in a similar way to Palese and Palese (1991) and Orlandi and Perna (1968).

2. Methodology

All the samples of *T. mutabilis* (for a total of 26 batches) and 1 batch of *Bolinus brandaris* were evaluated for viability and smell. They were collected from January 2019 to February 2021 through the fishing period of *T. mutabilis* (from the beginning of autumn to the end of spring) [Polidori, 2015] and of *B. brandaris* (from June to September). The catch area was the Adriatic Sea (FAO zone 37.2.1), particularly along the coast from Ravenna to Rimini.

As we have already published [Serratore et al., 2019], to estimate the viability, the whole batch or 30 specimens were placed on a large tray (tried to form a monolayer) and then dusted with sea salt. Only individuals that react to it with body extroversion and escaping movements or bubble/foam production were considered viable. The smell was expressed by five descriptors: saltwater, neutral, slightly acrid, acrid, and nasty.

In addition, 11 batches of *T. mutabilis* and one of *B. brandaris* were re-immersed in clean seawater into a sterile basin for 30 minutes and then the viability was evaluated as described before. We have done it both to see what marine gastropods do after a re-immersion and to allow them to recover for a while because we considered that stressed gastropods might not react to stimuli and might provide a fake viability assessment.

Lastly, 6 batches of *T. mutabilis* were re-immersed for 18-24 hours in clean seawater into a tank of a Recirculating Aquaculture System (RAS) and then the viability was evaluated as described before.

Statistical analysis was performed on viability and smell. The five descriptors of smell were converted into 1 (saltwater), 2 (neutral), 3 (slightly acrid), 4 (acrid), and 5 (nasty). According to the results of the Shapiro-Wilk test, to evaluate the possible correlation the Spearman's *rho* was calculated. Statistical significance was set at $p \leq 0.05$.

3. Results

3.1 Viability

As mentioned earlier, Palese and Palese (1991) suggested to sting the gastropod's body with a pin to see if the specimen is alive, however, if the gastropod has already retreated into the shell, this method cannot be used. Therefore, as reported, this section aims to evaluate a method to assess the viability, that should be cheap and easy to be replicated by official authorities and food operators. In our study, the viability was evaluated by using sea salt, following the statement of Gäde et al. (1984). It is also important to underline that in our preliminary attempts we evaluated the viability on the whole batch, but later we decided to use only 30 specimens to both be more precise and cause less stress to gastropod involved in other experiments, like the re-immersion.

All the results of *T. mutabilis* are given in Table 2.1.

Table 2.1: Viability and smell in *Tritia mutabilis* and origin of the batches. C: commerce; PP: primary production; S: saltwater; N: neutral; SA: slightly acrid; A: acrid; NA: nasty.

Sample Number - Date	Days since packaging date	Origin	Viability (%)	Smell
1433 - 15/01/19	1	C	20	SA
1434 - 17/01/19	1	C	30	SA
1435 - 22/01/19	1	C	40	SA
1436 - 29/01/19	1	C	90	S
1437 - 31/01/19	1	C	90	S
1438 - 05/02/19	1	C	30	A
1439 - 13/02/19	1	C	70	S
1440 - 13/02/19	1	C	50	SA
1442 - 26/02/19	1	C	0	NA
1443 - 28/02/19	1	C	25	S
1446 - 07/03/19	3	C	0	SA
1447 - 12/03/19	1	C	20	N
1448 - 18/03/19	3	C	0	NA
1449 - 19/03/19	1	C	80	S
1450 - 21/03/19	1	C	70	SA
1451 - 25/03/19	0	C	100	S
1452 - 03/04/19	2	C	25	SA
1453 - 09/04/19	4	C	0	NA
1458 - 25/06/19	0	PP	88	S
1470 - 12/02/20	0	PP	100	S
1471 - 19/02/20	0	PP	100	S
1472 - 24/02/20	0	PP	100	S
1486 - 19/10/20	0	PP	100	S
1489 - 03/11/20	0	PP	90	S
1492 - 18/01/21	0	PP	100	S
1495 - 22/02/21	0	PP	100	S

As can be seen, 84% of samples from commerce showed viability lower than 90%, while all the samples from primary production (except one having the 88%) presented viability higher than 90%. This result is remarkable since only mortality of 10% is almost always accepted by official authorities at the market. Hence, most of the analyzed commercial samples should not have been sold. These results are in line with our previous data [Serratore et al., 2019] that have already revealed that the 63% of samples at retail had mortality more than 10%.

According to Regulation No 853/2004/EC, a dispatch center can repackage a batch received from another dispatch center, hence the packaging date reported on the label might be different from the landing date. In fact, the results show the viability is not related to the date of packaging: in fact, e. g. the sample 1442 had 100% of mortality, such as 1453, but they presented different ranges since packaging date, respectively one and four days. Moreover, most commercial samples were received and analyzed one day after the packaging but corresponded to various viability.

The sample of *B. brandaris* (1457 - 25/06/19) was obtained from primary production, was analyzed the same day, and presented 100% of viability. In our previous study [Serratore et al., 2019], seven samples were already analyzed and showed the same characteristics.

Figure 2.1: Response to NaCl stimulation. A: *Tritia mutabilis* shows escaping movements; B: *Bolinus brandaris* produces foam and bubbles by excreting purple mucus; C: *B. brandaris* extends its foot.

As can been seen in Fig. 2.1, our study confirms that gastropods respond to NaCl stimulation by escaping movements [Gäde et al., 1984]. However, we also observed that gastropods respond by retracting themselves into the shell and by excreting mucus with a foam/bubble production, as we already published [Serratore et al., 2019]. Although gastropods could react in both ways, the remarkable difference between *T. mutabilis* and *B. brandaris* is that changeable nassas commonly respond by escaping movements and the purple dry murices usually produce foam and bubbles. This difference may be explained by the fact that *B. brandaris* has an operculum that protects it from external stressors, while *T. mutabilis* not.

As reported, Kohn (1961) affirm that dry gastropods react to immersion into seawater and Ho et al. (2019) clarify that the water should be as close as possible to natural conditions, or gastropods might not react well, trying to recover. As mentioned before, we did the re-immersion of 30 minutes both to see the response of gastropods and to test if stressed individuals could give a fake viability assessment with sea salt. In fact, gastropods might be stressed by the harvesting, the long period outside the seawater, and the refrigeration and might not respond to stimuli. Therefore, shortly after the beginning of this study, half of 11 batches of *T. mutabilis* and one of *B. brandaris* were re-immerse into clean seawater. All the results are represented in Figure 2.2.

Figure 2.2: Viability evaluated immediately vs. after a short re-immersion (30 minutes). The samples of *T. mutabilis* are from 1437 to 1451, while sample 1457 is of *B. brandaris*.

Figure 2.2 reveals that all the re-immersed samples had equal or higher viability than the same batch evaluated only with sea salt. This result seems to confirm that stressed gastropod might react less to stimuli and might be a slight limitation of the method used to assess the viability. Nevertheless, only three batches (27%) of *T. mutabilis* should have been acceptable for sales by assessing the viability after a short re-immersion and not acceptable by assessing with sea salt. However, even in a very short re-immersion, gastropods recovery well, as evident in Fig. 2.3.

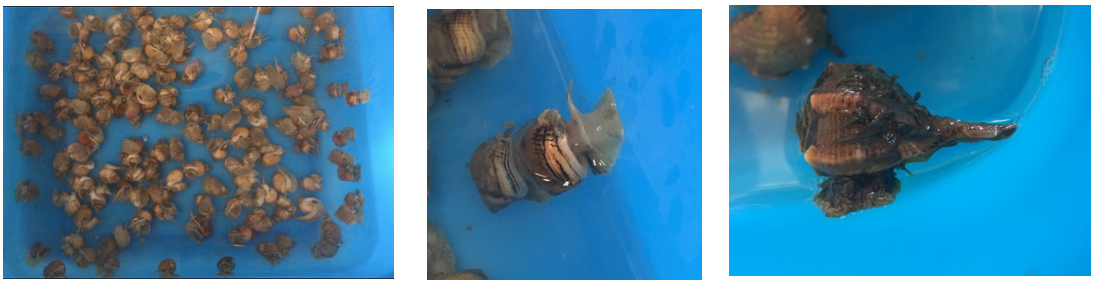

Figure 2.3: Short re-immersion (30 minutes) into a sterile basin in clean sea water.

Subsequently, we tried a long re-immersion (18-24 hours) into a tank of a Recirculating Aquaculture System (RAS) (see Fig. 2.4) to evaluate the possible viability change. Our first two preliminary attempts into an uncooled tank/aquarium were failed and are not discussed. The following 6 batches of *T. mutabilis* were re-immersed at ~ 15-16°C. All the results are shown in Figure 2.5.

Figure 2.4: Long re-immersion (18-24 hours) into a tank. A: before the immersion; B and C: during the re-immersion using two types of baskets.

As illustrated in Fig. 2.5, the viability was equal or higher in samples of re-immersed gastropod. However, it did not seem to be a big difference regarding the viability after a short and a long re-immersion. This result may indicate that gastropods in clean seawater could recovery and/or preserve and then react to stimuli. In fact, the 1452 batch was kept re-immersed up to 48 hours and the viability reached

54%. Obviously, as evident in Fig. 2.5, if gastropods are already dead at the T0, remain dead even after the re-immersion.

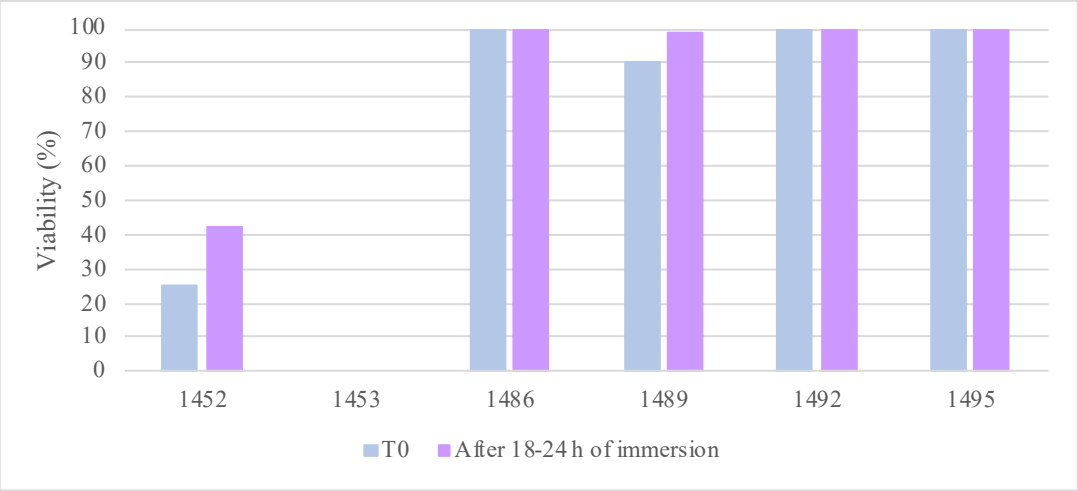

Figure 2.5: Viability evaluated immediately vs. after a long re-immersion (18-24 hours).

The method to evaluate the viability using sea salt is easy to apply even in gastropods retreated into the shell and could give more precise results than the punching, although the results obtained in the re-immersion condition seemed a slight limitation because gastropods might be stressed by the harvesting, the long period outside the seawater, and the refrigeration and might not respond to stimuli

3.2 Smell

The aim of this section was to evaluate if the smell can be another indicator of freshness. Following Palese and Palese (1991) and Orlandi and Perna (1968), we used five descriptors: saltwater (very fresh), neutral (freshness), slightly acrid (initial loss of freshness), acrid (loss of freshness), nasty (spoiled). All the results of *T. mutabilis* are showed in Table 2.1.

As can be seen in Table 2.1, the smell was slightly correlated to viability (Spearman's *rho* = -0.82; $p<0.05$). However, all the samples from primary production (including the one of *B. brandaris*) had a saltwater smell, as also reported by previous studies [Serratore et al., 2019]. In addition, the batches with 100% of mortality had a nasty smell, as we already published. Therefore, even if not parallel to viability, the smell could be a good freshness indicator.

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Section 3 - Bacteriological analysis

1. Introduction

As previously mentioned in the legislation section, gastropods must comply with safety criteria (including the amount of *Escherichia coli*) according to Reg. 2285/2015/EU.

Vibrio species are ubiquitous in the marine environment, which represents their main reservoir. They may be accumulated into mollusks' flesh but are usually harmless to humans except the "big four": *V. parahaemolyticus*, *V. vulnificus*, *V. cholerae*, and *V. alginolyticus* [Barbieri et al., 1999; Passalacqua et al., 2016; Zago et al., 2020]. However, marine gastropods are usually consumed after long cooking, which does not allow the bacteria to survive, but it is well known that *Vibrio* spp. may produce biogenic amines and tetrodotoxins, that are thermostable and truly dangerous for humans [Biessy et al., 2019; Leão et al., 2018; Magarlamov et al., 2017; Noguchi et al., 1987]. Moreover, marine gastropods can be contaminated by tetrodotoxins not only produced by accumulated *Vibrio* spp. in their flesh but also acquired from feeding, especially bivalves [Hort et al., 2020], that have been found to be contaminated even in the Adriatic Sea [Bordin et al., 2021].

In this chapter, only targets, that have been analyzed, will be discussed.

2. *Escherichia coli*

Escherichia coli is a gram-negative, motile, non-spore-forming, rod-shaped bacterium, belonging to the family Enterobacteriaceae [Al Humam, 2016; Mainil, 2013; Nataro and Kaper, 1998]. It was first isolated by Theodor Escherich in 1885 and it was originally called *Bacterium coli commune* [De Sousa, 2006; Méric et al., 2016]. It is the most abundant facultative anaerobe of the human intestinal flora (10^7-10^8 CFU per gram of feces) [Ruppé et al., 2013] and it colonizes the gastrointestinal tract within hours of life [Jaureguy et al., 2008; Kaper et al., 2004; Nataro and Kaper, 1998]. Nevertheless, highly adapted *E. coli* strains have acquired specific virulence factors and may cause three general clinical syndromes: sepsis/meningitis, urinary tract infection, and enteric/diarrheal disease [Kaper et al., 2004; Nataro and Kaper, 1998].

Pathogenic *E. coli* is classified into different serotypes based on its O (somatic) and H (flagellar) surface antigens [Kaper et al., 2004; Nataro and Kaper,

1998]; nowadays, a total of 187 serogroups (based only on O antigens) are recognized [Ludwig et al., 2020]. In addition, *E. coli* is classified in specific pathotypes ("*a group of strains of a single species that cause a common disease using a common set of virulence factors*" [Kaper et al., 2004]): enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), diffusely adhering *E. coli* (DAEC), uropathogenic *E. coli* (UPEC), neonatal meningitis *E. coli* (NMEC) and, even if not well described, necrotoxigenic *E. coli* (NTEC) and adherent invasive *E. coli* (AIEC) [Chaudhuri and Henderson, 2012; Croxen and Finlay, 2010; Kaper et al., 2004; Mainil, 2013]. Moreover, *E. coli* pathotypes that cause extraintestinal infections are called ExPEC [Jaureguy et al., 2008].

Each pathovar has specific virulence factors and causes different diseases, hence, they will be discussed individually.

ETEC pathovar contains *E. coli* strains that express at least one of two groups of enterotoxins ST (heat-stable toxins) and LT (heat-labile toxins) and is the main cause of travelers' diarrhea in adults from industrialized countries and children in developing ones [Kaper et al., 2004; Nagy and Fekete, 2005; Nataro and Kaper, 1998; Scheutz, 2019; Torres et al., 2005]. ETEC disease has a short incubation period (14-48 h) [Chao et al., 2006; Dalton et al., 1999; Yoder et al., 2006] and is characterized by watery diarrhea without mucus, blood, or pus, that commonly is self-limiting and rarely may be severe (cholera-like illness) [De Sousa, 2006; Fleckenstein and Kuhlmann, 2019; Nataro and Kaper, 1998; Scheutz, 2019]. ETEC strains colonize the proximal intestine by using fimbriae and fibrillae adhesins and then, as mentioned before, secrete their enterotoxins ST and LT, which cause, respectively, ion secretion and stimulation of secretion and inhibition of absorption [Croxen and Finlay, 2010; De Sousa, 2006; Kaper et al., 2004; Mainil, 2013; Nagy and Fekete, 2005; Nataro and Kaper, 1998; Torres et al., 2005].

EPEC is the oldest *E. coli* pathovar to be described and is a major cause of diarrhea in infants (potentially fatal under 2 years of age) in developing countries [Croxen and Finlay, 2010; De Sousa, 2006; Kaper et al., 2004; Lee et al., 2012; Nataro and Kaper, 1998; Scheutz, 2019]. EPEC causes acute, persistent watery diarrhea (which may contain mucus), vomiting, and low-grade fever [De Sousa, 2006; Nataro and Kaper, 1998; Scheutz, 2019] and its illness has a shorter incubation period than ETEC (6 – 32 h) [Chao et al., 2006; Lee et al., 2012]. It is characterized

by attaching and effacing (A/E) lesions, that are marked by localized degeneration of brush-border microvilli and formation of an actin-rich cytoskeletal structure in the epithelial cells at bacterial attachment's sites [De Sousa, 2006; Torres et al., 2005] and are encoded by genes localized to the locus of enterocyte effacement [Cleary et al., 2004]. EPEC strains colonize the small intestine by using adhesins, among which bundle-forming pili (BFP), EspA filaments, and intimin; the latter binds the translocated intimin receptor (Tir) and both form A/E lesions and an intimate attachment to host cells [Cleary et al., 2004; Girón et al., 2002; Nougayrède et al., 2003]. In addition, the pathology of EPEC strains is due to different secreted proteins (EPEC-secreted proteins, Esps, mitochondrial-associated protein, Map, and non-LEE effectors, Nle), that are translocated into the host cells by the type III secretion system (T3SS) [Croxen and Finlay, 2010; Dean and Kenny, 2009; Kaper et al., 2004; Nataro and Kaper, 1998]. Lastly, some, but not all, EPEC strains produce EspC, an enterotoxin that increases short circuit current in Ussing chambers and causes apoptosis, necrosis, and cytoskeletal damage in epithelial cells [Kaper et al., 2004; Mellies et al., 2001; Serapio-Palacios and Navarro-García, 2016; Vidal and Navarro-García, 2008].

EHEC causes severe gastroenteritis in developed countries and includes *E. coli* strains that express Shiga-like toxin (Stx), cause A/E lesions, hemorrhagic colitis (HC), and hemolytic uremic syndrome (HUS) [Croxen and Finlay, 2010; Nataro and Kaper, 1998]. The infectious dose of EHEC is very low (< 100 CFU) and its disease follows 3-9 days after ingestion of bovine-derived products as meat and unpasteurized milk and dairy products or contaminated water like vegetable and unpasteurized apple juice; in fact, animal reservoirs include cattle, sheep, goats, chickens, pigs, cats, dogs and gulls [Chao et al., 2006; Kaper et al., 2004; Karch et al., 2005; Nataro and Kaper, 1998; Nguyen and Sperandio, 2012; Welinder-Olsson and Kaijser, 2005]. *E. coli* O157:H7 is the most common member of EHEC pathovar, particularly in North America, Japan, and the United Kingdom [Croxen and Finlay, 2010; Kaper et al., 2004; Mead and Griffin, 1998; Pennington, 2010]. EHEC illness is characterized by initial non-bloody diarrhea (which becomes bloody within 1-2 day), abdominal pain and HUS, that is defined by a triad of acute renal failure, hemolytic anemia, and thrombocytopenia [Chao et al., 2006; Karch et al., 2005; Nataro and Kaper, 1998; Nguyen and Sperandio, 2012; Welinder-Olsson and Kaijser, 2005]. EHEC strains colonized the large intestine by using fimbriae and intimin

(similarly to EPEC) and express Stx, which induces apoptosis in colon epithelial cells (which results in HC) and cause renal inflammation (which leads to HUS) [Croxen and Finlay, 2010; Kaper et al., 2004; Nataro and Kaper, 1998; Nguyen and Sperandio, 2012; Torres et al., 2005].

EAEC strains are defined as *E. coli* that do not secrete LT or ST enterotoxins and that adhere to HEp-2 cells in an aggregative adherence (AA) pattern, which is an autoagglutination of the bacterial cells to each other, also called "stacked-brick" formation [Kaper et al., 2004; Okhuysen and DuPont, 2010; Nataro and Kaper, 1998; Nataro, 2004; Weintraub, 2007]. EAEC is a causative agent of persistent watery diarrhea (occasionally with mucus or blood) in children and adults in both developed and developing countries (is a cause of travelers' diarrhea) and its illness is also characterized by low-grade fever, vomiting, and abdominal pain [Chao et al., 2006; Harrington et al., 2006; Kaper et al., 2004; Okeke and Nataro, 2001; Nataro and Kaper, 1998; Weintraub, 2007]. EAEC strains colonize the small and large bowel by using AA fimbriae and dispersin (which sores bacteria association and diffusion across the mucosa) and then, produce different toxins: Pic (which has a mucinase activity), *Shigella* enterotoxin 1 (ShET1, which has an enterotoxin activity), EAST 1 (which is the ETEC ST toxin's homolog), Pec (which has an enterotoxin activity) [Harrington et al., 2006; Kaper et al., 2004; Nataro, 2004; Torres et al., 2005; Weintraub, 2007].

EIEC strains possess some, but not all, of biochemical characteristics of *E. coli* and are closely related to *Shigella*, they cause watery diarrhea or dysentery by using the same invasion method [Escobar-Páramo et al., 2003; van den Beld and Reubsaet, 2012]. EIEC pathogenesis comprises epithelial cell penetration, lysis of the endocytic vacuole, intracellular multiplication, directional movement through the cytoplasm, and dissemination into adjacent epithelial cells [Kaper et al., 2004; Nataro and Kaper, 1998; Pasqua et al., 2017; Torres et al., 2005; van den Beld and Reubsaet, 2012].

DAEC strains cause diarrhea and are defined by their diffuse adherence (DA) pattern on Hep-2 and HeLa epithelial cells [Le Bouguénec and Servin, 2006; Javadi et al., 2020; Kaper et al., 2004; Servin, 2005]. DAEC is divided into two groups, according to the adhesin expression (both of which are responsible for DA pattern and promote cell lesion, inflammation, and loss of microvilli): Afa/Dr DAEC and AIDA-I DAEC [Javadi et al., 2020; Jesser and Levy, 2020; Servin, 2005; Torres et

al., 2005]; moreover, these strains are associated with the secreted autotransporter toxin (Sat), that damages on epithelial cells and alters tight junction integrity [Guignot et al., 2007; Meza-Segura and Estrada-Garcia, 2016; Meza-Segura et al., 2020].

UPEC is the main cause (70-90%) of community-acquired and the large portion (50%) of nosocomial urinary tract infections (UTIs) [Bien et al., 2012; Shah et al., 2019; Terlizzi et al., 2017; Wiles et al., 2008]. UPEC illness is characterized by painful and frequent urination, a sudden compelling desire to urinate, cystitis, and pyelonephritis [Bien et al., 2012; Terlizzi et al., 2017; Wiles et al., 2008]. UPEC strains colonize the host by using fimbriae, flagella, capsular lipopolysaccharide (LPS), pili, non-pilus adhesins, curli, and outer membrane proteins (OMPs) and then, produce several toxins, including hemolysin (HlyA, which is a pore-forming toxin and causes renal damage and scarring), cytotoxic necrotizing factor 1 (CNF1, which is involved in kidney invasion), vacuolating autotransporter toxin (Vat, which induces cytopathic effects) and Sat [Bien et al., 2012; Kaper et al, 2004; Shah et al., 2019; Terlizzi et al., 2017; Wiles et al., 2008].

NMEC pathotype is the most common cause of Gram-negative neonatal meningitis, which occurs during the first month of life (only 10% between 1 and 3 months of age) with a case fatality rate of 10-30% and neurological sequelae in many of the survivor (like ventriculitis and intracerebral abscess) [Bonacorsi and Bingen, 2005; Kaper et al, 2004; Wijetunge et al., 2015]. NMEC strains can survive in blood and invade the meninges without damages to the blood-brain barrier, but even if this ability is not fully understood, some virulent factors have been described: fimbriae (for colonization and biofilm formation), colony stimulation factor V, Ibe ABC (for cell invasion), OMPs (which protect the bacterium from host defenses), Mat (associated with meningitis), K1 capsular antigen and Iss (both of which protect the bacterium against phagocytosis) [Antão et al., 2009; Kaper et al, 2004; Sarowska et al., 2019; Wijetunge et al., 2015].

NTEC strains cause dysentery and UTIs in humans and animals and originally were defined as *E. coli* producing a cytotoxic necrotizing factor (CNF) [de Rycke et al., 1999; Kaper et al, 2004; Rahman and Deka, 2014]. Two groups of NTEC have been reported: NTEC 1, which produces CNF 1 toxin and can be found in both human and all species of domestic animals, and NTEC 2, which produces CNF 2 toxin and have been reported only in ruminants [de Rycke et al., 1999; Van Bost et al., 2003].

Moreover, NTEC strains can produce other toxins, including cytolethal distending toxin (CDT, with two variants: CDT-IV in NTEC1 and CDT-III in NTEC2), hemolysin (Hly), P-fimbriae, S-fimbriae, and afimbrial adhesins [de Rycke et al., 1999; Rahman and Deka, 2014; Van Bost et al., 2003].

AIEC strains are defined as *E. coli* that can adhere to and invade intestinal epithelial cells (IEC), can survive and replicate extensively in macrophages without inducing host cell death, and can induce the release of large amounts of tumor necrosis factor (TNF)-α by infected macrophages, which may lead to intestinal inflammation, typical of Crohn's disease [Kaper et al, 2004; Martinez-Medina et al., 2009a; Martinez-Medina et al., 2009b; Pamela et al., 2018; Rolhion and Darfeuille-Michaud, 2007; Shawki and McCole, 2017]. In addition, AIEC strains can form biofilm on IEC, have long polar fimbriae (LPF), and may carry many virulence-associated genes characteristic of ExPEC [Martinez-Medina et al., 2009a; Pamela et al., 2018].

It should be underlined that, according to EN/ISO 16649-3, the quantified *E. coli* are only the ß-glucuronidase positive strains, that represent the fecal contamination. On the other hand, it is well known that, e.g., O157:H7 (an EHEC strain) is ß-glucuronidase negative [Kim et al. 2001], hence only the intestinal *E. coli* are monitored to ensure compliance with the legal limits.

3. Vibrio *species*

The genus *Vibrio* belongs to the family Vibrionaceae and consists of over 140 species of Gram-negative, asporogenous bacteria, among which thirteen are pathogenic to humans, but the most significant agents are *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus* [Drake et al., 2007; Gonzáles-Castillo et al., 2020; Lamon et al., 2019; Passalacqua et al., 2016].

3.1 Vibrio parahaemolyticus

Vibrio parahaemolyticus is a halophilic, straight or curved, rod-shaped bacterium [Su and Liu, 2007; Yeung and Boor, 2004]. It has commonly a single polar flagellum, but if it grows in a semi-solid media or on surfaces, it switches in a swarmer cell covered in lateral flagella [Broberg et al., 2011; Farmer III et al., 2005; McCarter, 1999]. It inhabits marine or estuarine environments in warm climates [Letchumanan et al., 2014; Yeung and Boor, 2004; Ottaviani et al., 2010; Zhang and

Orth, 2013] and it is found free-swimming or commensally associated with shellfish species [Broberg et al., 2011; McCarter, 1999].

V. parahaemolyticus is classified by serotyping by antibodies specific to O (somatic) and K (capsular) antigens; to date, there are 11 O and 69 K antigen serotypes [Chen et al., 2020; Drake et al., 2007] and at least 20 serovars can cause infections, including O3:K6, O1:K25, O4:K68, O4:K8, O1:KUT (K untypeable) [Broberg et al., 2011; Chen et al., 2011; Chen et al., 2020; Chowdhury et al., 2004; Drake et al., 2007; Nair et al., 2007].

This bacterium was first discovered in 1950 by Tsunesaburo Fujino as a causative agent of a seafood poisoning outbreak in Osaka, Japan, which recorded 272 illnesses with 20 deaths [Fujino et al., 1953]. Nowadays, *V. parahaemolyticus* commonly causes foodborne gastroenteritis associated with the consumption of raw or undercooked seafood in the United States of America and Asian countries [Caburlotto et al., 2008; Letchumanan et al., 2014; Ottaviani et al., 2005; Su and Liu, 2007]. Clinical characteristics of the infections include diarrhea, nausea, vomiting, abdominal cramps, headache, low-grade fever, and chills [Baker-Austin et al., 2010; Broberg et al., 2011; Yeung and Boor, 2004]. Infection occurs 4 - 96 h after the ingestion, is self-limited in immunocompetent individuals, and lasts up to three days [Baker-Austin et al., 2010; Broberg et al., 2011; Di Pinto et al., 2008]. Less commonly, *V. parahaemolyticus* can also cause wound infections and septicemia [Broberg et al., 2011; Daniels et al., 2000; Drake et al., 2007; Wang et al., 2015]. Wound infections are defined as "*those where a patient incurred a wound before or during exposure to seawater, seafood drippings or punctures from spines or bones, and from which the bacterium was subsequently cultured from that wound, blood or otherwise normally sterile site*" [Drake et al., 2007]. Primary septicemia is a systemic illness where the bacterium was isolated from blood or other sterile site but no wound infection preceding illness [Strom and Paranjpye, 2000]. Wound infections are common in fishermen and seafood processors and are usually limited to cellulitis, but may progress to necrotizing fasciitis [Broberg et al., 2011; Drake et al., 2007; Johnson et l., 1984], while septicemia may occur in individuals with underlying medical conditions (including diabetes, cancer, liver disease) and results in hypovolemic shock, multisystem organ failure and death [Broberg et al., 2011; Hally et al., 1995; Rabinowitch et al., 1993].

Virulence of *V. parahaemolyticus* is associated with multiple factors, including adhesins, iron acquisition, urea hydrolysis, thermostable direct hemolysin (TDH) and TDH related hemolysin (TRH), two Type 3 Secretion Systems (T3SS1 e T3SS2), and two Type VI Secretion System (T6SS1 e T6SS2) [Broberg et al., 2011; Ghenem et al., 2017; Letchumanan et al., 2014; Li et al., 2019; Wang et al., 2015; Zhang and Orth, 2013]. First, the bacterium needs to adhere to the host cells and, for this reason, on the bacterial surface, *V. parahaemolyticus* has a multivalent adhesion molecule (MAM), made of six or seven mammalian cell entry domains [Broberg et al., 2011; Ghenem et al., 2017; Krachler et al., 2011; Letchumanan et al., 2014; Zhang and Orth, 2013]. Secondly, the bacterium has to acquire the iron, which is an essential element for all the organisms and coordinates virulent factors; to do so, it uses siderophores, in particular vibroferrin, or acquires siderophores made by different bacteria (such as ferrichrome and aerobactin) or uptakes heme [Broberg et al., 2011; Ghenem et al., 2017; León-Sicairos et al., 2015; Li et al., 2019; Miethke and Marahiel, 2007; Wong et al., 1996]. Additionally, some *V. parahaemolyticus* strains can hydrolyze urea and often belong to the O4 group; hence, urea hydrolysis has been proposed as a virulence factor (also because is linked to the *trh* gene), but its role is still unclear [Drake et al., 2007; Kaysner et al., 1994; Okitsu et al., 1997; Ottaviani et al., 2010; Yeung and Boor, 2004]. Historically, the pathogenicity of *V. parahaemolyticus* has been associated with the Kanagawa phenomenon, which is the lysis of red blood cells on Wagatsuma agar, but now it is known that this reaction is caused by TDH [Drake et al., 2007; Miyamoto et al., 1969; Su and Liu, 2007; Wang et al., 2015]. Nowadays, it is well-known that TDH and TRH cause cells toxicity and have hemolytic activity and, hence, they are regarded as major virulence factors, and their genes have been considered molecular screening markers to discriminate avirulent and virulent strains [Baker-Austin et al., 2010; Broberg et al., 2011; Casandra et al., 2013; Drake et al., 2007; Yeung and Boor, 2004]. In addition, thermolabile hemolysis (TLH) is another toxin that is found in both clinical and environmental strains and seems to cause cytotoxicity and lysis of human erythrocytes like TDH and TRH [Broberg et al., 2011; Wang et al., 2015]. Although these toxins are considered major virulence factors, it has been demonstrated that deletion of *tdh* or *trh* does not affect the cytotoxicity and the enterotoxicity [Park et al., 2004; Raghunath, 2015]. Makino et al. (2003) analyzed the complete genome of *V. parahaemolyticus* and identified the presence of T3SSs in the bacterium. There are

two distinct T3SSs: T3SS1 and T3SS2, on chromosomes 1 and 2, respectively [Baker-Austin et al., 2010]. T3SSs are transmembrane apparatuses that deliver proteins, called effectors, into the cytoplasm of host cells [Broberg et al., 2011; Ghenem et al., 2017; Wang et al., 2015]. T3SS1 is found in all *V. parahaemolyticus* strains and is cytotoxic, causing autophagy and cell rounding, while T3SS2 is commonly associated with virulent strains and produces enterotoxicity [Letchumanan et al., 2014; Li et al., 2019; Wang et al., 2015; Zhang and Orth, 2013]. Lastly, T6SSs are structurally and functionally similar to T3SSs and in *V. parahaemolyticus* there are two T6SSs (T6SS1 and T6SS2) located on each chromosome [Ghenem et al., 2017; Letchumanan et al., 2014; Li et al., 2019; Raghunath, 2015; Wang et al., 2015]. Recently, it appears that T6SSs are necessary for the adhesion (in particular T6SS2) and seems to induce autophagy in macrophages [Ghenem et al., 2017; Letchumanan et al., 2014; Li et al., 2019; Raghunath, 2015; Wang et al., 2015].

3.2 Vibrio vulnificus

Vibrio vulnificus is a halophilic, alkaliphilic, motile, pleomorphic, rod-shaped bacterium with a single polar flagellum [Horseman and Surani, 2011; Leng et al., 2019; Park et al., 1999; Strom and Paranjpye, 2000; Wickboldt and Sanders, 1983]. It inhabits marine, estuarine and costal water located in tropical, sub-tropical and temperate areas [Hernández-Cabanyero and Amaro, 2020; Heng et al., 2017; Horseman and Surani, 2011].

V. vulnificus was first isolated in 1964 by the US Centers for Disease Control (CDC) [Strom and Paranjpye, 2000], but was first characterized by Roland in 1970 [Roland, 1970] and was defined as a new bacterial species in 1979 by Farmer III [Farmer III, 1979]. Historically, this bacterium was subdivided into three biotypes: Bt1 was the first described, is found worldwide in salt and brackish water and is responsible for the majority of human infection; Bt2 was thought to be pathogenic only to eels, but, on rare occasions, has been isolated from human cases in Eastern and Western Europe; Bt3 is a hybrid of biotypes 1 and 2 and has only been isolated in Israel and in freshwater fish, e.g. tilapia [Bisharat et al., 1999; Drake et al., 2007; Heng et al., 2017; Hernández-Cabanyero and Amaro, 2020; Horseman and Surani, 2011; Jones and Oliver, 2009; Oliver, 2015]. Afterward, there have been several attempts to classify *V. vulnificus*, utilizing molecular methods, and all the studies

divide the bacterium into two clusters: Environmental (avirulent) and Clinical (virulent) [Baker-Austin et al., 2010; Hernández-Cabanyero and Amaro, 2020; Oliver, 2015]. Recently, Roig et al. (2018) subdivide the species into five phylogenetic lineages and a pathovar, that can infect fish and claim, according to the Food and Agricultural Organization of the United Nations and the World Health Organization [FAO/WHO, 2005], that all strains of *V. vulnificus* may be pathogenic for humans.

V. vulnificus is transmitted via consumption of raw or undercooked seafood or contact with seawater or seafood handling [Heng et al., 2017; Hernández-Cabanyero and Amaro, 2020] and may cause septicemia, wound infections, and, uncommonly, gastroenteritis [Gulig et al., 2005]. Most cases occur in males (due to the protection of estrogen against the bacterium's endotoxin) over the age of 40-50 [Baker-Austin and Oliver, 2018; Leng et al., 2019; Oliver, 2015] with immunocompromising conditions, e. g. alcoholic liver disease (primarily cirrhosis), hepatitis B and C, metastatic cancer, liver transplantation, hemochromatosis, thalassemia major, diabetes, chronic renal failure, low gastric acid, chronic intestinal disease, steroid dependency, or other serum iron-elevating conditions [Baker-Austin et al., 2010; Bross et al., 2007; Hernández-Cabanyero and Amaro, 2020; Chiang and Chuang, 2003; Li and Wang, 2020; Linkous and Oliver, 1999; Jones and Oliver, 2009; Oliver, 2015; Strom and Paranjpye, 2000]. Nevertheless, healthy people have a self-limiting disease, that rarely leads to septicemia [Hernández-Cabanyero and Amaro, 2020]. They usually have gastroenteritis or wound infections [Passalacqua et al., 2016], even if the latter occurs more often in people with at least one predisposing condition [Strom and Paranjpye, 2000]. Generally, *V. vulnificus* has a short incubation period, averaging only 24-26 h [Baker-Austin and Oliver, 2018; Oliver, 2015] and most patients die within 24-48 h after hospitalization [Chiang and Chuang, 2003; Leng et al., 2019] with a case-fatality of 60-75% due to septicemia and 20-50% due to wound infections [Gulig et al., 2005; Heng et al., 2017; Li and Wang, 2020; Linkous and Oliver, 1999; Strom and Paranjpye, 2000]. Symptoms are different, depending on the type of infection. In primary septicemia patients present usually fever, chills, nausea, abdominal pain, vomiting, diarrhea, mental status changes, hypotension, and, within the first 24 h after onset of illness, development of secondary metastatic lesions on extremities, following by hemorrhagic blisters, cellulitis, ecchymosis, necrotic cutaneous ulcers and necrotizing fasciitis [Bross et

al., 2007; Gulig et al., 2005; Heng et al., 2017; Leng et al., 2019; Li and Wang, 2020; Linkous and Oliver, 1999; Oliver, 2015; Strom and Paranjpye, 2000]. Symptoms of wound infection and septicemia are similar, but different in their severity and timing and include fever, chills, mental status changes, hypotension, localized pain, edema, erythema, secondary cutaneous lesions, which may develop in hemorrhagic bullae, and necrosis of surrounding tissue, and secondary bacteremia [Bross et al., 2007; Chiang and Chuang, 2003; Heng et al., 2017; Linkous and Oliver, 1999; Strom and Paranjpye, 2000]. Lastly, in gastroenteritis (which requires hospitalization) patients present fever, dyspnea, abdominal cramps, nausea, vomiting, and diarrhea [Chiang and Chuang, 2003; Leng et al., 2019; Strom and Paranjpye, 2000].

In the same way as *V. parahaemolyticus*, the virulence of *V. vulnificus* is associated with multiple factors. Firstly, the bacterium needs to adhere and colonize the host surface: therefore, *V. vulnificus* has flagellum-bases motility and, on its surface, has pili (also called fimbriae) and outer membrane proteins (OmpU, able to bind fibronectin, and IlpA, able to stimulate the immune response), that are all involved in cytotoxicity too [Gulig et al., 2005; Horseman and Surani, 2011; Jones and Oliver, 2009; Leng et al., 2019; Strom and Paranjpye, 2000]. Afterward, *V. vulnificus* uses different ways to survive host defenses: e. g. OmpU prevents bile salts from entering the bacterium [Hernández-Cabanyero and Amaro, 2020]; against the acid pH of the stomach, manganese superoxide dismutase (MnSOD) reduce oxidative stress and neutralize acid and CadA (lysine/cadaverine anti-carrier), CadB (lysine decarboxylase) and CadC (transcription activator) synthesize cadaverine, which functions as both an acid neutralizer and a superoxidase radical scavenger [Hernández-Cabanyero and Amaro, 2020; Horseman and Surani, 2011; Jones and Oliver, 2009; Leng et al., 2019; Rhee et al., 2004]; capsular polysaccharide (CPS) provide resistance to phagocytosis by macrophages [Gulig et al., 2005; Jones and Oliver, 2009; Li and Wang, 2020; Linkous and Oliver, 1999; Pettis and Mukerji, 2020; Oliver, 2015; Strom and Paranjpye, 2000]. CPS is absolutely required for pathogenicity of *V. vulnificus*: in fact, all virulent strains are encapsulated and show opaque colonies in solid medium (in contrast, non-encapsulated colonies are translucent and avirulent) [Gulig et al., 2005; Jones and Oliver, 2009; Li and Wang, 2020; Linkous and Oliver, 1999; Pettis and Mukerji, 2020; Oliver, 2015]. Other virulent factors are extracellular proteins (ECPs), which are useful both to survive in the marine environment (e. g. chitinase is used to adhere to the chitin exoskeletons of

zooplankton) and to colonize the host (e. g. hemolysin, amylase, chondroitinase, elastase, collagenase, DNase, elastase, lipase, mucinase, gelatinase, lecithinase, phospholipase, hyaluronidase, fibrinolyse) [Linkous and Oliver, 1999; Strom and Paranjpye, 2000]. Among the exotoxins, hemolysin/cytolysin (VvhA) is the most studied and the most potent one: in fact, it lyses mammalian erythrocytes (to facilitate the uptake of iron), is involved in bacterium's cytotoxic activity and hypotensive shock [Chiang and Chuang, 2003; Horseman and Surani, 2011; Jones and Oliver, 2009; Lee et al., 2004; Li and Wang, 2020; Strom and Paranjpye, 2000]. Another possible virulence factor, particularly in skin lesions, is a metalloprotease (VvpE), because is involved in bacterium's colonization, induces edema and hemorrhagic damages, cause necrosis, enhances vascular permeability, which results in bradykinin generation (which promotes the spread of bacteria into the host) and protects bacteria from host defenses; nevertheless, VvpE is produced by virulent and avirulent strains, hence, it may not be an essential virulent factor [Hernández-Cabanyero and Amaro, 2020; Jones and Oliver, 2009; Lee et al., 2016; Li and Wang, 2020; Strom and Paranjpye, 2000]. One of the most virulent and cytotoxic factors is RtxA1, which belongs to multifunctional auto-processing repeats-in-toxin (MARTX) and induce sepsis, produce pores in the host cell membrane, promoting cell lysis, apoptosis, and necrosis, and is associated with actin depolymerization, induction of reactive oxygen species (ROS), and activation of caspase-1 [Horseman and Surani, 2011; Kwak et al., 2011; Li and Wang, 2020; Liu et al., 2007; Strom and Paranjpye, 2000]. Additionally, *V. vulnificus* can produce lipopolysaccharides (LPS), which are pyrogens e mediate the endotoxic shock, but estrogen and low-density lipoprotein (LDL) cholesterol may be a protection against LPS and this may be the reason why there is a sex difference in susceptibility to the bacterium between males and female [Horseman and Surani, 2011; Jones and Oliver, 2009; Linkous and Oliver, 1999]. Furthermore, in the same way as *V. parahaemolyticus*, *V. vulnificus* needs to scavenge iron from transferrin and lactoferrin or to obtain it from hemoglobin; so it produces two siderophores (vulnibactin and hydroxamate-type compound), that binds iron and transfers it into the bacterium [Barnes et al., 2020; Chiang and Chuang, 2003; Jones and Oliver, 2009; Strom and Paranjpye, 2000; Wright et al., 1981]. Lastly, the virulence of *V. vulnificus* is coordinated by multiple regulators [Elgaml and Miyoshi, 2017; Li and Wang, 2020]: Fur is involved in iron acquisition [Barnes et al., 2020; Jones and Oliver, 2009; Leng et al., 2019]; quorum-sensing (QS) system regulates virulence genes and

community behaviors [Kim et al., 2003; Jones and Oliver, 2009; Milton, 2006; McDougald et al., 2006]; cAMP-cAMP receptor protein (CRP) system play an important role in virulence and general metabolism [Alice and Crosa, 2012; Choi et al., 2006; Kim et al., 2018; Leng et al., 2019; Oh et al., 2009]; AphB promotes adhesion and host colonization, regulates acquisition and metabolism of nutrients and is involved in acid tolerance, cytotoxicity and lethality [Jeong and Choi, 2008; Jones and Oliver, 2009; Park et al., 2017; Rhee et al., 2006]; HlyU is a regulator of virulence, cytotoxicity and toxins and participates in sepsis [Imdad et al., 2018; Jones and Oliver, 2009; Liu et al., 2007]; LeuO is a master regulator of the cyclo(Phe-Pro)-mediated signaling pathway and plays an important role in pathogenesis, virulence, bacteria survival and host colonization [Park et al., 2019; Park et al., 2020]; ToxRS promote hemolysin production [Elgaml and Miyoshi, 2017; Lee et al. 2000]; leucine-responsive protein (Lrp) is a global regulator, involved in chemotaxis, cytotoxicity, virulence and growth in host body [Alice and Crosa, 2012; Ho et al., 2017 Jeong et al., 2003]; IscR regulates hemolytic activity, motility, adhesion, and survival under oxidative stress [Choi et al., 2020; Lim and Choi, 2014].

3.3 Vibrio cholerae

Vibrio cholerae is a comma-shaped bacillus with a polar flagellum [Faruque et al., 1998; Pant et al., 2020; Zhang et al., 2020] and it is ubiquitous in the aquatic environment [Gallego-Hernandez et al., 2020; Rivera et al., 2001]. In 1854 it was first recognized by John Snow and, in the same year, described and termed by Filippo Pacini; lastly, in 1883 Robert Koch isolated a pure culture and explained the transmission [Broeck et al., 2007; Lippi and Gotuzzo, 2013].

V. cholerae is classified into nearly 210 serogroups based on the heat-stable somatic O antigen, but only O1 and O139 (emerged in 1993) can cause cholera epidemics [Childers and Klose, 2007; Daboul et al., 2020; Fan et al., 2019; Faruque et al., 1998; Olaniran et al., 2011; Safa et al., 2020]. Since 1817, seven cholera pandemics have been occurred, among which the seventh began in 1961 and it continues today [Fan et al., 2019; Faruque et al., 1998; Olaniran et al., 2011]. The first six pandemics were caused by the O1 classical biotype, but the current one is caused by the O1 El Tor biotype [Childers and Klose, 2007; Fan et al., 2019; Faruque et al., 1998; Lee et al., 2020]. The O1 serogroup has two distinct biotypes classical and El Tor, that, historically, were distinguished by a hemolysis test [Fan et al., 2019;

Kim et al., 2017] and now by their susceptibility to polymyxin B and phage infection pattern [Lekshmi et al., 2020; Safa et al., 2020], while O139 serogroup would appear to be derived from O1 El Tor strains by the horizontal transfer of OAg (a specific lipopolysaccharide O antigen) genes [Benitez and Silva, 2016; Childers and Klose, 2007; Manning, 1997].

The ingestion of water and food and exposure of skin wounds to fresh and marine water contaminated by *V. cholerae* cause cholera, which is an acute diarrheal disease [Gallego-Hernandez et al., 2020; Gutierrez-Rodarte et al., 2019; Raskin et al., 2020; Zago et al., 2017]. Typical symptoms are vomiting, fever, electrolyte imbalance (due to the inhibition of the sodium and chloride transport) [Broeck et al, 2007; Galen et al., 1992] and profuse watery diarrhea, which result in severe dehydration, acidosis, hypovolemic shock, and, without appropriate treatment, death [Benitez and Silva, 2016; Childers and Klose, 2007; Manning, 1997]. Also, non-O1/non-O139 strains might cause gastroenteritis, extraintestinal infections, and primary septicemia [Ichinose et al., 1987; Rivera et al., 2001; Zago et al., 2017; Zhang et al., 2020], but they are usually asymptomatic and commonly isolated from the environment [Faruque et al., 1998]. Outbreaks of cholera have a seasonal pattern, probably due to *V. cholerae* association with aquatic organisms and its VNC (viable but nonculturable) form, which is triggered by specific environmental factors [Faruque et al., 1998]. The incubation period of *V. cholerae* has a duration of 1-5 days [Azman et al., 2013] and the infectious dose is 10^3-10^{11} [Childers and Klose, 2007, Yoon and Waters, 2019].

The virulence of *V. cholerae* is a combination of multiple factors. First of all, the bacterium needs to adhere to host cells and be protected against antigens and stomach acid, hence it can produce biofilms and has different surface components including proteins, pilus, flagella, and lipopolysaccharides (LPS) [Chatterjee and Chaudhuri, 2006; Gallego-Hernandez et al., 2020; Hsiao et al., 2006; Lekshmi et al., 2020; Manning, 1997; Raskin et al., 2020]. Among these adhesion factors, the toxin coregulated pilus (TCP), which is a type IV bundle-forming pilus, is the most important and is necessary for intestinal colonization [Childers and Klose, 2007; Gutierrez-Rodarte et al., 2019; Manning, 1997; Prouty et al., 2005; Rivera, 2001]. Secondly, the bacterium produces the main virulence factor: the cholera toxin (CT), an AB_5-subunit toxin, which causes typical diarrhea that defines the disease [Broeck et al., 2007; Childers and Klose, 2007; Faruque et al., 1998; Olaniran et al., 2011;

Prouty et al., 2005; Raskin et al., 2020]. The toxin is carried by a cholera toxin phage (CTXφ), that may be integrated into the genome of *V. cholerae* strains and allows them to produce CT [Childers and Klose, 2007; Gutierrez-Rodarte et al., 2019; Kim et al., 2017; Pant et al., 2020; Safa et al., 2020]. In addition, there are different factors, that may be found even in non-O1/non-O139 strains [Zhang et al., 2020]: OmpU and OmpT (outer membrane proteins) increase bile resistance and cholera toxin's expression and are involved in intestinal colonization [Faruque et al., 1998; Lekshmi et al., 2020; Simonet et al., 2003; Sperandio et al., 1995]; hemolysin (HlyA) has hemolytic activity and plays an important role in lethality and cardiotoxicity [Fan et al., 2019; Ichinose et al., 1987; Yamamoto et al., 1984]; neuraminidase (NANase) promotes the binding of CT to intestinal GM_1 ganglioside and, therefore, bacteria penetration [Fan et al., 2019; Galen et al., 1992]; hemagglutinins enhance intestinal fluid secretion and bacteria penetration and help *V. cholerae*'s dissemination along the gastrointestinal tract [Benitez and Silva, 2016; Broeck et al., 2007] and, in particular, mannose-sensitive hemagglutinin (MSHA) is mostly expressed by El Tor strains and is an essential colonization factor [Faruque et al., 1998; Sperandio et al., 1995]; zonula occludens toxin (Zot), accessory cholera toxin (Ace) are two additional toxin and, respectively, increase intestinal permeability and the short-circuit current in Ussing chambers (which cause fluid secretion) [Baudry et al., 1992; Faruque et al., 1998; Johnson et al., 1993; Shi et al., 1998; Trucksis et al., 1993; Uzzau et al., 1999]; Shiga-like toxin and heat-stable enterotoxin (stn/sto) are involved in cytotoxicity and cause dysentery [Acheson et al., 1993; Arita et al., 1986; Guglielmetti et al., 1994; O'Brien et al., 1984]; repeats-in-toxin (*rtx*) has a cytotoxic, hemolytic, leucotoxic and leucocyte-stimulating activity [Boardman et al., 2007; Chow et al., 2001; Lin et al., 1999]. Lastly, these virulence factors are regulated particularly by ToxR, ToxT, and the quorum sensing system [Childers and Klose, 2007; Faruque et al., 1998; Milton, 2006; Prouty et al., 2005; Raskin et al., 2020].

3.4 Vibrio alginolyticus

Vibrio alginolyticus is a halophilic, motile bacterium with a polar flagellum and it is ubiquitous in marine and estuarine waters especially in bathing areas [Carroll et al., 2020; Jacobs Slifka et al., 2017; Liu et al., 2015; Mustapha et al., 2013; Schmidt et al., 1979; Zhu et al., 2017]. Miyamoto (1961) first described a bacterium called *Oceanomonas alginolytica*, but then Sakazaki (1968) was the first to propose that the

biotype 2 of *V. parahaemolyticus* should have been excluded and it became a new species, *V. alginolyticus*.

Unlike other *Vibrio* species, *V. alginolyticus* is predominantly transmitted by water [Beshearse et al., 2021; Mustapha et al., 2013] and rarely causes gastroenteritis, but can usually cause ear, conjunctival, and wound infections [Citil et al., 2015; Jacobs Slifka et al., 2017; Pezzlo et al., 1978; Uh et al., 2001]. Its infections can usually be treated in a short time, but, in immunocompromised people, may lead to sphenoiditis, necrotizing fasciitis, intracranial infections, and septic shock [Citil et al., 2015; Jacobs Slifka et al., 2017]. In addition, *V. alginolyticus* is a potential reservoir of many virulence genes known in other *Vibrio* species, which may contribute to the development of its infections [Mustapha et al., 2013].

The pathogenicity is not fully understood, but it is known that *V. alginolyticus* has different virulence factors, including flagella, outer membrane protein (OmpA, OmpK, OmpU, and OmpW), siderophores, hemolysins (Thermostable Direct Hemolysin, TDH; Thermostable Related Hemolysin, TRH and Thermolabile Direct Hemolysin, TLH) [Bunpa et al., 2020; Hernández-Robles et al., 2016; Lv et al., 2020; Qian et al., 2008]. The adhesion of *V. alginolyticus* is regulated by RpoS [Huang et al., 2019], LuxS quorum-sensing system [Ye et al., 2008], *flrA*, *flrB*, and *flrC* [Luo et al., 2016]. It is also linked with flagella, which have been associated with the formation of biofilm, *swimming*, and *swarming* [Hernández-Robles et al., 2016]. OMPs help bacteria to adapt to external environmental changes and to acquire iron [Bunpa et al., 2020; Lv et al., 2020; Qian et al., 2008], which is also obtained with siderophores [Wang et al., 2007]. In addition, *V. alginolyticus* produce hemolysins, which are exotoxins that lyse erythrocyte membranes with the liberation of hemoglobin [Hernández-Robles et al., 2016; Jia et al., 2010] and are regulated by ToxR [Chang et al., 2012]. Lastly, it is known that *vppC* is a key virulence gene contributing to its pathogenicity [Hernández-Robles et al., 2016].

4. Methodology

A total of 25 batches of *Tritia mutabilis* and one batch of *Bolinus brandaris* were investigated. All the samples were collected as already described in the previous section.

Partially following the ISO 6887-3:2017/Amd.1:2020 method, sample units (SUs) were prepared by rising gastropods with sterilized seawater, then by cutting

aseptically their shells and finally by removing the whole body. Each SU was constituted by 10-20 individuals of *T. mutabilis* and by 10 *B. brandaris*, which were enough to obtain 10 g of flesh or a random SU of the batch. Lastly, gastropods bodies were blended with sterilized salt solution (NaCl 3%), then homogenized, obtaining the first dilution and from this the additional ones.

E. coli enumeration was performed according to the ISO 16649-2:2001 method. The results were expressed as Colony Forming Units (CFU) g^{-1}.

According to Serratore et al. (2019), the abundance of *Vibrio* spp. was checked on thiosulfate-citrate-bile salts-sucrose (TCBS) agar (OXOID) NaCl 3% by the spread plate method, and incubation at 20 °C for 3–5 days. The results were expressed as $\log_{10}$ CFU g^{-1}.

On the same SUs, the isolation of *V. vulnificus*, *V. cholerae*, *V. parahaemolyticus*, and *V. alginolyticus* were performed on CHROMagar™ Vibrio (CAV) (PBI) incubated at 37°C for 24 h. The colonies were provisionally scored: mauve as *V. parahaemolyticus*, green-blue to turquoise blue colonies as *V. cholerae/V. vulnificus* and colorless as *V. alginolyticus*. Then, as is the normal practice, the number of suspected colonies tested was from 1 to 5 (if available). Lastly, selected colonies were purified on Tryptone-Soya-Agar (TSA) (OXOID) NaCl 3% and then tested to confirm the typical traits as reported in Table 1 of Serratore et al. (2019).

Table 1. Phenotypical traits utilized to characterize the suspected colonies grown on CHROMagar™ Vibrio: *V. alginolyticus* colorless, *V. parahaemolyticus* mauve, *V. cholerae/V. vulnificus* green blue to turquoise blue. Expected results.

	TCBS Agar	m-CPC Agar	Gram-roads	Oxidase	Nitrate reduction	O129/150 µg S/R O129/10 µg S/R	SIM motility/indole/H_2S	A/L/O	KIA
VA	Y	ng	+	+	+	SR	+/+/-	-/+/+ or -	GF+/L-/no H_2S/no gas
VC	Y	P/G	+	+	+	SS	+/+/-	-/+/+ or -	GF+/L-/no H_2S/no gas
VV	G/Y	Y/W/ng	+	+	+	SS	+/+/-	-/+/+ or -	GF+/L+ or L-/no H2S/no gas
VP	G	ng	+	+	+	SR	+/+/-	-/+/+ or -	GF+/L-/no H_2S/no gas

VA, *V. alginolyticus*; VP, *V. parahaemolyticus*; VC/VV, *V. cholerae/V. vulnificus*; Y, yellow; G, green; W, white; P, purple; ng, no growth; GF, glucose fermentation; L, lactose utilization; SIM, Sulfide Indole Motility medium; A, arginine dihydrolase; L, lysine decarboxylase; O, ornithine decarboxylase; KIA, Kligler Iron Agar.

Suspected *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae* strains were also genotyped by Polymerase Chain Reaction (PCR) as reported by Serratore et al. (2019), utilizing specific primers targeting *tox*RP, *tdh*, and *trh* genes for *V. parahaemolyticus*; *vvh*A, *hsp*, *vcg*C, vcgE, CPS operon allele 1, CPS operon allele 2, 16s-rRNA type A gene, 16srRNA type B gene for *V. vulnificus*; *tox*RC, *hlya*, *tcp*I,

*tcp*A, *ctx*A, *ctx*B, *stn/sto* for *V. cholerae*. The results were expressed as the presence or absence of the targets.

In addition, 10 batches of *T. mutabilis* and one of *B. brandaris* were re-immersed in clean seawater into a sterile basin for 30 minutes and then were analyzed as described before.

Lastly, 5 batches of *T. mutabilis* were re-immersed for 18-24 hours in clean seawater into a Recirculating Aquaculture System (RAS) and then were analyzed as described before.

Statistical analysis was performed on *Vibrio* spp. and viability. According to the results of the Shapiro-Wilk test, to evaluate the possible correlation the Spearman's *rho* was calculated. Statistical significance was set at $p \leq 0.05$.

5. Results

As mentioned before, gastropods are considered safe "*if all the* [E. coli] *five values observed are ≤ 230 MPN/100 g of flesh […] or if one of the five values observed is > 230 MPN/100 g of flesh […] but ≤ 700 MPN/100 g of flesh*", according to EN/ISO 16649-3 [Reg. 2285/2015/EU]. *E. coli* enumeration was performed according to the ISO 16649-2:2001 method. *E. coli* was never detected in all batches, hence, according to the LOD of the method, results were expressed as <10 CFU g^{-1}. This result confirms our previous data [Serratore et al., 2019].

As outline in the introduction, *Vibrio* spp. could be accumulated into mollusks' flesh, but, even if marine gastropods are commonly consumed after long cooking, it is well known that *Vibrio* spp. may produce tetrodotoxins, which are thermostable. Using the method described above, we obtained that the abundance of *Vibrio* spp. in *T. mutabilis* batches was variable within 4.04 and 6.29 $\log_{10}$ CFU g^{-1} with a mean value of 5.44 ± 0.70 $\log_{10}$ CFU g^{-1} (median = 5.66), as can be seen in Table 3.1. In *B. brandaris*, *Vibrio* spp. was 6.52 $\log_{10}$ CFU g^{-1}. These results are in line with our previous study [Serratore et al., 2019] and remain notable if compared with the average *Vibrio* spp. load registered on *Ruditapes philippinarum* belonging to the same sea-area (4.69 $\log_{10}$ CFU g^{-1}) [Serratore et al., 2016].

As can be seen in Table 3.1, the *Vibrio* spp. load did not show a statistically significant correlation with viability ($p > 0.05$). In fact, in *T. mutabilis* batches, samples with viability up to 90% had a mean value of *Vibrio* spp. of 5.07 ± 0.82 $\log_{10}$

CFU g^{-1}, while samples with viability lower than 20% had 5.53 ± 0.70 log_{10} CFU g^{-1}.

Table 3.1: *Vibrio* spp. load, viability, and smell in *Tritia mutabilis* batches. S: saltwater; N: neutral; SA: slightly acrid; A: acrid; NA: nasty.

Sample Number - Date	**Vibrio spp.** (log_{10} CFU g^{-1})	**Smell/Viability** (%)	**Sample Number - Date**	**Vibrio spp.** (log_{10} CFU g^{-1})	**Smell/Viability** (%)
1433 - 15/01/19	4.34	SA/20%	1450 - 21/03/19	5.79	SA/70%
1434 - 17/01/19	5.92	SA/30%	1451 - 25/03/19	4.07	S/100%
1435 - 22/01/19	6.29	SA/40%	1452 - 03/04/19	5.76	SA/25%
1436 - 29/01/19	5.03	S/90%	1453 - 09/04/19	5.66	NA/0%
1437 - 31/01/19	6.07	S/90%	1458 - 25/06/19	5.76	S/88%
1439 - 13/02/19	5.73	S/70%	1470 - 12/02/20	5.53	S/100%
1440 - 13/02/19	5.71	SA/50%	1471 - 19/02/20	5.70	S/100%
1442 - 26/02/19	6.16	NA/0%	1472 - 24/02/20	4.04	S/100%
1443 - 28/02/19	5.18	S/25%	1486 - 19/10/20	6.23	S/100%
1446 - 07/03/19	5.48	SA/0%	1489 - 03/11/20	4.98	S/90%
1447 - 12/03/19	5.29	N/20%	1492 - 18/01/21	4.99	S/100%
1448 - 18/03/19	6.26	NA/0%	1495 - 22/02/21	4.09	S/100%
1449 - 19/03/19	4.88	S/80%			

All the batches resulted negative for pathogenic vibrios, which is in line with previous data, except for *V. parahaemolyticus*, because, in preliminary results, one batch of *B. brandaris* was positive.

Afterward, we evaluated the abundance of *Vibrio* spp. before and after a short re-immersion (30 minutes) in clean seawater into a basin. The results are shown in Table 3.2 and Figure 3.1.

As can be seen in Fig. 3.1, the *Vibrio* spp. load was similar between samples evaluated immediately and after a short re-immersion. This result may be explained by the fact that even if gastropods could have an exterior cleaning into seawater, they accumulate bacteria into the flesh and the re-immersion of 30 minutes is too shot to change the abundance into the body.

Table 3.2: *Vibrio* spp. load (log_{10} CFU g^{-1}) evaluated immediately vs. after a short re-immersion (30 minutes). The samples of *T. mutabilis* are from 1437 to 1451, while sample 1457 is of *B. brandaris*.

	1437	**1439**	**1442**	**1443**	**1446**	**1447**	**1448**	**1449**	**1450**	**1451**	**1457**
T0	6,07	5,73	6,16	5,18	5,48	5,29	6,26	4,88	5,79	4,07	6,52
After a short re-immersion	6,05	5,91	6,18	5,96	5,96	5,61	6,32	5,56	5,93	4,12	7,10

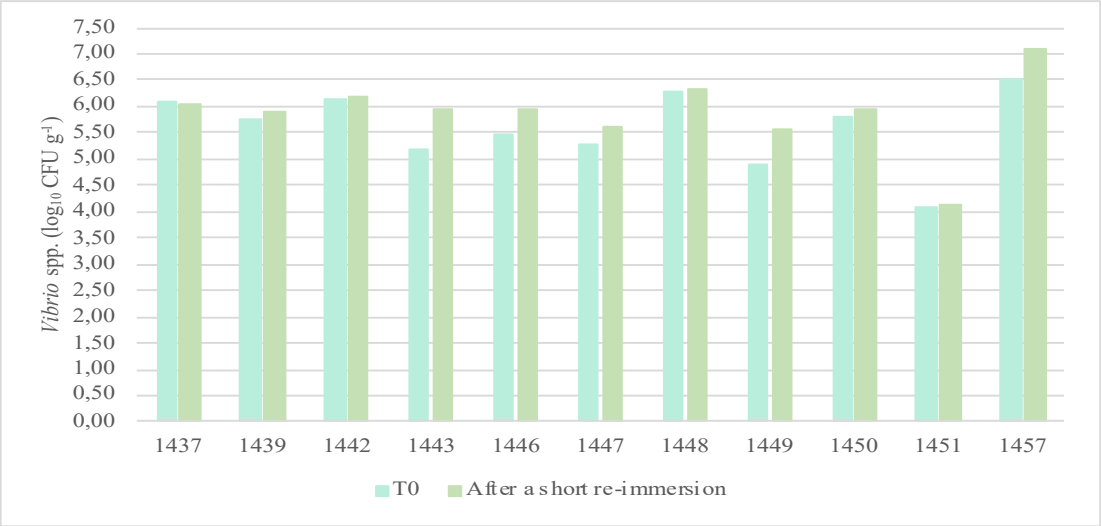

Figure 3.1: *Vibrio* spp. load evaluated immediately vs. after a short re-immersion (30 minutes). The samples of *T. mutabilis* are from 1437 to 1451, while sample 1457 is of *B. brandaris*.

Subsequently, five batches of *T. mutabilis* were analyzed immediately and after a long re-immersion (18-24 hours). As shown in Fig. 3.2, the *Vibrio* spp. load was variable: in fact, in 1452 and 1486 batches it was lower, on the contrary in 1489 and 1495 it was higher.

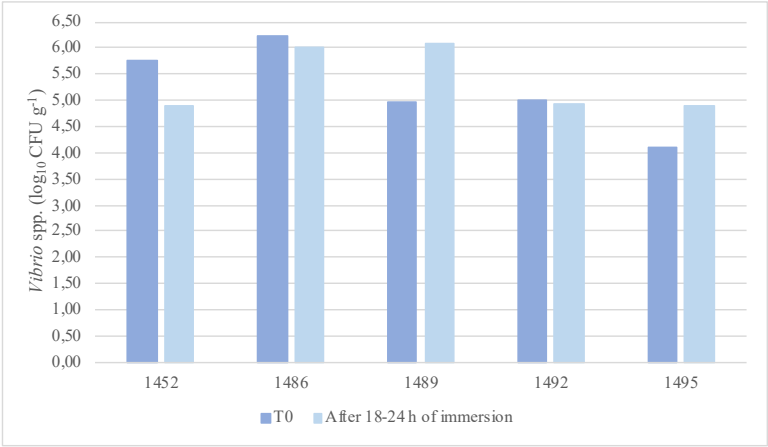

Figure 3.2: *Vibrio* spp. load evaluated immediately vs. after a long re-immersion (18 - 24 hours).

Overall, these results suggest that gastropods did not accumulate fecal contaminants, but vibrios due to their feeding. Moreover, by contrast with bivalves, *T. mutabilis* and *B. brandaris* are not filter-feeding and this might be a reason why bacteria might not have been eliminated during re-immersions.

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Section 4 - Shelf-life, indole-producing bacteria, and biogenic amines

1. Introduction

Shelf life is termed as "*the time, under defined storage conditions, during which food remains safe, retains desired sensory, chemical, physical and biological characteristics*" [Marzocco et al., 2010]. As previously mentioned, marine gastropods must be placed on the market alive [Reg. 624/2019/EU], however, there is not an official method to assess the viability and dispatch centers may have relabelled the package, which could lead to risks of these animals being sold dead as it is not known how many days gastropods survive in refrigerated conditions and what happens during their spoilage process.

The only example in literature of the shelf-life of *Tritia mutabilis* is Orlandi and Perna (1968). They valuated its shelf-life using response to stimuli, adhesiveness, water elimination, and body leak and have estimated a shelf-life of seven days at 0-2°C. However, after three days in refrigerated conditions, *T. mutabilis* has not reacted well to stimuli, has lost all its water, and has already had the 40% of the body exposed out of the shell.

Post-mortem changes in fish muscle could be summarized as catching – rigor mortis – resolution of rigor mortis – autolysis – spoilage [Hong et al., 2017]. "*Spoilage refers to any change in the condition of food in which the latter becomes less palatable, or even toxic; these changes may be accompanied by alterations in taste, smell, appearance, or texture*" [Cook, 1991]. The peculiarity of bivalves is that they have a higher content of free amino acid, lower levels of nitrogen, and a high amount of carbohydrates (particularly glycogen) in their flesh, which leads to fermentative spoilage characterized by a gradual decrease in pH and by the growth of lactic acid bacteria [Ashie et al., 1996; Cook, 1991; Gram, 2009; Jay, 1986]. In contrast, Orlandi and Perna (1968) affirm that the determination of pH for *T. mutabilis* is meaningless, even if gastropods have a high amount of glycogen, similar to Bivalvia [Livingstone and De Zwaan, 1983]. Moreover, marine gastropods have an open circulatory system, like bivalves [Jones, 1983; Narain, 1976; Smolowitz, 2012], hence, in their bloody flesh, they accumulated viruses and bacteria, which, in cold waters, are predominantly psychrophilic Gram-negative microbes, that lead to quicker spoilage than warm-water or tropical shellfish [Ashie et al., 1996].

Furthermore, it is well known that the refrigeration process does not kill the microorganisms, but it initially reduces the heterogeneous microbial population and then increases a selective one, depending on biochemical activities in tissues [Ashie et al., 1996; Ghaly et al., 2010], but it is unknown which one rises in marine gastropods and the microbial activity may produce indole and biogenic amines, which might be toxic for human [Ashie et al., 1996].

1.1 Indole-producing bacteria

Indole is produced in considerable amounts by both Gram-positive and Gram-negative bacteria, by using tryptophanase (TnaA), which can convert tryptophan into indole, ammonia, and pyruvate [Han et al., 2011; Lee and Lee, 2010; Li and Young, 2013; Roager and Licht, 2018]. It has been considered a pollutant, on account of its toxicity and potential mutagenicity and may cause hemolysis, glomerular sclerosis, hemoglobinuric nephrosis, improper oviduct functioning, temporary skin irritation, tumor formation, and chronic arthritis [Arora et al., 2015; Ma et al., 2018; Megna et al., 2016; Ochiai et al., 1986].

Indole has also been suggested as a spoilage indicator [Gram, 2009] in prawn [Thomas et al., 1995], crab [Sarnoski et al., 2010] and shrimp [Alexander, 1956; Mendes et al., 2002] and, nowadays, it is used to evaluate the shrimp decomposition with a maximum of 250 μg kg^{-1} by some regulatory agencies, including the US Food & Drug Administration (FDA) [Mendes et al., 2005].

1.2 Biogenic amines

Biogenic amines (BA) are low-molecular-weight nitrogenous compounds and derived from microbial decarboxylation of amino acids or by reductive amination and transamination of aldehydes or ketones by amino acid transaminases (Figure 4.1) [Kim et al., 2009; Park et al., 2010; Ruiz-Capillas and Jiménez-Colmenero, 2009; Visciano et al., 2012; Wójcik et al., 2021]. They can be divided by (i) the number of amine groups: monoamines (histamine, tyramine, tryptamine, dopamine, octopamine, serotonin, norepinephrine), diamines (cadaverine, putrescine), and polyamines (spermine, spermidine, agmatine) or (ii) the chemical structure: aromatic (tryptamine, ß-phenylethylamine, octopamine, dopamine, norepinephrine), aliphatic (putrescine, cadaverine, agmatine, spermine, spermidine), and heterocyclic ones

(serotonin, histamine, and tryptamine) [Önal, 2007; Özogul and Özogul, 2020; Vidal-Carou et al., 2009; Wójcik et al., 2021].

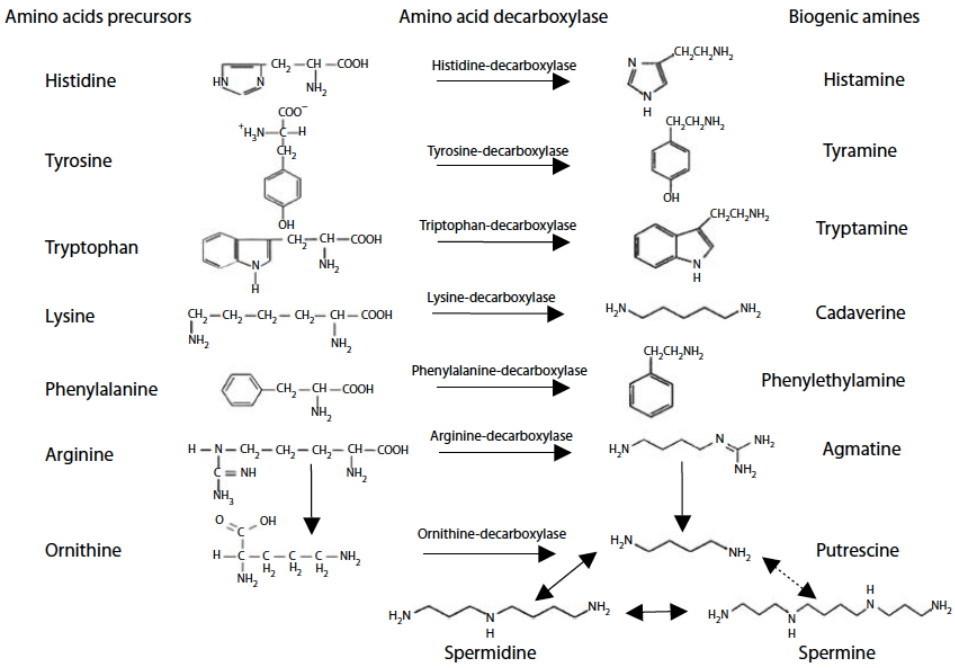

Figure 4.1: Biogenic amines formation from amino acid precursors [Ruiz-Capillas and Jiménez-Colmenero, 2009].

BAs can be produced by both Gram-negative (Enterobacteriaceae family, *Pseudomonas*, *Vibrio*, *Photobacterium*, *Aeromonas*, *Acinetobacter*) and Gram-positive bacteria (*Staphylococcus*, *Clostridium*, *Bacillus*) [Houicher et al., 2021; Refai et al., 2020; Ruiz-Capillas and Jiménez-Colmenero, 2009; Visciano et al., 2012; Visciano et al., 2020] and they are usually utilized as a spoilage indicator [Dabadè et al., 2021], by using mainly two indexes: one of Mietz and Karmas (called quality index QI, calculated as (histamine + putrescine + cadaverine)/(1 + spermine + spermidine)) and another one (biogenic amines index, BAI) that is the sum of the contents of histamine, cadaverine, putrescine and tyramine [Biji et al., 2016; Özogul and Özogul, 2020; Ruiz-Capillas and Herrero, 2019; Vidal-Carou et al., 2009; Wójcik et al., 2021]. According to Mietz and Karmas (1978), the limit of QI was set at 10, whilst scores of 0 and 1 indicate a good quality fish and between 1 and 10 are tolerable [Ruiz-Capillas and Herrero, 2019; Vidal-Carou et al., 2009], but nowadays it changes between seafood, e. g. the assessment below 2 for fish fillets, >0.8 for salmon steak and >5 for shrimps and lobsters determinates an acceptable product and scores between 2 and 10 for fish fillets, 0.8–8 for salmon steak, 5–25 for shrimps and 5–50

for lobster indicate the beginning of spoilage; above these values, seafood are unacceptable [Wójcik et al., 2021]. Hernández-Jover et al. (1996) proposed the BAI and suggested that <5 mg/kg indicating good quality, between 5 and 20 mg/kg for acceptable products but with signs of initial spoilage, between 20 and 50 mg/kg for low quality, and >50 mg/kg for spoilage [Ruiz-Capillas and Herrero, 2019; Wójcik et al., 2021], although some authors affirmed lower acceptable (15-20 mg kg^{-1}) limits for anchovy [Pons-Sánchez-Cascado et al., 2006; Prester, 2011] and Mediterranean hake [Baixas-Nogueras et al., 2005].

Despite the strong influence of BAs on food quality, only the histamine has a specific regulation in fishery products, but its limits change throughout the world. European Commission Regulations (2073/2005, 1441/2007, 1019/2013) regard only species within the Scombridae, Clupeidae, Eugraulidae, Coryphenidae, Pomatomidae, and Scomberesocidae families and affirm that the average histamine of nine samples should be below 100 mg kg^{-1}, but two of them are allowed to have a concentration between 100 and 200 mg kg^{-1} but no fish samples must exceed the limit of 200 mg kg^{-1}. These limits duplicate for fish products that have undergone enzyme maturation treatment in brine, manufactured from fish species associated with a high amount of histidine. On the other hand, the US FDA suggests a safe level of 50 mg kg^{-1} of histamine in fish meat [FDA, 2020], while South Korea, Australia, and New Zealand Food Standards Code states similarly to Europe [Biji et al., 2016; Özogul and Özogul, 2020; Ruiz-Capillas and Herrero, 2019] and South Africa sets a limit of 100 mg kg^{-1} [Houicher et al., 2021].

Histamine is a heterocyclic monoamine and high amounts of it cause an intoxication, called “scombroid poisoning” or “histamine fish poisoning”, of which common symptoms include a drop in blood pressure, skin irritation, tingling tongue, vomiting, nausea, diarrhea, headaches, dizziness, edemas, vasodilatation, intracranial bleeding, palpitation, breathing difficulties and rashes typical of allergic reactions [Comas-Basté et al., 2020; Feng et al., 2016; Hungerford, 2010; Özogul and Özogul, 2020; Ruiz-Capillas and Herrero, 2019; Ruman, 2020; Wójcik et al., 2021]. The histamine poisoning is usually self-limited with a short duration: indeed, symptoms appear within 10- 30 minutes after fish ingestion and resolve in 6 – 8 hours [Comas-Basté et al., 2020; Feng et al., 2016; Ruman, 2020]; only in rare cases, they last for days [Hungerford, 2010; Wójcik et al., 2021]. It should be underlined that other amines, like cadaverine and putrescine, are associated with this intoxication, because

they enhance the toxicity of histamine [Ruiz-Capillas and Herrero, 2019; Tabanelli, 2020; Wójcik et al., 2021]. Moreover, histamine is a mediator of allergic disorders and each and every person has a different histamine tolerance, hence, the same content of histamine could cause or not typical symptoms [Comas-Basté et al., 2020; Prester, 2011; Ruiz-Capillas and Herrero, 2019].

Tyramine is an aromatic monoamine and its poisoning is called "cheese reaction", which is characterized by a short incubation period (within 1 – 2 hours after ingestion) and by migraine, nausea, vomiting, gastrointestinal complaints, respiratory disorders, an increase in blood sugar, noradrenaline ejection and tachycardia; at worst, the increase in blood pressure could cause a heart attack, a stroke or symptoms of shock [Andersen et al., 2018; Marcobal et al., 2012; Özogul and Özogul, 2020; Ruiz-Capillas and Herrero, 2019; Tabanelli, 2020; Wójcik et al., 2021]. In addition, histamine, at concentrations below the legal limit, may increase the cytotoxicity of tyramine at normal concentrations reached in some foods, causing the cheese reaction [del Rio et al., 2017].

ß-phenylethylamine is an aromatic monoamine with an estimated toxic level of 30 mg kg^{-1} and may cause migraine and hypertensive crisis; moreover, it enhances histamine toxicity, by the inhibition of enzymes diamine oxidase and histamine methyl-transferase [Biji et al., 2016; Bilgin and Gençcelep, 2015; Marcobal et al., 2012; Özogul and Özogul, 2020; Ruiz-Capillas and Jiménez-Colmenero, 2009; Wójcik et al., 2021].

Cadaverine and putrescine are aliphatic diamines, that not only enhance the toxicity of histamine, interfering with the detoxification system, but also have their own cytotoxicity and may react with nitrites producing nitrosamines (putrescine is converted in N-nitrosopyrrolidine and cadaverine in N-nitrosopiperidine), that are carcinogenic compounds [Biji et al., 2016; del Rio et al., 2019; Prester, 2011; Tabanelli, 2020; Visciano et al., 2020; Wójcik et al., 2021].

Spermine and spermidine are aliphatic polyamines produced from putrescine and may enhance histamine toxicity, react with nitrites, and have their own toxicity, resulting in a decrease in blood pressure, respiratory symptoms, and nephrotoxicity [Biji et al., 2016; Özogul and Özogul, 2020].

Agmatine is an aliphatic polyamine, is used as a spoilage indicator particularly in cephalopods, increases histamine toxicity, and is neurotoxic enhancing

the glutamate release and causing uremia [Galgano et al., 2012; Halaris and Plietz, 2007; Visciano et al., 2020; Uzbay et al., 2017].

2. Methodology

A total of 8 batches of *T. mutabilis* and one batch of *Bolinus brandaris* were investigated to value how some parameters of previous sections change during refrigerated conditions. On the other hand, 19 batches of *T. mutabilis* were assessed for indole-producing bacteria and 22 batches for biogenic amines.

Firstly, a batch was evaluated for viability, smell, and microbiological analysis (as described in previous sections), and then the remaining part was placed inside a bowl covered with aluminum into a refrigerator at 2-8°C. Every day of sampling, some specimens were removed from the bowl and analyzed. There is only an important remark: throughout shelf-life trials, the smell was evaluated immediately after the sample was removed from the refrigerator, while the viability was evaluated 20 minutes later, because gastropods do not react well if assessed before.

In addition, in 7 batches of *T. mutabilis*, the total viable count (TVC) of heterotrophic bacteria and the enumeration of H_2S-producing bacteria were performed on Iron Agar Lyngby (Atlas, 1995), incubated at 25°C for 48 h. Black colonies are scored as H_2S-producing bacteria on the media, while TVC is derived from the sum of black and white colonies. Moreover, in 3 batches, *Pseudomonas* spp. enumeration was performed according to the ISO 13720:2010 method. The results were expressed as $\log_{10}$ CFU g^{-1}.

For indole-producing bacteria, test tubes were prepared with 5 or 10 ml of tryptone water 1% NaCl 3%, were distributed to make a 3-tube MPN (dilutions change during shelf-life tests) and were incubated for 3-5 days at 20°C. The results were expressed as Most Probable Number (MPN) g^{-1} following the table of ISO 7218:2007.

Biogenic amines analysis was performed by an external service (FoodMicroTeam s.r.l., Academic Spin-off of the University of Florence, Italy), using an HPLC method employing a UV detector after derivatization by Dansyl chloride.

Statistical analysis was performed on indole-producing bacteria and *Vibrio* spp., and viability. According to the results of the Shapiro-Wilk test, to evaluate the

possible correlation Pearson's correlation coefficient and Spearman's *rho* were calculated. Statistical significance was set at $p \leq 0.05$. Paired samples *t*-test was used to compare viability, *Vibrio* spp., TVC and SSOs load, BAI and IPB content between T0 and T3 and T0 and T6-7 within treatment groups (with or without immersion). Statistical significance was set at $p \leq 0.05$.

3. Results

As outlined in the introduction, there is not an official method to assess the viability and it is not known how many days gastropods survive in refrigerated conditions and what happens during their spoilage process. Hence, initially, we observed in *T. mutabilis* how viability and smell change during refrigerated conditions (see Table 4.1 and Figure 4.2).

Table 4.1: Viability, and smell in *Tritia mutabilis* batches during refrigerated conditions. S: saltwater; N: neutral; SA: slightly acrid; A: acrid; NA: nasty.

	Viability (%)	Smell		Viability (%)	Smell
1451 - 25/03/19	100	S	**1486** - 19/10/20	100	S
T1	33	N	T1	100	S
T2	0	N	T2	94	S
T3	0	A	T3	84	A
T4	0	A	T7	10	NA
1470 - 12/02/20	100	S	**1489** - 03/11/20	90	S
T1	74	S	T1	80	S
T2	32	S	T2	35	A
T3	73	N	T3	16	A
T4	26	SA	T6	0	NA
T5	34	A	**1492** - 18/01/21	100	S
T6	14	NA	T1	100	N
1471 - 19/02/20	100	S	T2	97	SA
T1	86	S	T3	51	A
T2	44	S	T7	0	NA
T3	54	SA	**1495** - 22/02/21	100	S
T4	17	NA	T1	100	N
T5	0	NA	T2	100	N
1472 - 24/02/20	100	S	T3	100	SA
T1	73	S	T7	10	NA
T2	69	SA			
T3	59	SA			
T4	40	NA			
T5	25	NA			

As evident from Figure 4.2, the overall response was a downward trend: after four days, at least half of each batch consisted of dead gastropods and within a week almost all individuals were not viable. Our results are comparable to the outcome of

Orlandi and Perna (1968). Moreover, all batches started from a 100% of viability (expect from 1489), and after one day in refrigerated conditions, they showed an 80,8% (SD= 22,4) on average. This result is interesting since only mortality of 10% is almost always accepted by official authorities at the market: 62,5% of the batches should not have been sold after only one day in refrigerated conditions, and almost all the samples then three days. Additionally, only 1451 had a different trend with a rapid decrease (see Fig. 4.2) probably because it was the only batch from retail, and gastropods may have been stressed more than individuals of samples from primary production.

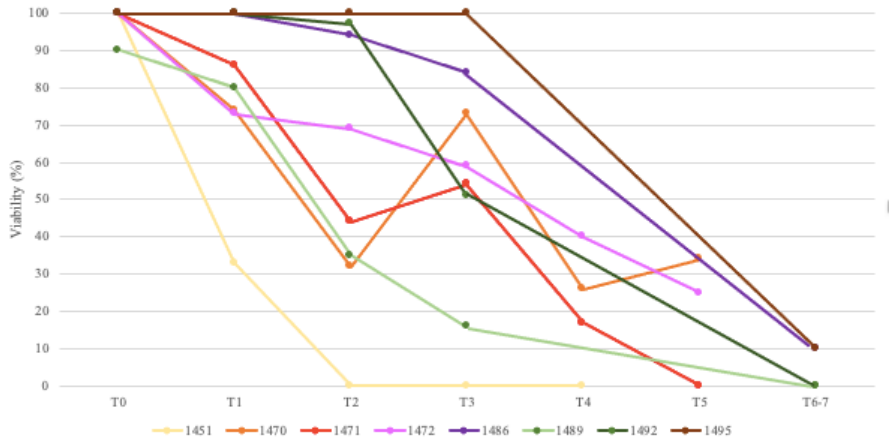

Figure 4.2: Viability of *T. mutabilis* evaluated during refrigerated conditions.

The smell had also a downward trend, following our chosen five descriptors. All the batches began with a saltwater smell and finished with a nasty odor. Hence, the smell could be a good freshness indicator.

Afterward, we evaluated the abundance of *Vibrio* spp. in refrigerated conditions. All the results of *T. mutabilis* are given in Table 4.2 and illustrated in Figure 4.3.

Table 4.2: *Vibrio* spp. load (log_{10} CFU g^{-1}) in *Tritia mutabilis* batches during refrigerated conditions.

Sample Number	T0	T1	T2	T3	T4	T5	T6-7
1451	4.07	5.66	5.65	6.76	6.80	/	/
1470	5.53	4.74	5.71	4.00	5.68	6.46	/
1471	5.70	5.49	6.12	5.08	5.20	6.76	/
1472	4.04	4.41	4.67	5.38	5.12	5.63	/
1486	6.23	4.63	5.25	5.88	/	/	6.49
1489	4.98	4.00	5.64	4.60	/	/	6.81
1492	4.99	5.09	5.75	5.45	/	/	6.99
1495	4.09	3.81	4.86	5.30	/	/	6.30
M ± SD	**4.95** ± 0.84	**4.73** ± 0.66	**5.46** ± 0.49	**5.31** ± 0.82	**5.70** ± 0.77	**6.28** ± 0.58	**6.65** ± 0.31

Contrary to viability and smell, *Vibrio* spp. load on average tended to increase over time. In fact, except for 1486, all the samples showed a difference of about one logarithm between the initial and the terminal load. It also appears that the abundance of *Vibrio* spp., on average, was slightly reduced after one day, then remained approximately constant between T_2 and $T_{3\text{-}4}$, and lastly increased. In fact, there was not a statistically significant difference between T_0 and T_3 ($p > 0.05$) but in the last four samples (1486, 1489, 1492, and 1495) the *Vibrio* spp. load was significantly higher at T_6 than at T_0 (paired-*t*: -3.538; p=0.038). It is well known that refrigeration slows (or even stops) bacterial growth, so it may be an explanation for these results.

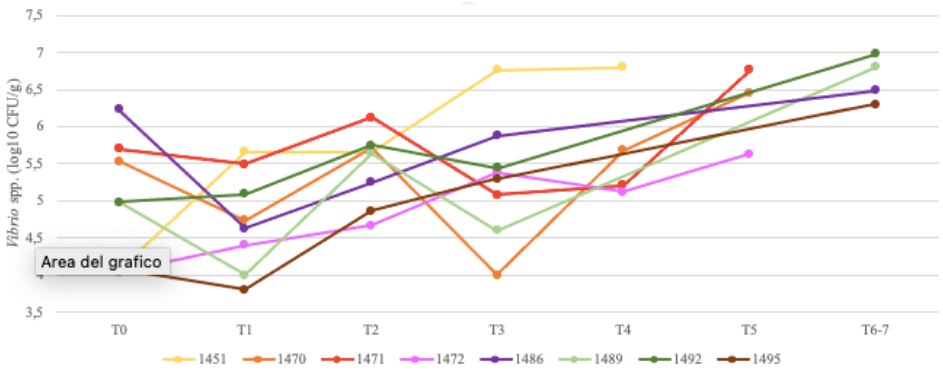

Figure 4.3: *Vibrio* spp. load evaluated during refrigerated conditions in *Tritia mutabilis*.

The total viable count (TVC) and the enumeration of H_2S-producing bacteria were also determined to estimate the abundance of heterotrophic bacteria and specific spoilage organisms (SSOs), respectively. Results of TVC are given in Table 4.3 and are displayed in Figure 4.4.

Table 4.3: Total viable count ($\log_{10}$ CFU g^{-1}) in *Tritia mutabilis* during refrigerated conditions.

Sample Number	T0	T1	T2	T3	T4	T5
1470	5.74	5.09	6.13	5.00	6.43	6.74
1471	6.10	6.11	6.69	6.06	6.29	7.36
1472	4.61	4.81	5.23	6.11	5.66	6.41
1486	6.42	5.05	5.53	6.40	/	/
1489	/	4.73	5.66	6.15	/	/
1492	/	5.60	6.03	6.31	/	/
1495	/	4.77	5.33	5.57	/	/
M ± SD	**5.72** ± 0.79	**5.17** ± 0.51	**5.80** ± 0.51	**5.94** ± 0.49	**6.13** ± 0.41	**6.84** ± 0.49

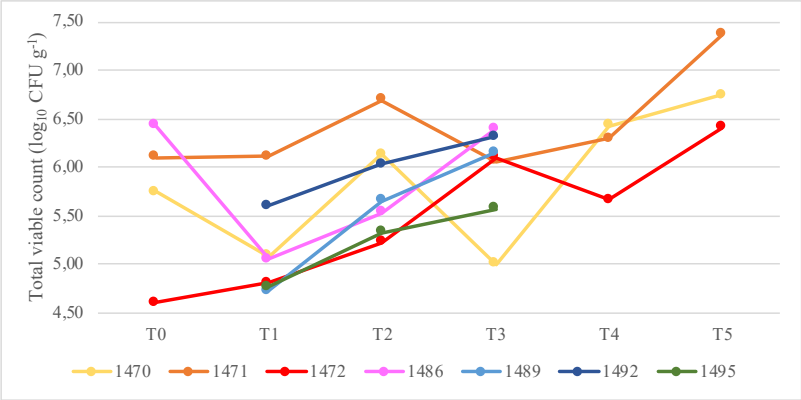

Figure 4.4: Total viable count in *Tritia mutabilis* during refrigerated conditions.

T. mutabilis showed an average TVC higher than 5 logarithms since the beginning, which remained approximately constant until T_3. A slight increase was presented from T_4. It is also notable that TVC, on average, was slightly reduced after one day in refrigerated conditions, identical to the *Vibrio* spp. load. All these data resulted in a statistically significant difference between T_1 and T_3 (paired-*t*: -3.214; p=0.018). Additionally, 54,8% of the samples (n= 31) exceeded recommended maximum limits for fish and shellfish of 5 x 10^5 CFU g^{-1} [ICMSF, 1986].

The SSOs may produce off-odors (in particular, trimethylamine in seafood spoilage) and spoilage metabolites (biogenic amines, ammonia, organic acids, and sulfur compounds from amino acids, acetate from lactate and hypoxanthine from ATP degradation products) [Gram and Dalgaard, 2002]. As can be seen in Table 4.4, the SSOs had a similar trend to TVC and were approximately one logarithm less than it. In fact, data presented a statistically significant difference (paired-*t*: -3.076; p=0.022) between T_1 and T_3 as TVC. The load was considerably high from the beginning probably due to gastropods' feeding: in fact, they are scavengers and dead fishes may have a high presence of SSOs.

Table 4.4: Specific spoilage organism (log_{10} CFU g^{-1}) in *Tritia mutabilis* during refrigerated conditions.

Sample Number	T0	T1	T2	T3	T4	T5
1470	5.08	4.06	5.22	4.00	5.49	5.70
1471	5.46	5.15	5.90	5.33	5.38	6.61
1472	3.44	3.78	4.48	4.90	4.54	5.30
1486	5.57	3.88	4.04	5.27	/	/
1489	/	3.90	5.31	5.02	/	/
1492	/	4.70	5.23	4.90	/	/
1495	/	3.65	4.65	4.30	/	/
M ± SD	**4.89** ± 0.99	**4.16** ± 0.55	**4.98** ± 0.62	**4.82** ± 0.49	**5.14** ± 0.52	**5.87** ± 0.67

We also analyzed *Pseudomonas* spp. load to evaluate its presence as a spoilage indicator. According to Table 4.5, *Pseudomonas* spp. load resulted approximately 4 logarithms (total samples average = 4.20 $\log_{10}$ CFU g^{-1}). It seems that the load was poorly reduced between T_1 and T_2 and then slightly raised. In fact, there was not a statistically significant difference ($p>0.05$) between both T_0 and T_3 and T_0 and T_5. The high load of *Pseudomonas* spp. is probably due to the same reason of the abundance of SSOs.

Table 4.5: *Pseudomonas* spp. load ($\log_{10}$ CFU g^{-1}) in *Tritia mutabilis* during refrigerated conditions.

Sample Number	T0	T1	T2	T3	T4	T5
1470	3.65	4.21	3.40	4.45	3.65	4.58
1471	4.56	4.39	3.18	3.40	4.13	4.59
1472	3.99	3.36	4.56	4.88	4.96	5.75
M ± SD	**4.07** ± 0.37	**3.99** ± 0.55	**3.71** ± 0.74	**4.24** ± 0.76	**4.25** ± 0.66	**4.97** ± 0.67

For what to concerns *B. brandaris*, one batch (1457 – 25/06/19) was analyzed for viability, smell, *Vibrio* spp. and divided into two parts as follows: half of the batch was evaluated immediately and during refrigerated conditions, while the other half was re-immersed into a basin for 30 minutes and then analyzed both right after the re-immersion and during refrigerated conditions. All the data are illustrated in Figure 4.5 and Table 4.6.

Figure 4.5: Viability of *Bolinus brandaris* evaluated immediately and after a short re-immersion (30 minutes) during refrigerated conditions.

As can be observed in Figure 4.5, the viability of *B. brandaris* decreased over time, but slower than *T. mutabilis*. The trends were similar between the half of the batch analyzed "normally" and the other half re-immersed, even if the latter was

always slightly higher. As already discussed, viability lower than 90% is not accepted by official authorities, and the re-immersed half batch gained an additional day of shelf-life. The smell was almost identical between the two parts of the batch. The abundance of *Vibrio* spp. had an initial decrease and then raised, but the trends of the two halves of the batch are different: in fact, the re-immersed part reached the minimum value at T_2, while the other part had it at T_1, and then both increased. Comparing Table 4.6 and 2 shows that *T. mutabilis* increased by about one logarithm within a week, while *B. brandaris* showed a *Vibrio* spp. load comparable with the original abundance.

Table 4.6: *Vibrio* spp. load, viability, and smell in *Bolinus. brandaris* evaluated immediately and after a short re-immersion (30 minutes) during refrigerated conditions batch. S: saltwater; N: neutral; SA: slightly acrid; A: acrid; NA: nasty.

	Viability (%)	**Smell**	***Vibrio* spp.** (log_{10} CFU g^{-1})
1457 - 25/06/19	100	S	6.52
After a short re-immersion	100	S	7.10
T1	91	S	5.44
T1 after re-immersion	98	S	6.36
T2	90	N	6.49
T2 after re-immersion	94	N	5.18
T3	85	N	6.18
T3 after re-immersion	94	SA	5.80
T6	36	NA	6.88
T6 after re-immersion	41	NA	7.60

As resulted from the viability section, gastropods in clean seawater could recovery and/or preserve viability, but a short re-immersion did not seem enough to make a difference in shelf-life. Hence, we analyzed (for viability, smell, *Vibrio* spp., TVC, SSO) four batches of *T. mutabilis* divided into two parts as follows: half of the batch was evaluated immediately and during refrigerated conditions, while the other half was re-immersed into a tank for 18-24 hours and then analyzed both right after the re-immersion and during refrigerated conditions.

According to Table 4.7, re-immersed samples had acceptable viability (except for 1489) even after three days in refrigerated conditions, while the others were unacceptable: hence, a long re-immersion resulted in an extra day of shelf-life. However, there was not a statistically significant difference both in re-immersed and non-re-immersed samples between T_0 and T_3. On the contrary, the viability at T_0 is significantly higher than at $T_{6\text{-}7}$ with (paired-*t*: 6.489; p=0.007) or without (paired-*t*:

37; $p<0.05$) the re-immersion. The smell followed the evolution described by Orlandi and Perna (1968), so our chosen descriptors could be adequate.

Table 4.7: Viability (%) and smell in *Tritia mutabilis* batches evaluated immediately (yellow) and after a long re-immersion (18 – 24 hours) (orange) during refrigerated conditions. S: saltwater; N: neutral; SA: slightly acrid; A: acrid; NA: nasty.

	T0	T1	T2	T3	T6-7
1486	100 - S	100 - S	94 - S	84 - A	10 - NA
	100 - S	100 - S	100 - S	93 - N	22 - NA
1489	90 - S	80 - S	35 - A	16 - A	0 - NA
	99 - S	84 - N	84 - SA	84 - A	56 - NA
1492	100 - S	100 - N	97 - SA	51 - A	0 - NA
	100 - S	100 - N	100 - N	100 - SA	58 - NA
1495	100 - S	100 - N	100 - N	100 - SA	10 - NA
	100 - S	100 - S	100 - SA	100 - SA	35 - NA

Table 4.8: *Vibrio* spp. load (log_{10} CFU g^{-1}) in *Tritia mutabilis* batches evaluated immediately (yellow) and after a long re-immersion (18 – 24 hours) (orange) during refrigerated conditions.

	T0	T1	T2	T3	T6-7
1486	6.23	4.63	5.25	5.88	6.49
	6.03	5.79	4.64	5.53	5.89
1489	4.98	4.00	5.64	4.60	6.81
	6.08	4.76	5.36	5.43	4.66
1492	4.99	5.09	5.75	5.45	6.99
	4.92	5.23	5.86	5.58	6.25
1495	4.09	3.81	4.86	5.30	6.30
	4.91	5.48	5.87	5.31	5.90
M ± SD	**5.07** ± 0.88	**4.38** ± 0.59	**5.38** ± 0.40	**5.31** ± 0.53	**6.65** ± 0.31
	5.48 ± 0.66	**5.31** ± 0.44	**5.43** ± 0.58	**5.46** ± 0.12	**5.67** ± 0.69

The mean *Vibrio* spp. load (see Table 4.8) of non-re-immersed samples showed the same trend described before: it decreased initially, then was steady between T_2 and T_3, and finally increased; instead, in re-immersed samples, it remained similar during refrigeration. In fact, there was not a statistically significant difference ($p>0.05$) in re-immersed samples, whereas the *Vibrio* spp. load was significantly higher at $T_{6\text{-}7}$ than at T_0 in non-re-immersed batches (paired-*t*: -3.538; $p=0.038$). No significant difference ($p>0.05$) was observed in *Vibrio* spp. load between T_0 and T_3.

As regards TVC (see Table 4.9), non-re-immersed samples had a significant difference between T_1 and T_3 (paired-*t*: -5.834; $p=0.010$), while re-immersed samples

also showed an increase, but not statistically significant ($p>0.05$), and did not have a slight reduction after one day in refrigerated conditions.

Table 4.9: Total viable count (log_{10} CFU g^{-1}) in *Tritia mutabilis* batches evaluated immediately (yellow) and after a long re-immersion (18 – 24 hours) (orange) during refrigerated conditions.

	T0	T1	T2	T3
1486	6.42	5.05	5.53	6.40
	/	5.89	5.31	6.07
1489	/	4.73	5.66	6.15
	6.00	5.73	6.12	6.23
1492	/	5.60	6.03	6.31
	5.68	5.94	6.34	6.29
1495	/	4.77	5.33	5.57
	4.85	5.66	6.10	6.06
M ± SD	/	**5.04** ± 0.40	**5.64** ± 0.29	**6.11** ± 0.37
	5.51 ± 0.60	**5.80** ± 0.13	**5.97** ± 0.45	**6.16** ± 0.11

Table 4.10: Specific spoilage organism (log_{10} CFU g^{-1}) in *Tritia mutabilis* batches evaluated immediately (yellow) and after a long re-immersion (18 – 24 hours) (orange) during refrigerated conditions.

	T0	T1	T2	T3
1486	5.57	3.88	4.04	5.27
	/	5.23	4.45	5.19
1489	/	3.90	5.31	5.02
	5.90	5.04	5.54	5.46
1492	/	4.70	5.23	4.90
	4.48	4.81	5.18	5.18
1495	/	3.65	4.65	4.30
	3.88	4.54	5.04	5.16
M ± SD	/	**4.03** ± 0.46	**4.81** ± 0.59	**4.87** ± 0.41
	4.75 ± 1.04	**4.91** ± 0.30	**5.05** ± 0.45	**5.25** ± 0.14

As can be seen in Table 4.10, the SSOs had a similar trend to TVC but were approximately one logarithm less. In fact, non-re-immersed samples had a significant difference between T_1 and T_3 (paired-*t*: -3.201; $p=0.049$), whereas in re-immersed samples SSOs had not a significant difference ($p>0.05$). The only variation was that, on average, TVC had a more linear increase, while SSOs showed a strong increment between T_1 and T_2 and then were constant at T_3.

As mentioned before, indole is both a pollutant (on account of its toxicity and potential mutagenicity) and a spoilage indicator. It is produced by bacteria, by using tryptophanase. In our study, we measured the abundance of indole-producing bacteria (IPB), which resulted on average 2.40 ± 0.93 log_{10} MPN g^{-1} at T_0 (see Table 4.11).

They did not show a statistically significant correlation with viability and *Vibrio* spp. load ($p > 0.05$).

Table 4.11: Indole-producing bacteria (log_{10} MPN g^{-1}) in *Tritia mutabilis*.

Sample Number - Date	IPB log_{10} MPN g^{-1}	Sample Number - Date	IPB log_{10} MPN g^{-1}
1435 - 22/01/19	1.08	1450 - 21/03/19	4.04
1436 - 29/01/19	2.18	1451 - 25/03/19	2.97
1437 - 31/01/19	1.58	1470 - 12/02/20	1.66
1438 - 05/02/19	1.63	1471 - 19/02/20	3.04
1439 - 13/02/19	2.18	1472 - 24/02/20	2.18
1442 - 26/02/19	4.04	1486 - 19/10/20	4.04
1443 - 28/02/19	2.20	1489 - 03/11/20	2.81
1447 - 12/03/19	1.63	1492 - 18/01/21	1.63
1448 - 18/03/19	1.38	1495 - 22/02/21	2.30
1449 - 19/03/19	3.08		

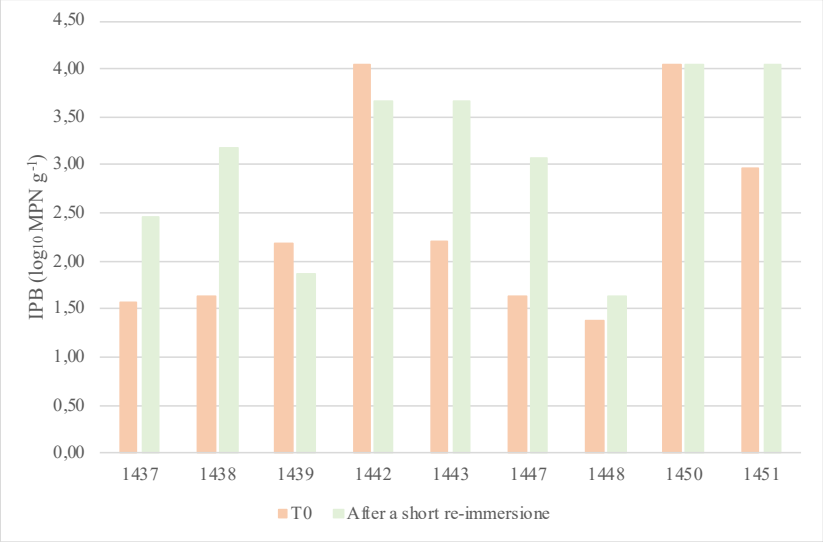

Figure 4.6: Indole-producing bacteria in *Tritia mutabilis* evaluated immediately and after a short re-immersion (30 minutes).

Subsequentially, 9 samples of *T. mutabilis* were evaluated immediately and after a short re-immersion (30 minutes) into a basin. As can been observed in Fig. 4.6, in 5 batches (56%) IPB load was higher than one logarithm in re-immersed samples compared to others.

Next, IPB load was evaluated during refrigerated conditions in 8 batches of *T. mutabilis*. As evident in Table 4.12, the abundance of IPB showed a difference of more than one logarithm on average between T_0 and $T_{5\text{-}7}$. It increased gradually within a week, but it reduced at T_3, on average, in fact, there was not a statistically

significant difference between T_0 and T_3 ($p>0.05$), but there was between T_0 and T_{5-7} (paired-*t*: -5.264; p=0.006)

Finally, we analyzed four batches of *T. mutabilis* divided into two parts as follows: half of the batch was evaluated immediately and during refrigerated conditions, while the other half was re-immersed into a tank for 18-24 hours and then analyzed both right after the re-immersion and during refrigerated conditions. Results are given in Table 4.13. IPB load seemed floaty both in re-immersed and non-re-immersed samples, even if it increased by one logarithm on average within a week. In fact, there was not a statistically significant difference ($p>0.05$) between T_0 and T_3 both in re-immersed and non-re-immersed samples.

Table 4.12: Indole-producing bacteria (log_{10} MPN g^{-1}) in *Tritia mutabilis* during refrigerated conditions.

	T0	**T1**	**T2**	**T3**	**T4**	**T5-7**
1451	2.97	3.66	4.18	1.36	3.56	/
1470	1.66	2.58	5.04	4.63	5.18	5.18
1471	3.04	4.04	4.18	5.18	4.96	5.04
1472	2.18	3.18	3.63	3.20	3.88	5.04
1486	4.04	/	/	2.96	/	/
1489	2.81	2.04	4.18	1.56	/	/
1492	1.63	1.36	3.04	2.54	/	2.56
1495	2.30	3.81	2.88	3.18	/	4.45
M ± SD	**2.58** ± 0.80	**2.95** ± 1.00	**3.87** ± 0.75	**3.08** ± 1.33	**4.39** ± 0.80	**4.45** ± 1.10

Table 4.13: Indole-producing bacteria (log_{10} MPN g^{-1}) in *Tritia mutabilis* batches evaluated immediately (yellow) and after a long re-immersion (18 – 24 hours) (orange) during refrigerated conditions.

	T0	**T1**	**T2**	**T3**	**T6-7**
1486	4.04	/	/	2.96	/
	3.30	/	/	3.38	/
1489	2.81	2.04	4.18	1.56	/
	3.66	1.36	2.63	1.56	/
1492	1.63	1.36	3.04	2.54	2.56
	1.63	2.97	3.15	3.63	4.18
1495	2.30	3.81	2.88	3.18	4.45
	3.66	1.88	2.81	4.32	4.18
M ± SD	**2.70** ± 1.02	**2.40** ± 1.26	**3.36** ± 0.71	**2.56** ± 0.72	**3.50** ± 1.34
	3.07 ± 0.97	**2.07** ± 0,82	**2.86** ± 0.26	**3.22** ± 1.18	**4.18** ± 0.00

As outlined in the introduction, BAs are toxic for humans and are a spoilage indicator. We evaluated histamine (Hist), putrescine (Put), cadaverine (Cad), tyramine (Tyr), spermidine (Spermid), spermine (Sperm), agmatine (Agma), phenylethylamine (Phenyl), ethanolamine (Etha) and we used the BAI index (BAI=

histamine + putrescine + cadaverine + tyramine) to compare our results to spoilage limits repoted in literature. In this part, we also analyzed samples previously collect from October 2017.

Table 4.14: Viability of the batches (%), Origin (Or: primary production, PP, and commerce, C), *Vibrio* spp. load (log_{10} CFU g^{-1}), BAs content (mg Kg^{-1}): histamine (Hist), putrescine (Put), cadaverine (Cad), tyramine (Tyr), spermidine (Spermid), spermine (Sperm), agmatine (Agma), phenylethylamine (Phenyl), ethanolamine (Etha). BAI= Histamine + Putrescine + Cadaverine + Tyramine (mg Kg^{-1}). SP: spreaders.

Sample Number	Or	Viability	*Vibrio* spp.	Agma	Etha	Phenyl	Put	Cad	Hist	Tyr	Spermid	Sperm	BAI
1389	PP	100%	5.92	4.4	<1	9.0	38.3	13.3	15.6	6.5	7.4	20.7	73.7
1406	C	70%	6.32	8.5	<1	10.4	14.5	15.9	6.0	5.7	9.1	22.7	42.1
1433	C	20%	4.34	3.5	<1	5.5	9.6	19.1	2.0	3.3	5.9	17.7	34.0
1434	C	30%	5.92	2.7	<1	4.3	16.9	21.1	5.4	4.6	6.3	17.7	48.0
1435	C	40%	6.29	5.7	<1	3.8	29.6	43.6	7.0	6.7	5.5	18.3	86.9
1436	C	90%	5.03	4.8	<1	4.2	12.6	27.9	4.0	5.4	6.9	17.7	49.9
1437	C	90%	6.07	13.5	<1	5.0	16.0	27.9	2.3	4.6	10.6	26.5	50.8
1438	C	30%	SP	<1	<1	6.0	172	24.9	2.5	25.7	13.9	30.6	225.1
1439	C	70%	5.73	10.8	<1	5.6	19.7	19.3	4.6	2.5	10.1	28.2	46.1
1442	C	0%	6.16	8.0	<1	17.6	24.1	36.5	16.9	13.2	12.3	28.4	90.7
1443	C	25%	5.18	5.0	<1	9.4	8.0	12.6	1.7	8.5	9.5	33.7	30.8
1446	C	0%	5.48	7.8	23.5	9.2	18.2	26.4	5.9	9.6	9.6	22.9	60.1
1447	C	20%	5.29	6.5	<1	9.3	8.2	16.3	5.0	7.8	9.4	31.2	37.3
1448	C	0%	6.26	5.9	<1	7.7	20.7	27.4	4.2	10.9	7.8	21.2	63.2
1449	C	80%	4.88	9.8	<1	6.8	8.1	28.5	39.0	7.7	9.9	37.7	83.3
1470	PP	100%	5.53	10.6	<1	3.5	5.1	17.1	19.0	5.5	7.8	30.5	46.7
1471	PP	100%	5.70	4.9	<1	8.8	15.8	33.5	3.8	5.1	7.8	31.0	58.2
1472	PP	100%	4.04	8.8	<1	1.8	6.3	8.7	3.9	4.3	6.1	17.5	23.2
1486	PP	100%	6.23	7.0	<1	<1	16.0	13.0	14.0	1.0	8.0	28.0	44.0
1489	PP	90%	4.98	15.0	<1	<1	47.0	48.0	51.0	2.0	11.0	25.0	115.0
1492	PP	100%	4.99	8.0	<1	2.0	5.0	18.0	18.0	2.0	6.0	26.0	43.0
1495	PP	100%	4.09	10.0	<1	<1	5.0	15.0	14.0	2.0	7.0	30.0	36.0

Generally speaking, BAI resulted 63.1 mg kg^{-1} on average (see Table 4.14) and did not present a statistically significant correlation with viability and *Vibrio* spp. load ($p > 0.05$). More specifically, while not differing significantly ($p > 0.05$), batches from commerce showed a mean of 67.7 mg kg^{-1}, whereas samples from primary production showed 55.0 mg kg^{-1} on average. As mentioned before, Hernández-Jover et al. (1996) suggested that a BAI > 50 mg kg^{-1} indicates a spoilage product, although some authors affirmed lower acceptable (15-20 mg kg^{-1}) limits for anchovy [Pons-Sánchez-Cascado et al., 2006; Prester, 2011] and Mediterranean hake [Baixas-Nogueras et al., 2005]. All the results were higher than 20 mg kg^{-1} and on average higher than 50 mg kg^{-1}. Nevertheless, in all the samples histamine resulted lower than the acceptable legal European limit (100 mg kg^{-1}) and only one batch exceeded the

US safe level (50 mg kg^{-1}). But it should also be underlined that other amines, like cadaverine and putrescine, enhance the toxicity of histamine [Ruiz-Capillas and Herrero, 2019; Tabanelli, 2020; Wójcik et al., 2021]. Ethanolamine was never detected, except from one sample (1446-19).

Table 4.15: BAs evaluated immediately (yellow) and after a long re-immersion (18 – 24 hours) (orange) during refrigerated conditions. Viability of the batches (%), *Vibrio* spp. load (log_{10} CFU g^{-1}), BAs content (mg Kg^{-1}): histamine (Hist), putrescine (Put), cadaverine (Cad), tyramine (Tyr), spermidine (Spermid), spermine (Sperm), agmatine (Agma), phenylethylamine (Phenyl), ethanolamine (Etha). BAI= Histamine + Putrescine + Cadaverine + Tyramine (mg Kg^{-1}).

Sample Number	Viability	Vibrio spp.	Agma	Etha	Phenyl	Put	Cad	Hist	Tyr	Spermid	Sperm	BAI
1486	100%	6.23	7.0	<1	<1	16.0	13.0	14.0	1.0	8.0	28.0	44.0
T18-24h Post IMM	100%	6.03	8.0	<1	<1	6.0	10.0	12.0	<1	5.0	20.0	28.0
T3	84%	5.88	9.0	<1	<1	14.0	13.0	14.0	<1	9.0	23.0	41.0
T3IMMF	93%	5.53	31.0	<1	<1	8.0	8.0	20.0	1.0	6.0	15.0	37.0
T6-7	10%	6.49	53.0	<1	<1	49.0	61.0	21.0	5.0	10.0	25.0	136.0
T6-7IMMF	22%	5.89	13.0	<1	<1	16.0	15.0	11.0	<1	10.0	14.0	42.0
1489	90%	4.98	15.0	<1	<1	47.0	48.0	51.0	2.0	11.0	25.0	115.0
T18-24h Post IMM	99%	6.08	5.0	<1	<1	17.0	16.0	25.0	1.0	10.0	30.0	59.0
T3	16%	4.60	6.0	<1	<1	30.0	31.0	46.0	<1	12.0	32.0	107.0
T3IMMF	84%	5.43	8.0	<1	<1	14.0	15.0	24.0	1.0	11.0	34.0	54.0
T6-7	0%	6.81	18.0	<1	<1	79.0	63.0	29.0	9.0	20.0	25.0	180.0
T6-7IMMF	56%	4.66	5.0	<1	<1	36.0	40.0	39.0	<1	15.0	23.0	115.0
1492	100%	4.99	8.0	<1	2.0	5.0	18.0	18.0	2.0	6.0	26.0	43.0
T18-24h Post IMM	100%	4.92	12.0	<1	<1	9.0	13.0	19.0	2.0	6.0	19.0	43.0
T3	51%	5.45	5.0	<1	1.0	26.0	40.0	23.0	5.0	10.0	38.0	94.0
T3IMMF	100%	5.58	4.0	<1	<1	5.0	19.0	24.0	3.0	9.0	32.0	51.0
T6-7	0%	6.99	9.0	<1	3.0	43.0	64.0	25.0	7.0	13.0	34.0	139.0
T6-7IMMF	58%	6.25	18.0	<1	3.0	19.0	40.0	35.0	9.0	7.0	17.0	103.0
1495	100%	4.09	10.0	<1	<1	5.0	15.0	14.0	2.0	7.0	30.0	36.0
T18-24h Post IMM	100%	4.91	6.0	<1	<1	8.0	13.0	17.0	<1	9.0	35.0	38.0
T3	100%	5.30	14.0	<1	<1	12.0	19.0	17.0	2.0	7.0	22.0	50.0
T3IMMF	100%	5.31	11.0	<1	<1	7.0	20.0	15.0	1.0	10.0	28.0	43.0
T6-7	10%	6.30	19.0	<1	<1	26.0	61.0	17.0	5.0	11.0	21.0	109.0
T6-7IMMF	35%	5.90	4.0	<1	<1	10.0	15.0	18.0	2.0	10.0	24.0	45.0

Lastly, we analyzed four batches of *T. mutabilis* divided into two parts as follows: half of the batch was evaluated immediately and during refrigerated conditions, while the other half was re-immersed into a tank for 18-24 hours and then analyzed both right after the re-immersion and during refrigerated conditions. As can be seen in Table 4.15, BAI was slightly correlated to viability (Spearman's *rho* = -0.70; $p< 0.05$) but did not show a statistically significant correlation with *Vibrio* spp. load ($p> 0.05$). It should be notice that putrescine, cadaverine, spermidine, and

tyramine hardly increased within a week in non-re-immersed samples, whereas in re-immersed batches (except 1492) they remained approximately equal. In fact, there was a statistically significant difference in BAI between T_0 and $T_{6\text{-}7}$ (paired-*t*: 10.948; p=0.002) and there was not between $T_{18\text{-}24h}$ Post IMM and $T_{6\text{-}7}$IMMF (p> 0.05). Moreover, putrescine (Spearman's *rho* = 515.14; p< 0.05), cadaverine (Spearman's *rho* = 527.45; p< 0.05), spermidine (Spearman's *rho* = 538.66; p< 0.05) and tyramine (Spearman's *rho* = 472.3; p< 0.05) were correlated with viability. In non-re-immersed batches, there was a statistically significant difference in putrescine (paired-*t*: -8.653; p=0.003), cadaverine (paired-*t*: -4.886; p=0.0164), spermidine (paired-*t*: 3.538; p=0.038) and tyramine (paired-*t*: -5.5626; p=0.011) between T_0 and $T_{6\text{-}7}$. Instead, only spermine had a statistically significant decrease in re-immersed samples between T_0 and $T_{6\text{-}7}$ (paired-*t*: 3.517; p=0.039). Furthermore, the other BAs taken individually were approximately constant (p> 0.05).

On the whole, these data showed that non-re-immersed gastropods exceeded the acceptable mortality (10%) after three days in refrigerated conditions, but the *Vibrio* spp. load did not show a significant increase within three days. The TVC was already high from the beginning (more than 5 $\log_{10}$ CFU g^{-1}) and its major part consisted of SSOs, which could be explained by gastropods' feed, such as the *Pseudomonas* spp. load and the abundance of IPB. The BAs amount partially could be explained in the same way, but it was also correlated with viability and had a statistically significant difference within a week on refrigerated conditions, principally due to the rise of putrescine, tyramine, spermidine, and cadaverine. It also should be noted that the BAs amount was higher on average than the recommendation of literature (50 mg kg^{-1}). Moreover, re-immersed batches showed acceptable viability even after 3 days, and the *Vibrio* spp. load, TVC, SSOs, and biogenic amines remained almost constant (except for a decrease of spermine) within a week contrary to non-re-immersed samples.

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Section 5 - Virological analysis

1. Introduction

Two human pathogens were examined in virological analysis and will be discussed in this section: Norovirus and Hepatitis A virus.

It is well known that bivalve mollusks may become contaminated by bioaccumulating human pathogens, but the latter do not infect the mollusks themselves and do not replicate in them, however, they remain infected for several days and even weeks [Khora, 2018; Lopatin et al., 2020]. The prevalence of hepatitis A and norovirus is ∼5% and 12%–47%, respectively [Khora, 2018; Velebit, 2020].

It is well known that microbiological requirements do not include some human viral pathogens [Terio et al., 2010], but even if sea snails have been considered to present little risk of NoVs infection, Ozawa et al. (2015) have found it in *Umbonium giganteum*, which is a filter-feeding marine gastropod. In addition, a sea urchin (*Paracentrotus lividus*) has been found positive too [Santos-Ferreira et al., 2020].

1.1 Norovirus (NoV)

Noroviruses belong to the family Caliciviridae and are non-enveloped viruses with a single-stranded positive-sense RNA genome approximately 7.5 kb in length [Chhabra et al., 2019; Guadagnucci Morillo and Timenetsky, 2011; Karst et al., 2014; Ozawa, 2015]. NoVs were divided into six genogroups and only genogroups GI, GII, and GIV are known to infect humans [de Graaf et al., 2016], however in 2019 the classification has been updated into ten genogroups (GI-GX) and 48 genotypes [Chhabra et al., 2019].

Human norovirus, previously known as Norwalk virus, was first identified in 1972 during an outbreak of gastroenteritis in Norwalk, OH, but, in 1929, its illness had already been described as “winter vomiting disease” due to its seasonal predilection [Robilotti et al., 2015]. Nowadays, NoVs are the main cause of acute nonbacterial human gastroenteritis and may be transmitted from contaminated food and water, from contaminated environmental surfaces, and from person to person via the fecal-oral and vomit-oral (which might generate an infectious aerosol) routes [Atmar and Estes, 2006; Guadagnucci Morillo and Timenetsky, 2011; Hennechart-Collette, 2021]. The virus is highly infectious on account of its low infecting dose

(less than 10 to 100 virions) and the high excretion level (10^8 to 10^{10} copies of RNA per gram of feces) [Atmar and Estes, 2006; Guadagnucci Morillo and Timenetsky, 2011]. The incubation period is on average 1-2 days and the illness normally lasts within 1-3 days [Glass et al., 2009; Robilotti et al., 2015]. NoVs infections are usually self-limiting in healthy people, but some patients (immunocompromised individuals, the elderly, and young children) may have severe forms [de Graaf et al., 2016]. Clinical features are characterized by abdominal pain, nausea, vomiting, non-bloody diarrhea, headache, fever, and chills [Glass et al., 2009; Robilotti et al., 2015].

1.2 Hepatitis A virus (HAV)

Hepatitis A virus belongs to the Picornaviridae family, is classified under the *Hepatovirus* genus, and is a small (27–32 nm diameter) virus, existing in a non-enveloped and quasi-enveloped form, with a single-stranded positive-sense RNA genome approximately 7.5 kb in length [Cuthbert, 2001; Randazzo and Sánchez, 2020; Wang et al., 2015]. HAV is classified into six genotypes: I, II and III infect humans, while IV, V and VI cause infections in nonhuman primates [Randazzo and Sánchez, 2020; Smith and Simmonds, 2018].

Even if it has been available a successful vaccine since 1995, HAV causes 1.4 million cases worldwide annually, particularly where low standards of sanitation promote its transmission [Martin and Lemon, 2006; Matheny and Kingery, 2012; Yin et al., 2020; Wang et al., 2015]. Indeed, HAV is primarily transmitted fecal-orally but can also be contracted from contaminated food (especially seafood), contaminated water, personal contact, sexual contact, and illicit drug use [Cuthbert, 2001; Foster et al., 2019; Matheny and Kingery, 2012]. The infective dose is very low (10-100 virions), the long incubation period is approximate 14-50 days, the illness usually lasts in 4-6 weeks and the excretion level is high (10^{11} copies of RNA per gram of feces) [Cuthbert, 2001; Khora, 2018; Matheny and Kingery, 2012]. Young children (< 6 years) are usually asymptomatic, while most adults have symptoms like nausea, vomiting, diarrhea, jaundice, dark urine, fever, chills, headache, weight loss, arthralgia, abdominal pain, and rashes, as well as a loss of desire for cigarette smoking or alcohol [Cuthbert, 2001; Jacobs et al., 2002; Jacobsen, 2018; Randazzo and Sánchez, 2020]. However, the typical symptom is acute liver failure, which may be fatal [Randazzo and Sánchez, 2020].

2. Methodology

A total of 27 batches of *Tritia mutabilis* and 5 batches of *Bolinus brandaris* were investigated. They were collected from October 2017 to February 2021 through the fishing period of *T. mutabilis* (from the beginning of autumn to the end of spring) [Polidori, 2015] and during summer for *B. brandaris*. The catch area was the Adriatic Sea (FAO zone 37.2.1), particularly along the coast from Ravenna to Rimini.

Sample units (SUs) were prepared by rising gastropods with sterilized seawater, then by cutting aseptically their shells, and finally by removing the inner part of the body. The hepatopancreas is small and mixed with the digestive apparatus, hence it is almost impossible to extract individually, so we decided to collect all the final portion of the gastropod's body. We analyzed a pool of picked hepatopancreases (1.5–2 g), that subsequentially underwent a viral purification with the proteinase K [Comelli et al., 2008; Jothikumar et al., 2005]. From homogenates, RNA was extracted according to the manufacturer's instructions with Nucleospin® RNA II (Macherey-nagel, Düren, Germany). RNA samples were stored at -80°C until use.

For NoV, a specific analysis of genogroups was performed with an RT-Real Time PCR method: genogroup I followed the method described by da Silva et al. (2007), and genogroup II was analyzed according to Loisy et al. (2005). If positive, an RT-seminested PCR method was performed as an RT-PCR one-step [Vinjé e Koopmans, 1996] followed by two amplification cycles with internal primers [Boxman et al., 2006; Green et al., 1998]; then, if also positive, an external sequencing (Bio-fab Sequencing Service, Roma, Italy) was made to confirm.

The presence of HAV was investigated by an RT-seminested PCR method, performed according to Le Guyader et al. (1994). The PCR products were detected with an agarose gel electrophoresis. If positive, PCR products were purified, and an external sequencing (Bio-fab Sequencing Service, Roma, Italy) was made to confirm the results.

3. Results

As mentioned earlier, HAV and NoVs are human pathogens that can be found in bivalves [Khora, 2018; Velebit, 2020]. All the results of *T. mutabilis* are reported in Table 5.1.

Table 5.1: Results of virological analysis of *Tritia mutabilis* batches. NoV: Norovirus; HAV: Hepatitis A virus; Neg: negative; Pos: positive.

Sample Number - Date	HAV	NoV
1384 - 25/10/17	Neg	Neg
1386 - 31/10/17	Neg	Neg
1389 - 21/11/17	Neg	Neg
1406 - 21/03/18	Neg	Neg
1407 - 18/04/18	Neg	Neg
1408 - 04/05/18	Neg	Neg
1414 - 10/05/18	Neg	Neg
1433 - 15/01/19	Neg	Neg
1434 - 17/01/19	Neg	Neg
1435 - 22/01/19	Neg	Neg
1436 - 29/01/19	Neg	Neg
1437 - 31/01/19	Neg	Neg
1438 - 05/02/19	Neg	Neg
1439 - 13/02/19	Neg	Neg
1442 - 26/02/19	Neg	Neg
1443 - 28/02/19	Neg	Neg
1446 - 07/03/19	Neg	Neg
1447 - 12/03/19	Neg	Neg
1448 - 18/03/19	Neg	Neg
1449 - 19/03/19	Neg	Neg
1470 - 12/02/20	Neg	Neg
1471 - 19/02/20	Neg	Neg
1472 - 24/02/20	Neg	Neg
1486 - 19/10/20	Neg	Neg
1489 - 03/11/20	Neg	Neg
1492 - 18/01/21	Pos	Neg
1495 - 22/02/21	Neg	Neg

As can be seen from Table 5.1, all the samples of *T. mutabilis* were negative for NoVs. Instead, one sample (1492) was positive for HAV with a total prevalence of 3.7%. This result raises a suspicion that changeable nassas may transmit human disease.

Table 5.2: Results of virological analysis of *Bolinus brandaris* batches. NoV: Norovirus; HAV: Hepatitis A virus; Neg: negative; Pos: positive.

Sample Number - Date	HAV	NoV
1415 - 10/05/18	Neg	Neg
1416 - 25/05/18	Neg	Neg
1417 - 31/05/18	Neg	Neg
1418 - 31/05/18	Neg	Neg
1419 - 31/05/18	Neg	Neg

We also analyzed 5 batches of *B. brandaris* (see Table 5.2) that did not reveal the presence of human pathogens.

Overall, these results suggest that the changeable nassa and the purple dye murex did not accumulate NoVs. Instead, the one positivity of the HAV sample needs further investigation.

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Section 6 - Toxicological analysis

1. Introduction

Most marine biotoxins can bioaccumulate up trophic levels [Faber, 2012] and are a serious hazard to public health because they are resistant to freezing and cooking [Daguer et al. 2018; Nicolas et al., 2017]. In literature, both herbivorous and carnivorous gastropods have been reported to accumulate high levels of toxins, especially PSP (paralytic shellfish poisoning) toxins [Costa et al., 2017]. Furthermore, toxin levels are more diet-related in gastropods instead of bivalves (in which they are related to the surrounding habitat) [García et al., 2016]: in fact, Noguchi et al. (2011) suggest that the origin of TTX in scavenger gastropods are dead pufferfish organs. For example, tetrodotoxin poisoning was usually reported in Asia (especially Japan, China e Bangladesh) both in bivalves and gastropods, but, after the arrival of pufferfish in the Mediterranean Sea due to climate changes, a recent emergence has been notified in European coasts [Bordin et al., 2021; Hort et al., 2020; Leão et al., 2018; Rodrigues et al., 2019; Tamele et al., 2019a].

Saxitoxin (STX) and tetrodotoxin (TTX) were examined and discussed in this section. We chose these toxins because: (1) they have been reported to usually co-occur in bivalves and gastropods [Biessy et al., 2019]; (2) PSP toxins have been the most common toxins notified in marine gastropods [Costa et al., 2017]; (3) TTX has been appeared recently in the Mediterranean Sea, as previously mentioned; (4) TTX is produced by symbiotic bacteria including *Vibrio* spp. [Nicolas et al., 2017]; in previous bacteriological section, the *Vibrio* spp. load has resulted high and TTX is linked to the presence of high contents of this bacteria genus [Blanco et al., 2019].

1.1 Saxitoxin (STX)

Paralytic shellfish toxins (PSTs) are a group of more than 50 water-soluble tetrahydropurine derivates, with STX as the parent compound [Dell'Aversano et al., 2019; Tamele et al., 2019a]. They are produced by marine dinoflagellates especially *Alexandrium tamarense*, *A. fundyense*, *A. catenella*, *Gymnodinium catenatum* and *Pyrodinium bahamense* and by cyanobacteria including *Anabaena circinalis*, *A. lemmermannii*, *Aphanizomenon gracile*, *Aphanizomenon issatschenkoi*, *Cylindrospermopsis raciborskii*, *Lyngbya wollei*, *Planktothrix* spp., and *Rivularia* spp. [Etheridge, 2010].

STX and analogs act by binding voltage-gated sodium channels (VGSCs), inhibit Na+ influx, and consequently blocking ion conduction in nerves and muscles fibers leading to paralysis [Daguer et al. 2018]. PSP is characterized by nausea, vomiting, shortness of breath, dizziness, numbness of extremities, headache, tingling of the mouth, lips, and tongue, paresthesia, weakness, ataxia, slurred speech, and paralysis [Etheridge, 2010; Tamele et al., 2019a]. Symptoms can generally occur within 30 minutes post-ingestion of contaminated seafood and death is commonly observed within 3-12 hours following consumption [Etheridge, 2010; Faber, 2012]. The maximum acceptable limit in Europe is 800 micrograms of saxitoxin equivalents diHCl per kilogram (μg STXdiHCl-eq kg^{-1}) of shellfish meat [Reg. 853/2004/EC; 624/2019/EU; 1374/2921/EU], however EFSA (European Food Safety Authority) suggests a level of 75 μg STX-2HCl-eq kg^{-1} of shellfish meat [EFSA, 2009].

1.2 Tetrodotoxin (TTX)

Tetrodotoxins are a group of approximately 30 highly hydro-soluble zwitterions [Biessy et al., 2019; Tamele et al., 2019b]. TTX is mostly found in pufferfish but can then accumulate in various edible marine species including fish, gastropods, and bivalves [Dell'Aversano et al., 2019]. It is produced by bacteria such as *Serratia marcescens*, *Vibrio* spp. (especially *V. alginolyticus* and *V. parahaemolyticus*), *Aeromonas* spp., *Microbacterium arabinogalactanolyticum*, *Pseudomonas* spp., *Bacillus* spp., *Shewanella putrefaciens*, *Micrococcus* spp., *Alteromonas* spp. (particularly *A. tetraodonis*), *Pseudoalteromonas* spp., and *Nocardiopsis dassonvillei* [Kudo et al., 2014; Magarlamov et al., 2017; Tamele et al., 2019a].

This toxin shares the same mode of action as STX, activating the VGSCs site [Nicolas et al., 2017; Noguchi et al., 2011]. Symptoms are vomiting, headache, lingual numbness, muscle weakness, incoordination, respiratory failure, hypotension, bradycardia, and even death due to respiratory and/or heart failure [Blanco et al., 2019; Bordin et al., 2021; Leão et al., 2018; Tamele et al., 2019a]. TTX poisoning may either have rapid onset (within 45 minutes) or delayed onset (generally within 3-6 hours) and death usually occurs within the first 4 to 8 hours [How et al., 2003; Kotipoyina et al., 2021]. However, there is not a current limit in Europe for TTX. EFSA claims that 44 mg of TTX eq kg^{-1} of shellfish meat is expected not to harm

humans and only a large portion (400 g or more) might be a concern for consumers, especially oysters [EFSA, 2017].

2. Methodology

A total of 23 batches of *Tritia mutabilis* and one batch of *Bolinus brandaris* were investigated. They were collected from November 2017 to February 2021 through the fishing period of *T. mutabilis* (from the beginning of autumn to the end of spring) [Polidori, 2015] and during summer for *B. brandaris*. The catch area was the Adriatic Sea (FAO zone 37.2.1), particularly along the coast from Ravenna to Rimini.

Two commercial kits using ELISA were utilized to search saxitoxin and tetrodotoxin: respectively, Perkin Elmer and CD Creative Diagnostics. These kits are specifically developed for toxin detection in tissues of bivalve mollusks.

Sample units were prepared by following the manual of the kits: 3 individuals were homogenized and processed.

3. Results

As mentioned earlier, gastropods have been reported to accumulate high levels of toxins [Costa et al., 2017]. All the results of *T. mutabilis* and *B. brandaris* are reported in Table 6.1.

As can be seen from Table 6.1, all the samples of TTX resulted to be below the limit of quantification of the methodic. On the other hand, even if all the results were below the detection limit (3 ng/g), minimal traces of SST were found in 9 batches (39%) of *T. mutabilis* and one batch of *B. brandaris* (100%). Hence, all the results remain abundantly below the maximum acceptable limit in Europe (800 μg STXdiHCl-eq kg^{-1}) and the EFSA suggested limit (75 μg STX-2HCl-eq kg^{-1}, equivalent to 30 μg equivalent STX per serving of 400g for an adult of 60 kg).

In conclusion, we could confirm that *T. mutabilis* and *B. brandaris* can accumulate a little amount of STX, although not at levels toxic to humans. Therefore, the presence of this toxin is more widespread than previously thought, even in the Adriatic Sea. Instead, the two species of gastropods seem not to accumulate TTX in the Northern Adriatic, probably because the pufferfish, which is the origin of TTX in scavenger gastropods [Noguchi et al., 2011], can be found in the Mediterranean Sea due to climate changes, but not in the Adriatic Sea yet.

Table 6.1: Results of toxicological analysis of *Tritia mutabilis* and *Bolinus brandaris* (highlighted in blue) batches. STX: Saxitoxin; TTX: Tetrodotoxin.

Sample Number - Date	**TTX** ng/ml	**STX** ng/g
1389/17 - 21/11/17	<10 ng/ml	<3 ng/g
1406/18 - 21/03/18	<10 ng/ml	<3 ng/g
1408/18 - 04/05/18	<10 ng/ml	0,62 ± 0,13
1433/19 - 15/01/19	<10 ng/ml	<3 ng/g
1434/19 - 17/01/19	<10 ng/ml	<3 ng/g
1435/19 - 22/01/19	<10 ng/ml	0,551 ± 0,001
1436/19 - 29/01/19	<10 ng/ml	<3 ng/g
1437/19 - 31/01/19	<10 ng/ml	<3 ng/g
1438/19 - 05/02/19	<10 ng/ml	0,94 ± 0,03
1439/19 - 13/02/19	<10 ng/ml	<3 ng/g
1442/19 - 26/02/19	<10 ng/ml	0,59 ± 0,03
1443/19 - 28/02/19	<10 ng/ml	0,56 ± 0,03
1446/19 - 07/03/19	<10 ng/ml	0,74 ± 0,18
1447/19 - 12/03/19	<10 ng/ml	0,58 ± 0,07
1448/19 - 18/03/19	<10 ng/ml	0,81 ± 0,25
1449/19 - 19/03/19	<10 ng/ml	0,96 ± 0,03
1457/19 - 25/06/19	<10 ng/ml	0,72 ± 0,17
1470/20 - 12/02/20	<10 ng/ml	<3 ng/g
1471/20 - 19/02/20	<10 ng/ml	<3 ng/g
1472/20 - 24/02/20	<10 ng/ml	<3 ng/g
1486/20 - 19/10/20	<10 ng/ml	<3 ng/g
1489/20 - 03/11/20	<10 ng/ml	<3 ng/g
1492/21 - 18/01/21	<10 ng/ml	<3 ng/g
1495/21 - 22/02/21	<10 ng/ml	<3 ng/g

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Section 7 - Discussion and conclusions

1. Discussion

Live marine gastropods are assimilated to live bivalve mollusks in terms of safety criteria for human consumption [Reg. 853/2004/EC]. According to Reg. 624/2019/EU, marine gastropods must be placed on the market alive, but the official method applies only to bivalve mollusks. Palese and Palese (1991) suggest stinging the gastropod's body with a pin to see if the specimen is alive because if it is viable, the body retreats quickly inside the shell. However, if the gastropod has already retreated into the shell, this method cannot be used. The lack of an official method to assess viability could be seen by the fact that 84% of samples from commerce showed viability lower than 90% (only mortality of 10% is almost always accepted by official authorities at the market).

Gäde et al. (1984) affirm that *Tritia mutabilis* responds to NaCl stimulation with escape movements. Following this statement, we evaluated gastropods' viability by dusting sea salt on the whole batch (as we have already published [Serratore et al., 2019]) or 30 specimens. Our results are in partial agreement with Gäde and colleagues, but we also observed that gastropods respond both by retracting themselves into the shell and excreting mucus with a foam/bubble production. However, it should be underlined that *T. mutabilis* commonly responds by escaping movements and *Bolinus brandaris* usually produces foam and bubbles. This difference may be explained by the fact that *B. brandaris* has an operculum that protects it from external stressors, while *T. mutabilis* does not have it. Nevertheless, the method to evaluate the viability is easy to apply even in gastropods retreated into the shell and could give more precise results than the punching.

However, gastropods might be stressed by the harvesting, the long period outside the seawater, and the refrigeration and might not respond to stimuli. Kohn (1961) affirm that dry gastropods react to immersion into seawater with the extension of the foot, head, and tentacles and, if the immersion persists, locomotion. Hence, we verified the accuracy of this method by re-immersing the gastropods into a sterile basin for 30 minutes. All the re-immersed samples had equal or higher viability than the same batch evaluated only with sea salt. However, as mentioned before, considering that at most mortality of 10% is almost always accepted by official authorities at the market, only the 27% (3/11) of batches showed such a discrepancy

to make the samples unacceptable if evaluated with sea salt and acceptable after a short re-immersion. Subsequentially, we verified if gastropods could recovery better after a longer re-immersion (18-24 hours). We obtained results like the short re-immersion outcomes, but, in this case, unacceptable batches remained so and vice versa. These results seem a slight limitation to the method and further investigations are needed.

According to Reg. 853/2004/EC, individual costumer-size packages could be opened and relabeled between dispatch centers. Nevertheless, the label reports only the packaging date, hence the final customer can only see the last date of packaging and cannot know the harvesting one. We also noticed this aspect because, e.g., after one day of packaging, some of the analyzed samples from retail had a slightly acrid smell and 20% of viability, while others had a saltwater smell and only 10% of mortality. On the other hand, all the samples from primary production (except one having the 88%) presented viability higher than 90%.

The evolution of smell during storage has been described by Palese and Palese (1991), Orlandi and Perna (1968), and Regulation (EC) No 2406/96 (for fish, crustaceans, cephalopods, and selachii) in a similar way. Following these papers, we evaluated the smell using five descriptors and revealed that the smell is correlated to viability and could be a good freshness indicator.

Contrary to bivalves, gastropods seem to be incapable of concentrating fecal contaminants, such as *E. coli*, hence non-filter-feeders gastropods can be harvested into non-classified areas [Reg. 558/2010/EU; Reg. 624/2019/EU]. However, *E. coli* has been found in *Rapana venosa* [Altug and Güler, 2002], which is a carnivorous gastropod. We have not detected *E. coli* both in our previous study [Serratore et al., 2019] and this thesis; hence, our results confirm the suitability of the European legislation.

The microbiology of marine gastropods has been poorly investigated, except for Cheng et al. (1995) reporting high contamination of the muscle of the marine gastropod *Niotha clathrata* (nowadays *Nassarius conoidalis*) by *Vibrio* spp. (6-8 $\log_{10}$ CFU g^{-1}). It is well known that *Vibrio* spp. may produce biogenic amines and tetrodotoxins, that are thermostable and truly dangerous for humans [Biessy et al., 2019; Leão et al., 2018; Magarlamov et al., 2017; Noguchi et al., 1987]. In the present study, all the batches resulted negative for pathogenic vibrios (*V. parahaemolyticus*, *V. vulnificus*, *V. cholerae*, and *V. alginolyticus*). The *Vibrio* spp. load resulted on

average 5.44 ± 0.70 $\log_{10}$ CFU g^{-1} in *T. mutabilis* and 6.52 $\log_{10}$ CFU g^{-1} in *B. brandaris* at T_0, similarly to our previous data [Serratore et al., 2019]. Even in this thesis, the load was slightly higher in *B. brandaris* than *T. mutabilis*. Moreover, the abundance of *Vibrio* spp. was similar after both a short and long re-immersion and this may be explained by the fact that even if gastropods could have an exterior cleaning into seawater, they accumulate bacteria into the flesh.

Furthermore, we evaluated four bacteriological targets as spoilage indicators, because we expected a high load due to gastropods' feeding and fishing: they are scavengers and spoiled fish is preferred by fishermen. The mean total viable count (TVC) exceeded 5 $\log_{10}$ CFU g^{-1} since the beginning (5.72 ± 0.79 $\log_{10}$ CFU g^{-1}) and specific spoilage organisms (SSOs) were approximately one logarithm less than it (on average 4.89 ± 0.99 $\log_{10}$ CFU g^{-1}). The *Pseudomonas* spp. load was 4.07 ± 0.37 $\log_{10}$ CFU g^{-1}. All the loads resulted high if compared to literature: recommended maximum limits of TVC for fish and shellfish are 5 x 10^5 CFU g^{-1} [ICMSF, 1986]; SSOs (including *Pseudomonas* spp.) typically constitute only a very small fraction of the microflora on newly processed seafood and are present in low numbers [Gram and Dalgaar, 2002]. The abundance of indole-producing bacteria (IPB) resulted on average 2.40 ± 0.93 $\log_{10}$ MPN g^{-1} at T_0. Indole has a maximum limit of 250 μg kg^{-1} to identify shrimp in the first stage of decomposition [Mendes et al., 2005], and all species of *Vibrio* spp. can produce indole via tryptophanase activity [Mueller et al., 2009], particularly in a strongly alkaline environment [Kim et al., 1995], such as fish meat. Hence the resulting IPB load is certainly potentially able to produce a high level of indole. It should also notice that after a short re-immersion more than 50% of batches had an IPB load higher than one logarithm in re-immersed samples compared to others. All these microbiological criteria (including *Vibrio* spp.) are not investigated by Official Authorities and, together with the lack of viability control, represent a huge issue for the safety control of marine gastropods.

Biogenic amines are also used as spoilage indicators and, to our best knowledge, this is the first study conducted on a marine scavenger gastropod and comparative data are lacking. Considering the BAI limits of acceptability suggested for tuna (<50 mg kg^{-1}) [Ruiz-Capillas and Herrero, 2019; Wójcik et al., 2021] and for anchovy and Mediterranean hake (<15-20 mg kg^{-1}) [Baixas-Nogueras et al., 2005; Pons-Sánchez-Cascado et al., 2006; Prester, 2011], the values registered in *T. mutabilis* can be considered certainly high. All the results were >20 mg kg^{-1} and on

average >50 mg kg^{-1} (mean: 63.1 mg kg^{-1}). This high result is probably due to the feeding habits of the studied gastropods, that naturally accumulated such amount. It should be noticed that, even if it could be a "standard" amount, it could cause intoxication due to the synergy of the different amines.

The legislation does not include some human viral pathogens for gastropods [Terio et al., 2010], such as Norovirus (NoVs) and Hepatitis A virus (HAV), and the virology of marine gastropods has been poorly investigated. However, one sample was positive for HAV with a total prevalence of 3.7%, while all the batches were negative for NoVs. Further investigations will be needed for the presence of HAV.

In literature, both herbivorous and carnivorous gastropods have been reported to accumulate high levels of toxins, especially PSP (paralytic shellfish poisoning) toxins [Costa et al., 2017], however, we could confirm that *T. mutabilis* and *B. brandaris* can accumulate a little amount of STX, although not at levels toxic to humans: minimal traces of SST were found in 9 batches (39%) of *T. mutabilis* and one batch of *B. brandaris* (100%). Instead, the TTX was not detected in both investigated species. Hence, *T. mutabilis* and *B. brandaris* do not pose a risk for intoxications by PSP and TTX.

The study of Orlandi and Perna (1968) is the only example in literature on the shelf-life of *T. mutabilis*. They valuated its shelf-life using response to stimuli, adhesiveness, water elimination, and body leak and have estimated a shelf-life of seven days at 0-2°C. However, after three days in refrigerated conditions, gastropods did not react well to stimuli. In this thesis, we evaluated a shelf-life within one week at 2-8°C and partially confirmed these results. As already mentioned, if only mortality of 10% is almost always accepted by official authorities at the market, 62,5% of the batches should not have been sold after only one day in refrigerated conditions, and almost all the samples after three days. It should also be underlined that all the batches were from primary production except one, which had a more rapid decrease probably because gastropods may have been stressed more than individuals of samples from primary production.

As outlined previously, the microbiology of marine gastropods has been poorly investigated even during refrigerated conditions. The abundance of *Vibrio* spp. and IPB were stable within 3 days during refrigerated conditions ($T_{0Vibrio}$ = 4.95 ± 0.84 and $T_{3Vibrio}$ = 5.31 ± 0.82 log_{10} CFU g^{-1}; T_{0IPB} = 2.58 ± 0.80 and T_{3IPB} = 3.08 ± 1.33 log_{10} MPN g^{-1}) and significantly increased later by most a logarithm ($T_{6\text{-}7Vibrio}$ =

6.65 ± 0.31 $\log_{10}$ CFU g^{-1}; $T_{5\text{-}7IPB}$ = 4.45 ± 1.10 $\log_{10}$ MPN g^{-1}). The similar trend between *Vibrio* spp. and IPB is easy to explain because all species of *Vibrio* spp. can produce indole. The TVC and SSOs load resulted stable within 3 days, but significantly increased between T_1 (T_{1TVC} = 5.17 ± 0.51 and T_{1SSO} = 4.16 ± 0.55 $\log_{10}$ CFU g^{-1}) and T_3 (T_{3TVC} = 5.94 ± 0.49 and T_{3SSO} = 4.82 ± 0.49 $\log_{10}$ CFU g^{-1}): it should be noticed that refrigeration slows (or even stops) bacterial growth (in fact, there was a decrease between T_0 and T_1), so it may be an explanation for these results. Instead, the amount of *Pseudomonas* spp. was almost constant within 5 days (T_{0Ps} = 4.07 ± 0.37 and T_{5Ps} = 4.97 ± 0.67 $\log_{10}$ CFU g^{-1}), but, even if it seemed increasing by about one logarithm, there was not a significant difference. As mentioned before, SSOs and *Pseudomonas* spp. commonly constitute only a very small fraction of the microflora on newly processed seafood, but *T. mutabilis* is a scavenger, so probably the initial high amount is due to its feeding; however, the abundance of both targets gradually increased during refrigerated conditions due to the decrease of viability. The same line of reasoning may be applied for BAI: the stating amount is high, as commented previously, nevertheless the BAI is also correlated with viability, such as putrescine, cadaverine, spermidine, and tyramine. These should be considered the most important safety targets of marine gastropods because BAs cannot be eliminated by cooking and could cause intoxication.

The batch of *B. brandaris* showed a shelf-life of 2 days in refrigerated conditions and viability decreased slower than in *T. mutabilis*. Moreover, the abundance of *Vibrio* spp. was almost constant for six days, contrary to *T. mutabilis*. A short re-immersion (30 minutes) did not seem enough to make a difference in shelf-life or the *Vibrio* spp. load.

On the other hand, a long re-immersion (18-24 hours) resulted in an extra day of shelf-life. Moreover, the *Vibrio* spp. load did not show a statistically significant difference within a week ($T_{18\text{-}24hIMM}$ = 5.48 ± 0.66 and $T_{6\text{-}7IMM}$ = 5.67 ± 0.69 $\log_{10}$ CFU g^{-1}) contrary to non-re-immersed batches. Likewise, the TVC and SSOs load did not have a significant difference between T1 and T3 ($T_{1IMMTVC}$ = 5.80 ± 0.13 and $T_{3IMMTVC}$ = 6.16 ± 0.11 $\log_{10}$ CFU g^{-1}; $T_{1IMMSSO}$ = 4.91 ± 0.30 and $T_{3IMMSSO}$ = 5.25 ± 0.14 $\log_{10}$ CFU g^{-1}) and the BAI was constant within a week, even if spermine showed a statistical decrease. Instead, the abundance of IPB had the trend described above both in re-immersed and non-re-immersed batches.

2. Conclusions

The actual control by the Official Authority (OA) lacks an efficient method to evaluate the viability and effective criteria to assess the safety of marine gastropods. Moreover, dispatch centers can relabel the gastropod and only the packaging date is shown to the customer, hence it is hard to understand how many days have passed since the harvest. This thesis suggests that the usage of sea salt is easy and successful to assess the viability. Moreover, the BAI index, which now is not controlled by the OA, could be used as a safety target, because the synergy among amines could cause human intoxications. The present study evidenced high bacterial contamination of the gastropod flesh, even if pathogenic vibrios were never detected. In any case, the post-harvest treatment of re-immersion produced an extension of the shelf-life and keep constant the microbiological and toxicological parameters, particularly the BAs, which partially even decrease in time. Further studies are needed for *T. mutabilis* and other marine gastropods, to implement post-harvest treatments and official controls in order to protect consumers health.

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