Histological features of Rickettsia-like organisms in the European flat oyster (*Ostrea edulis* L.)

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Abstract

The European flat oyster (*Ostrea edulis* L.) represents an economically important oyster production in Southern Italy, widespread in natural beds along the coast. The practice to be eaten raw is an everlasting concern for possible health risk with a need to stringently monitor the health of aquatic environment. A screening survey using histopathological examination, was undertaken by harvesting *O. edulis* from different sites along the Apulian coast of Italy. Tissue samples of digestive gland, kidney, gonad and gill were provided for morphologic study in Light Microscopy (LM), Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM) analysis. The LM observations revealed spherical cytoplasmic inclusions, as basophilic prokaryote colonies in 13/250 oysters. The TEM and SEM confirmed the presence of intracytoplasmic inclusions of Rickettsia-like organisms (RLOs), merely in the epithelial cells of digestive gland tubule tissues in the 13 oysters. Within intracytoplasmic vacuoles, RLOs exhibited a prokaryotic

characteristic ultrastructure with transverse binary fission, a DNA zone full of chromatin fibers and a granular periplasmic ribosome zone. *O. edulis* were found positive for RLOs in wild oysters from Manfredonia, while the other sites were found free of pathological inclusions. Thus, we present the first report of a Rickettsia-like infection in the Apulian wild oyster (*O. edulis*) from Italy, including an ultrastructural description and pathological characterization.

Keywords: Rickettsia-like organisms (RLOs), *Ostrea edulis* L., oyster pathology, oyster histology, electron microscopy

Introduction

The European flat oyster (*Ostrea edulis* Linnaeus, 1758) has becoming a relevant marine profit supply in the Apulia region in the South of Italy, due to the presence along the coasts of both natural beds and offshore aquacultured populations. The harvesting of native oysters in Southern Italy is specifically directed to the production of high-value species of edible bivalve for human consumption. Though eating raw oysters may cause serious illness to the consumer, the report of bivalve pathologies is scarce. Even though the bulk of the total global production of all farmed oyster species is the cultured *Crassostrea gigas* (96.2% of world production), overall the production of the European flat oyster (less than 0.11% of world production), *Ostrea edulis* (Family: Ostreidae; Genus: Ostrea; Species: *O. edulis*), is the most valued fishery resource in marine coastal waters of the South Italy (Tacon 2003). The great aquaculture potential of *O. edulis*, with the development of hatchery techniques, has led to a significant natural breeding expansion; on the other hand the high host densities may increase the risk of disease by favoring the transmission of parasites, including intracellular organisms belonging to the Rickettsiales, which are reported to affect a wide variety of

other marine mollusks (Novack 2007). Overall, marine bivalve mollusks may be affected by numerous infectious diseases as reported in the extensive review article by Zannella et al. (2017), the most important diseases caused by viruses, bacteria and protistans, responsible for mortality outbreaks and substantial commercial losses. In recent investigations, along the Southern Italy coasts, protozoan parasites belonging to the phylum Haplosporidia, genera *Bonamia*, (*B. ostreae* and *B. exitiosa)* were found affecting natural beds of *O. edulis* in the Manfredonia Gulf (Adriatic Sea) (Narcisi et al. 2010). This obligate pathogen infects the hemocytes of oysters and clams, multiplies within blood cells, and spreads to all tissues inducing physiological disorders (Zannella et al. 2017). Specifically, *B. ostreae* and *B. exitiosa* are currently under surveillance and require mandatory notification by the World Organization for Animal Health.

Apart from recent detections of *Bonamia* parasites, it has been over twenty years since the description of diseases associated to bacterial species affecting bivalves, and mostly is caused by intracellular bacteria belonging to Rickettsia-like organisms (RLOs) as previously reviewed by Fryer and Lannan (1994). Further, RLOs have been reported by Zhu and Wu (2008) as important agent of massive mortality outbreaks in both native and cultured oysters all over the world. RLOs are small, pleomorphic, rod-shaped coccoid prokaryotes, most of which are obligate intracellular Gram-negative pathogens (Lauckner 1983; Chen et al. 2000; Bower & McGladdery 2003). Several authors have recognized the presence of RLOs in many aquatic animals, including fish, mollusc and crustacean (Gulka and Chang 1984; Wang & Gu 2002; Sun & Wu 2004; Gollas-Galvan et al. 2013). In recent time, RLOs have been increasingly documented as important fish pathogens, mostly in the salmonid aquaculture industry.

The first taxonomically identified RLO affecting fish is *Piscirickettsia salmonis*, the etiologic agent of a systemic infection known as piscirickettsiosis (Fryer and Hedrick 2003). *P. salmonis* was initially isolated in farmed Coho salmon, *Oncorhynchus kisutch*, during a high mortality epizootic

in Chile in 1989, which resulted in huge economic losses (Schafer et al. 1990; Fryer et al. 1992). Moreover, occurrences of rickettsial septicemia have been reported in farmed salmonids in many countries (Rodger and Drinan 1993; Olsen et al. 1997; Cusack et al. 2002; Birrell et al. 2003; Corbeil et al. 2005) and in various non-salmonid species too (Chen et al. 2000¸ Arkush et al. 2005, McCarthy et al. 2005, Contreras-Lynch et al. 2015). Formerly, *Piscirickettsia salmonis*, was just described by Mikalsen et al. (2008) as a serious pathogen of farmed salmonid and marine fish; later, in recent times, a high mortality event where RLOs were detected among Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), farmed in New Zealand, was reported (Brosnahan et al., 2017). Notwithstanding the importance of Rickettsiales as causative agents of massive mortality outbreaks, in farmed aquatic species, little is known about their life cycle and their host range perhaps in bivalves (Ferrantini et al. 2009; Gollas-Galvan et. al 2013). Since the first RLO infection investigated in the bivalve *Mya arenaria* (Harshbarger et al. 1977), almost another 25 species of marine mollusks throughout the world have been reported as infected with RLOs in the last decade, as well reviewed by Gollas-Galvan and colleagues (2013). Some RLOs were found to cause severe disease and mortality both in marine and freshwater mollusks, such as scallop (*Argopecten purpuratus*; Lamark, 1819) (Gulka et al. 1983, Lohrmann 2009), abalone (*Haliotis* spp.) (Moore et al. 2000, Crosson et al. 2014), giant clam (*Venerupis rhomboides*)(Norton et al. 1993), tropical pearl oyster (*Pinctada maxima*) and Suminoe oyster (*Crassostrea ariakensis*) (Wu and Pan 1999a,b; Wu and Pan 2000). In recent times, some populations of abalone (*Haliotis* spp.) in California experienced a strong population decline and dramatic economic losses due to the Withering syndrome, a fatal disease caused by the rickettsial bacterium *Xenohaliotis californiensis* (Haaker et al.1992; Friedman et al.2002), (Crosson et al. 2014). *Xenohaliotis californiensis* is an intracellular bacterium (family *Anaplasmataceae*) that replicates within intracytoplasmic vacuoles 14-56 µm in diameter, within gastrointestinal epithelia, causing abalone rickettsiosis in wild and farmed

abalones, *Haliotis* spp. (Friedman et al.2000; Dumler et al.2001). Some evidences, by water-borne transmission studies, demonstrated that the bacterium may survive outside the host for an undetermined period of time (Friedman et al. 2002; Braid et al. 2005; Friedman et al. 2007; Rosenblum et al. 2008). Basically, moderate to severe infections occurred in abalones at elevated water temperatures (above 18°C) with prolonged incubation period ranging between 3 and 7 months thus posing a serious risk for aquatic carry over, indeed exposure of abalones to seawater containing infectious material is sufficient for transmission of the bacterium (Friedman et al. 2000; Friedman et al. 2002; Braid et al. 2005; Balseiro et al. 2006; Friedman et al. 2007).

Recently, RLOs were detected for the first time infecting *Crassostrea gasar* oysters, in gills, cultivated in the Rio São Francisco estuary, Sergipe state in northeastern Brazil, in oysters with a high prevalence of *Perkinsus* sp. infections, a disease caused by protozoan parasites of the genus *Perkinsus* which is listed by the World Organisation for Animal Health (OIE notifiable diseases). Hence, more information on factors affecting the health of oysters is needed, especially concerning the pathogens listed in OIE (Da Silva et al. 2015).

Nevertheless, no effective measures are currently available to prevent and control these diseases in mollusks. After the first description of RLOs, many other studies have been undertaken to study these pathogens. While, few studies were done to investigate the ultrastructure, morphogenesis, biology and pathogenesis of several other marine RLOs (Cano et al. 2018), fewer studied outbreaks in cultured oysters worldwide, and none was performed in Italy (Arkush et al. 2005). Ultrastructularly, RLOs have been shown to form microcolonies within the epithelial cells of the mantle, digestive gland, gill, and hepatopancreas in both marine and freshwater mollusks (Gulka et al. 1983, Renault and Cochennec 1994, Wu and Pan 1999 a, b).

In Italy, studies on mollusk diseases are scarce; there is not enough knowledge about health status and parasites of bivalves, as well as on *Rickettsiales* as putative pathogens to humans. Besides, in

recent years in Italy, several rickettsia species, obligate intracellular Gram-negative bacteria, were found implicated as causative agents of human diseases (Parola et al. 2013). Almost all of the cases reported in Italy are considered as cases of Mediterranean spotted fever (MSF), which is endemic in the Mediterranean basin, predominantly in Southern Italy (Ciceroni et al. 2006). Though, this disease is due to *Rickettsia conorii* subsp. *conorii*, it is acknowledged to be transmitted by the bite of the brown dog tick, *Rhipicephalus sanguineus* (Parola et al. 2013). Henceforth, since RLOs were found in the South of Italy as a pathogen of concern due to the endemic diffusion of species potentially dangerous to human, RLOs occurring in commercially bivalve mollusks should deserve special attention and further studies.

Therefore, this study aimed to describe the histological features of RLOs in the oyster, *O. edulis* collected in natural coastal areas of Apulia region in the South of Italy.

Materials and Methods

Two hundred and fifty *O. edulis* (Mollusca, Bivalvia), adult oysters were collected during a routine screening program from natural populations placed at different sites of Apulia Region in the South of Italy. Native oysters, inhabiting the intertidal zone, were randomly sampled (sample volume/length × width × height, 63.4 x 50.3 x 120 mm) along the coastal seabed of the fishery sealines of Manfredonia (site 1; *n* = 42) (41° 38′ 0″ N, 15° 55′ 0″ E), Margherita di Savoia (site 2; *n* = 30) (41° 22′ 0″ N, 16° 9′ 0″ E), Monopoli (site 3; *n* = 70) (40° 57′ 17″ N, 17° 10′ 24″ E), Taranto (site 4; *n* = 73) (40° 25′ 5″ N, 17° 14′ 27″ E) and Tricase (site 5; *n* = 35) (39° 56′ 0″ N, 18° 22′ 0″ E) (Fig. 1). Oysters were held inside tanks of aerated seawater at temperatures near 25°C, up to 24 h from their sampling sites, until they reached the laboratory for obtaining tissue samples for histological and ultrastructural preparations. Ten adults of each harvesting group were randomly

sampled from collected oysters; the soft parts were dissected for obtaining digestive glands, kidneys, gonads and gills.

Microscopy Analysis

In order to proceed for laboratory testing, small fragments of all dissected tissues were compressed, and observed by using Nomarski differential interference contrast optics, to assess the presence of inclusion bodies symptomatic of a prokaryote infection.

For histological investigation, specimens were rinsed with 0.22 µm filtered seawater, fixed in Immunofix solution (paraformaldehyde/buffer phosphate 0.05M mix, pH 7.4; volume sample/fixative 1:50) (Immunofix solution, Bio-Optica Milan s.p.a.) and processed routinely according to the Immunofix Fixative Protocol. Afterward, they were dehydrated with an ascending series of ethanol solutions, embedded in paraffin, sectioned at 5 µm and stained with Harris' Hematoxylin and Eosin (H&E). Afterward, H&E positive specimens, which resulted with high levels of intranuclear inclusions by Light Microscopy (LM), were processed and fixed for transmission electron microscopy (TEM) and scanned electron microscopy (SEM).

Transmission Electron Microscopy (TEM)

For transmission electron microscopy (TEM), small pieces of paraffin blocks containing intracellular inclusions were cut at 1 mm^2, deparaffinized and extracted by de-waxing in xylene overnight, hydrating through 2 changes of 100, 90 and 70% ethanol. Next were fixed for 2 h with 2.5% glutaraldehyde and stored in 0.05M phosphate buffer (PBS), pH 7.4 at 4 °C. Tissues were post-fixed in 1% osmium tetroxide (OsO_4) buffered with 0.1M PBS, pH 7.2, for 1.0 to 1.5 h at 4°C, then rinsed in PBS, dehydrated in an ethanol series (30, 50, 70, 80, 95% and absolute for 30 min each) and lastly embedded in a mixture of Epon-812 resin. Ultrathin sections with silver interference were cut with an ultramicrotome (RMC-MT 6000-XL), picked up on copper grids, and

stained with 2% uranyl acetate for 20 min and with 1% lead citrate for 3 to 5 min. The sections were observed on a Zeiss transmission electron microscope operated at 60 kV (TEM)(EM 109 Zeiss) (Lighezan et al. 2009).

Scanning Electron Microscopy (SEM)

For scanning electron microscopy (SEM), small samples were microdissected and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, for 48 h. The samples were washed in tap water, dehydrated in a graded series of alcohol, dried by the method of the critical point and sputter coated with 20 nm gold-palladium (Barré et al. 2006). Samples were then examined under a Cambridge Stereoscan 240 to 20kV.

Results

Macroscopically, oysters collected revealed no evidence of gross lesions. Of all dissected tissues sampled (digestive glands, kidneys, gonads, gills), firstly compressed and observed by Nomarski differential interference contrast (DIC) optics, only fragments from digestive gland revealed the presence of several intracytoplasmic vacuoles, filled with inclusion bodies, more stringently symptomatic of a prokaryote infection; as well as largely hypertrophied cells like first signs of histopathological changes. The histopathological examination of collected oysters, revealed the occurrence of organisms exhibiting characteristic morphologically similar to those of prokaryotic rickettsia only in 2/42 adults of *O. edulis* collected from Manfredonia (N: 41° 35' 29.66"N - 15° 56' 30.00"E), containing intracytoplasmic vacuoles. The other surveyed oysters, collected from the other sites, were found free of pathological inclusions.

Light microscopic observation of H&E-stained tissues revealed the occurrence of numerous spherical intracytoplasmic inclusion bodies in sections of digestive gland tubule epithelium of *O. edulis* parasitized. Most inclusions, which appeared polymorphic, were observed in the form of

irregular basophilic inclusions, containing a great number of slightly amaranth or purple-red granules (~25-35 intracytoplasmic vacuoles) (Fig. 2). Some vacuoles appeared ellipsoidal, while other, with enlarged volume (~20 μm long and 0.5 μm wide; ~30/40 μm diameter) were in necrosis, undergoing to a lytic rupture, releasing an extensive lytic hollows formed by dead intestine epithelial cells and numerous inclusions dropped from lytic cells (Fig. 2).

The TEM observations revealed that oval-shaped intracellular inclusions of the digestive glands were full of spherical and/or rod shaped microorganisms, thus confirming the presence of intracytoplasmic colonies of RLOs, merely in 2 adults, all from one out of the five harvesting sites. According to the TEM finding, RLOs exhibited an ultrastructure characteristic of prokaryotic bacteria-like cells, with reproductive stages in transverse binary fission, including a DNA zone full of chromatin fibers and a granular periplasmic ribosome. Each intracytoplasmic colony (~ 8 μm long and 6 μm wide) counted a total of 260 microorganism particles (Fig. 3a). To a higher magnification of intracellular inclusions by TEM (Fig. 3b), microorganisms appeared coated with a bilayered envelope, while the cytoplasm showed a finely granular electron-dense material which mainly accumulated in the periphery and in the centre of the cell. The intracellular RLOs were approximately 2.5 μm long (range: 1.5 – 3.5 μm) and 1.5 μm wide (range: 1.5 – 2.5 μm) (Fig. 3b). The Fig. 3 shows the fine granular ribosomal materials, with chromatin fibers, filling the cytoplasm of the RLO organisms. Several RLO microorganisms in replicative stage and transverse binary fission were present in different stages of cell division, up to binary fission completed and reconstitution of wall envelope (Fig. 3b). At the highest RLO proliferations, within the intracellular inclusion, a break of cell envelope was observed by TEM and numerous free intracytoplasmic RLOs were seen degranulating inside the cell (Fig. 3b).

Ultrastructural analyses by scanning electron micrograph (SEM) confirmed the presence of roundish bodies (RLOs) (Fig. 4a). SEM revealed the particular characteristics of RLO inclusion

surface morphology, showing isolated (white arrow) or assembled (black arrows) roundish inclusion bodies, in *O. edulis* digestive gland tubule epithelium (Fig. 4).

Discussion

The present paper shows for the first time the occurrence of RLOs in the tissues of the flat oyster *O. edulis* collected in the South of Italy. Here, we report the first recorded case of rickettsiosis in edible bivalve mollusks from Apulian region of Italy, by studying the characteristics of the histology, ultrastructure, and morphogenesis of this RLO, along with the histological and ultrastructural pathology of RLO infection in oyster. Even though Apulian fisheries oyster landings are a mix of native harvest and aquaculture, only native samples were collected for the survey. The ultrastructural morphology of the RLOs found in the present study showed similarity to that of RLOs in other oysters as *C. ariakensis* (Sun and Wu 2004), *C. rizophorae* (Azevedo et al. 2005) and *C. gasar* (Da Silva et al. 2015) except that in our work inclusions were observed merely in digestive gland cells and not in gills. In *C. ariakensis* the rickettsia-like organisms usually were found in membrane-bound cytoplasmic vacuoles, as we observed in the present work and here referred as RLO microcolonies (Sun and Wu 2004). We found some inclusions, which presumably were mature, containing pure RLOs surrounded by a thick electron-dense membrane, as previously described by Sun and Wu 2004 (Fig 3b); also, in the same Figure we showed some RLOs escaping to the cytoplasm from a mature inclusion. A higher magnification of the intracellular inclusion by TEM revealed both the presence of fine granular ribosomal materials filling the cytoplasm of the RLO organisms and crushed to the wall envelope, typical of microorganism in replicative stage with chromatin fibers (Fig 3b). Such RLO inner description is similar to that found by Sun and Wu (2004), in fact the authors described in the cytoplasm the granular presence of tightly packed ribosomes at the periphery of RLO envelope and a compact DNA chromatin zone, with dense

filamentous structure seen in longitudinal section. In a recent study by Da Silva et al. (2015), it was described the occurrence of cytoplasmic colonies of RLOs among the epithelial cells of gills (3%–7%) and in digestive gland tubules (7%–20%) of *C. gasar* oysters cultivated in Brazil. RLO colonies, as we reported in this work, were basophilic and granular, with diameters of 20–25 mm (Fig. 3). Authors found that the intensity of RLO lesions, in both types of infected tissues was generally low (1-4 colonies per section), except in one native and one cultured oyster that showed 10 and 18 RLO colonies, respectively. By contrast, in parasitized *O. edulis* we found a higher intensity of pathogenic lesions due to the occurrence of 260/300 microorganisms per inclusion and 25-35 colonies per section. Usually, retrieving low intensities of such infections suggests that their mortality effects may also be low; as well as on the other hand, to find high intensities of infections advises for serious mortality effects as in our findings. Heavy and extensive cytopathological lesions have been reported in the epithelial cells of the gills, digestive gland, mantle, and digestive tube in several oyster species, and as from our results the parasitized cells were greatly hypertrophied and eventually ruptured (Wu and Pan 2000). As detected here, in a previous report Wu & Pan (1999a,b) revealed that in digestive gland and mantle tissues of pearl oysters, RLO infection was associated with acute tissue-altering inflammation, including cell disintegration and cell lytic necrosis. The degree of destruction of tissues and the cytopathological effects of RLO infection are associated with the number of RLO inclusions in tissues and with intracellular growth, as judged by the large numbers of RLO in cells (Wu & Pan 1999a,b). The currently most accepted hypothesis is that these RLO would be of low virulence and that the degree of the pathological alterations would be related with the infection degree (Cano et al. 2018; Hooper et al. 2019). Then, only massive infections would cause a significative pathology with lytic necrosis and production of enzymatic activities, such as catalase or acid phosphatase, which could be related with its severe pathogenicity (Le Gall et al. 1991). Likewise, since long time RLOs have been reported as potential

pathogens of bivalve mollusks (Comps and Bonami 1977), and have been associated with severe mortalities of mollusc species (Le Gall et al. 1991; Norton et al 1993; Villalba et al. 1999). Other reports suggest that RLOs cause no damage to the host cells or cause limited pathology (Fries and Grant 1991; Morrison and Shum 1983) or might act as reservoirs of infection for rickettsia infecting animals in the marine environment. It may be associated with different species of rickettsial organisms or different development stages of inclusions. This study represents the first histopathological record for *O. edulis* parasitized by RLOs in Italy, providing a reference starting point for expanded future surveys for health monitoring among both native and cultured oysters in the South of Italy. It is noteworthy to mention that near native *O. edulis* landings, extending offshore cultivation of *O. edulis* have been implemented by Apulian fisheries.

Conclusions

The aim of this study was to investigate the presence of putative pathogens in an economically important bivalve species, the European flat oyster, from the southern coast of Italy, to produce knowledge for assuring a healthy production, both for managing natural stocks and for oyster aquaculture cultivation. Given these results, an important aspect to consider for consumer's health is the possibility of infections caused by rickettsia species, as emerging diseases from aquatic environment. Also, recent findings described the possibility of serious human liver involvement in some other human RLOs infections, as much as to contemplate rickettsioses in the differential diagnosis of acute hepatitis (Madison et al. 2008, Tosoni et al. 2016). Additionally, since RLOs were found in the South of Italy as a pathogen of concern due to the endemic diffusion of species potentially dangerous to man, RLOs that occurs in commercially bivalve mollusks should deserve special attention, due to synergistic dangerous effects, because some bivalve species are habitually eaten raw.

In conclusion, this study represents a first histopathological inventory for the presence of Rickettsia-like organisms in the European flat oyster, *O. edulis*, providing a reference baseline survey for a presumptive diagnosis of the disease, which is based on the standard histopathological method combined with the confirmatory methods of scanning electron microscopy and transmission electron microscope. In the future, further molecular characterization by PCR and in situ hybridization (ISH) is recommended, for a better understanding of microbe taxonomical position. Expanded future epidemiological surveys on the real incidence of RLOs in Southern Italy oysters are also needed, to monitor adequate size representative sample, with planned strategies for large longitudinal studies as follow up in time, in order to avoid epizootic mortalities and ruinous economic losses.

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Figure 1. Map showing the location of the Apulian harvesting sites in the South of Italy. Wild oysters were collected from the fishery sealines of Manfredonia (site **1**) (41° 38′ 0″ N, 15° 55′ 0″ E), Margherita di Savoia (site **2**) (41° 22′ 0″ N, 16° 9′ 0″ E), Monopoli (site **3**) (40° 57′ 17″ N, 17° 10′ 24″ E), Taranto (site **4**) (40° 25′ 5″ N, 17° 14′ 27″ E) and Tricase (site **5**) (39° 56′ 0″ N, 18° 22′ 0″ E).

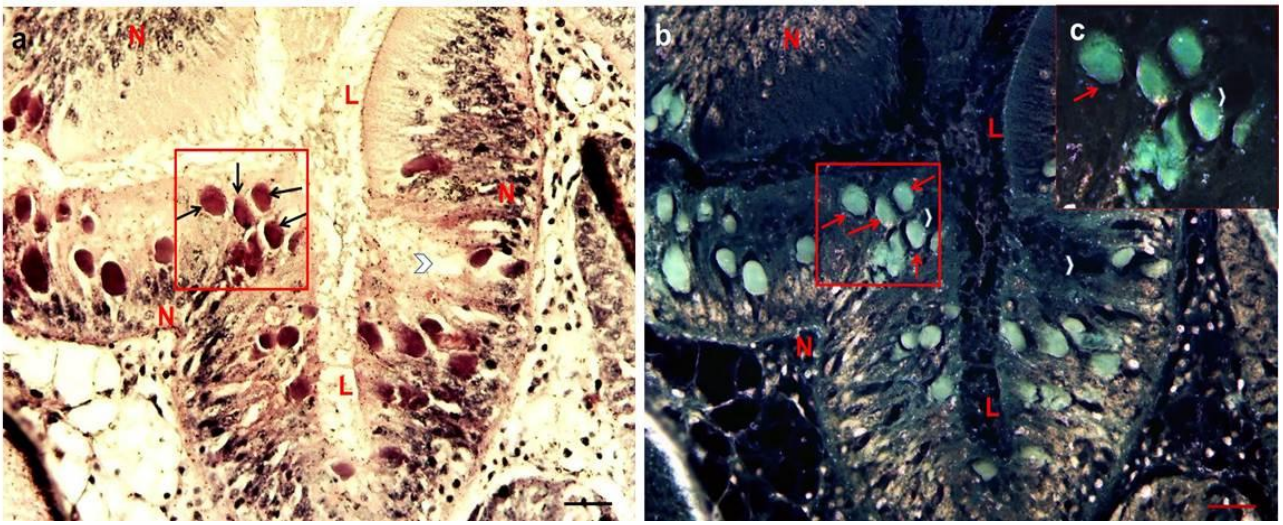

Figure 2. Haematoxylin and Eosin (H&E) stain picture. Section of epithelium of a digestive tubule of *Ostrea edulis* parasitized by RLOs. (**a**) Numerous intracytoplasmic inclusions (**I**) of RLO containing a great number of slightly amaranth or purple-red granules (black arrows). The inclusions in the digestive gland were polymorphic and irregular. Note the extensive lytic hollows (arrowheads) formed by lytic necrosis of intestine epithelial cells and numerous inclusions dropped from lytic cells. L, intestinal lumen; N, nuclei. H&E stain (40 ×). (**b**) Inverted image of (**a**) histological section showing some colonies of Rickettsia-like organisms (red arrows) with a smooth cell wall. Scale bar = 20 µm. (**c**) Higher magnification showing a disintegrated inclusions.

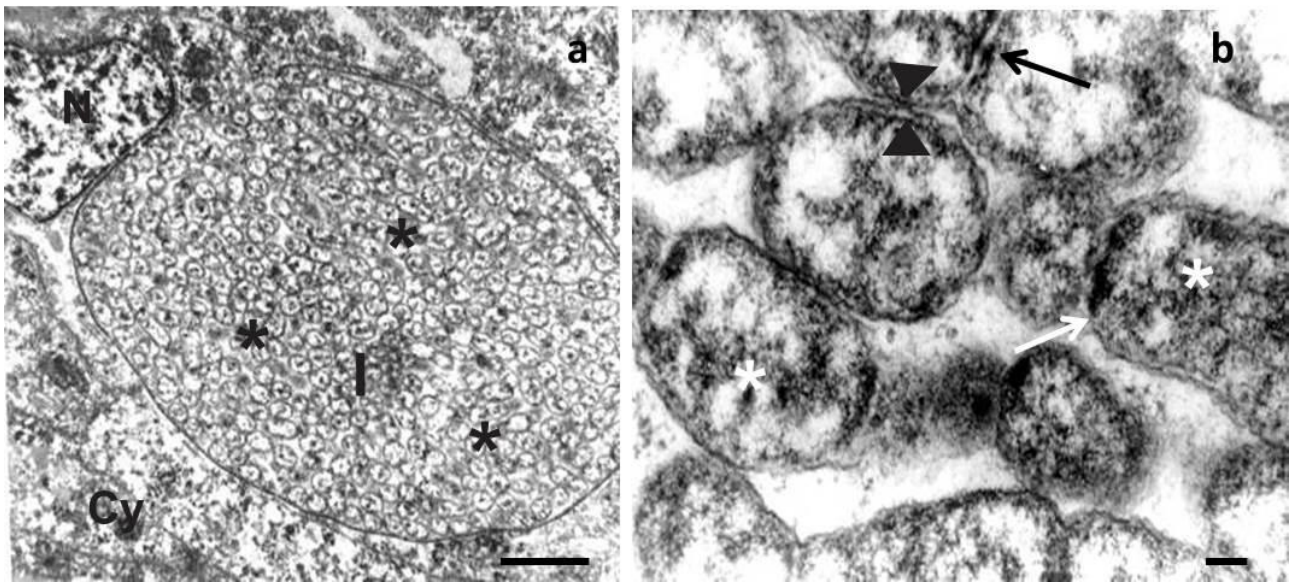

Figure 3. Transmission electron micrographs (TEM) of *Ostrea edulis* digestive gland tubule epithelium.

a) Intracytoplasmic colony (**I**) showing numerous rickettsia-like organisms parasitizing the epithelial cells of the digestive gland; a total of 260 microorganism particles (asterisks) were counted. Cytoplasm of digestive gland epithelial cell (**Cy**); intracellular inclusion (**I**); nucleus of digestive gland epithelial cell (**N**); (bar = 1 µm).

b) Higher magnification of **a**) intracellular inclusion. Note the fine granular ribosomal materials, with chromatin fibers (white asterisks), filling the cytoplasm of the RLO organisms. Several transverse binary fission of RLOs were present in different stages of organism division. White arrow: microorganism in replicative stage; black arrow: binary fission completed; black arrowheads: end of binary fission and reconstitution of wall envelope (bar = 0.5 µm).

The sections were observed under a transmission electron microscope (EM 109 Zeiss).

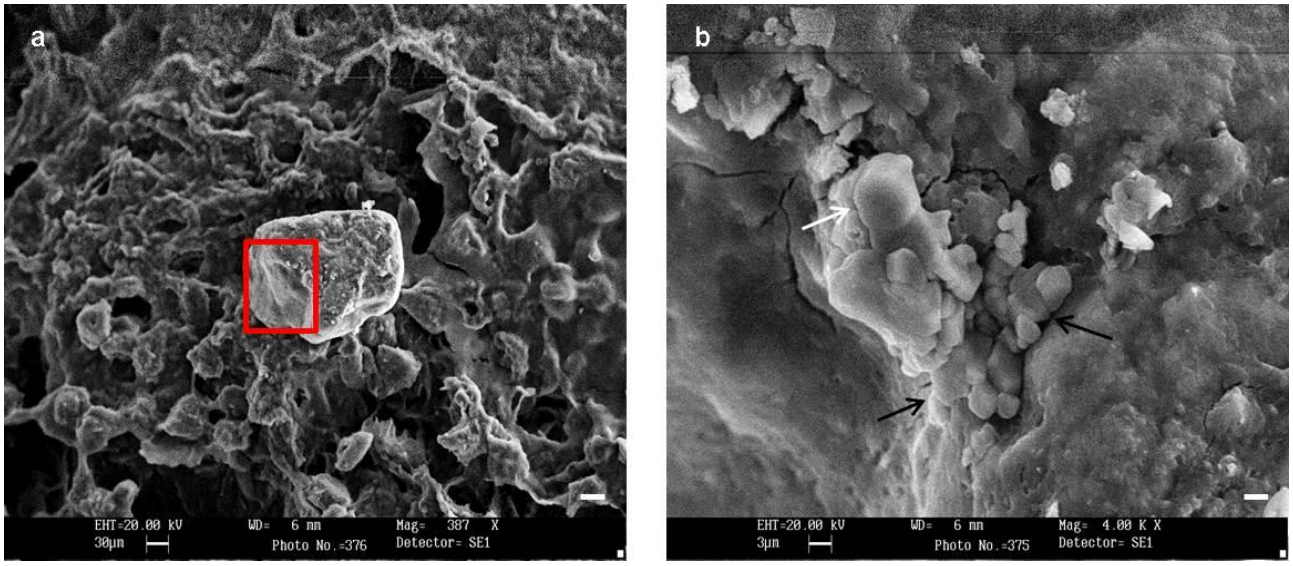

Figure 4. Ultrastructural analyses by Scanning electron micrograph (SEM) revealed particular characteristics of RLO inclusion surface morphology (**a**) Scanning electron micrograph of the spherical inclusion body in *Ostrea edulis* digestive gland tubule epithelium. Scale bar = 30 µm; (**b**) Detail of squared zone of (**a**) showing isolated (white arrow) or assembled (black arrows) roundish bodies (rickettsia-like organisms). Scale bar = 3 µm.