



New three-way symbiosis: an eukaryotic alga, a blue mussel, and an endolithic cyanobacteria

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Abstract

In several parts of the world, mytilid mussels, *Mytilus* spp., are infected with pathogenic, single-celled, photosynthetic algae belonging to the genus *Coccomyxa*. The posterior shell edge of heavily infected mussels becomes considerably thickened with an extra shell material. Also, the external shell surface is usually eroded as a result of the microboring activity of endolithic cyanobacteria. We compared the number of bioeroded shells, the bioerosion degree, and the number of badly eroded shells, in uninfected and *Coccomyxa*-infected *Mytilus* spp. from the Lower St. Lawrence Estuary, Québec, Canada. The thickness of prismatic and nacreous layers was measured. The epibionts (pink calcareous algae, crustose brown algae, and barnacles) which encrusted surface of studied shells, were counted. Epibionts did not occur frequently and their possible relationship with the partners of a three-way symbiosis, *Coccomyxa* sp. – *Mytilus* spp. – endolithic cyanobacteria, has been neglected. We suggest that the mussel provides the alga *Coccomyxa* a protected space and metabolic carbon for photosynthesis. The alga stimulates shell thickening, and this protects mussel against ocean acidification and predators. The endolithic cyanobacteria remove black-colored periostracum providing the mussel and alga with an increased ability to survive during sunny days when exposed at low tide. The eroded shells become more translucent which encourages alga photosynthesis. However, shell degradation caused by endolithic cyanobacteria is a possible reason for the death of the *Coccomyxa*-infected mussels at the studied sites.

Keywords Symbiosis · Blue mussels · Photosynthetic algae · Endolithic cyanobacteria · Bioerosion

1 Introduction

Symbiotic associations rarely occur with marine mytilid mussels. For example, it is known between barnacle (*Balanus*) and *Mytilus* (see in Laihonen and Furman 1986), as well as the deep-sea *Bathymodiolus* harbours two types of chemosynthetic bacterial endosymbionts (Ponnudurai et al. 2020). These associations involved commensalism and mutualism, respectively.

Blue mussels, *M. edulis* L. and *M. trossulus* Gould, (and their hybrids) are colonized by an alga belonging to the genus *Coccomyxa*. This genus of single celled green algae is known to be the symbiont of a number of terrestrial lichens (Nash 2008) and the alga produces the sugar alcohol ribitol, which moves to and supports the growth of the fungal symbiont (Richardson et al. 1968). The strain of *Coccomyxa* found in mussels, strain KJ372210, has been studied in the Estuary and the Gulf of St. Lawrence, Canada (Fig. 2a–c) since 2012 (Zuykov et al. 2014, 2018a). The algae colonize mussels' tissues (Fig. 1d) and are also present in the hemolymph and

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extrapallial fluid (Zuykov et al. 2014). The posterior shell edge of colonized mussels are often thickened significantly with an extra shell material (Fig. 2i) which forms a distinctive L-shaped shell deformity (Fig. 1g). This suggests, that the *Coccomyxa* alga can influence the calcification process in *Mytilus*. This phenomenon, light enhanced calcification, is known in tridacnid bivalve mollusks which have a symbiotic association with single-celled photosynthetic algae of the genus *Symbiodinium* (“zooxanthella”) (Zuykov et al. 2018b; Zhao et al. 2019; Rossbach et al. 2019). *Coccomyxa* occurs in mytilid mussels, and some other bivalves, in several areas around the world (Zuykov et al. 2018b, 2020). There is no clear understanding of the relationships between the alga and its host. We agree with the proposal by Sokolnikova et al. (2016) who called it a facultative parasitism after a detailed histopathological study of mytilid *Modiolus kurilensis* Bernard from the Sea of Japan.

The present study focuses on a third partner joining the *Coccomyxa-Mytilus* association in the form of endolithic cyanobacteria. Endoliths excavate a space in the external shell surface to live and grow that leads to its erosion. This can become badly eroded so that the periostracum, (thin organic outermost shell layer) and nearly all prismatic layer can be lost completely (Fig. 1c, f, h). Erosion leads to the thinning of shells in *Coccomyxa*-infected mussels to the point that the thickness cannot be correctly measured. This erosion, following shell colonisation by endolithic cyanobacteria was first reported by Zuykov et al. (2014) and investigated using scanning electron microscope (Fig. 1j). The endolithic assemblage includes *Leptolyngbya terebrans* (Bornet & Flahault ex Gomont) Anagnostidis & Komárek, *Pseudoscytonema endolithicum* (Ercegovic) Anagnostidis, and *Hyella caespitosa* (Bornet & Flahault). In addition, many other epizoic organisms are also known to use mussel shell as a substratum on which to settle. These include pink calcareous algae, crustose brown algae, and barnacles that are very common in lower tide pools and the uppermost sublittoral zone in the North Atlantic (Leclerc 1987). They encrust mussels shells and compete for space with a shell-degrading endoliths.

The aim of the present study was to analyze the potential three-way symbiosis between the pathogenic single-celled photosynthetic microalga *Coccomyxa* sp., the shoreline mussel *Mytilus* spp. and endolithic cyanobacteria. Attempts were made to answer four questions: (1) how often does the *Mytilus* - *Coccomyxa* association include a third partner, the endolith, (2) what is the degree of bioerosion and what proportion of valves have badly eroded surfaces, (3) how is bioerosion related to shell size and some environmental parameters, and (4) how often do epibiotic algae and barnacles occur on external shell surfaces?

2 Materials and methods

2.1 Sampling sites and mytilid mussels

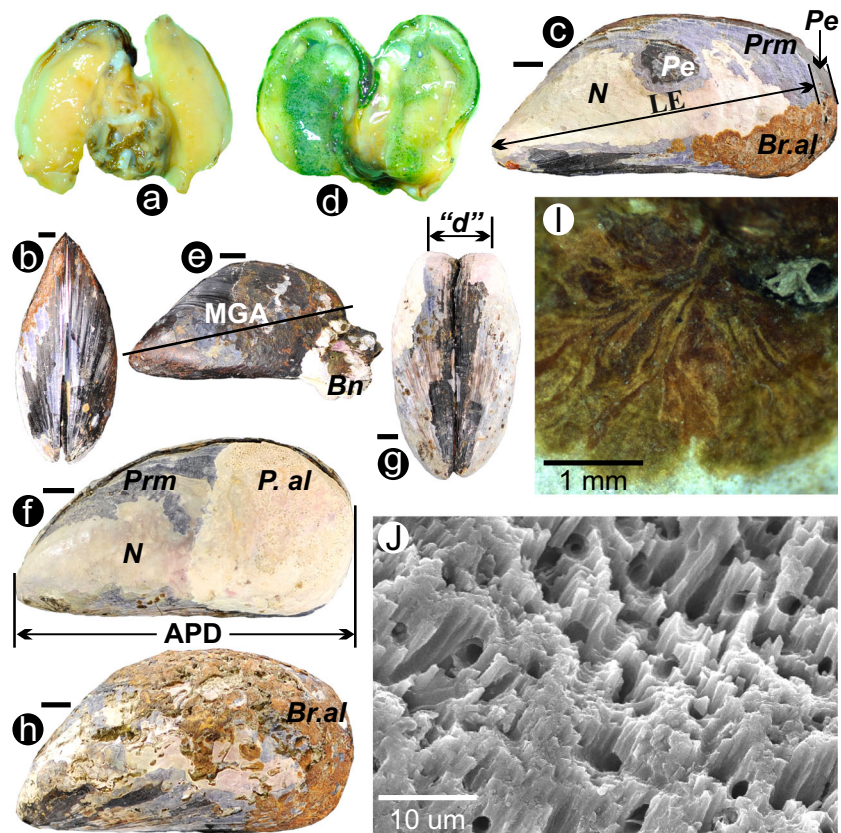
Sampling was conducted at low tide at *circa* 100 m from the shoreline in the site Métis-sur-Mer (48°40'46"N, 68°2'2"W) in September 2013, and in the site Longue-Rive (48°34'19"N, 69°11'36"W) in August 2018 (Fig. 2a–c). As part of our survey, we compared shells obtained from both uninfected (set 1) and *Coccomyxa*-infected (set 2) mussels of two sizes (small and large) from two sites which had a different hydrodynamic regime and bottom sediment characteristics. The average salinity in May 2017 was 25 PSU and 28 PSU for the Longue-Rive site and Métis-sur-Mer site, respectively, and the average pH of surface waters varied between 7.8 and 8 at both sampling sites (Dr. Y. Gélina, Concordia University, Canada, personal communication). At the Métis-sur-Mer site, the muddy bottom sediment is softer than in the Longue-Rive site where there is a sandy beach. The Longue-Rive and Métis-sur-Mer are open and closed to storm wave action, respectively (Fig. 2b, c). At both sampling sites, mussels occur in clusters but do not form dense beds. Their molecular analysis revealed the presence of *M. edulis*, *M. trossulus* and a hybrid; all are known as possible hosts of *Coccomyxa* (Zuykov et al. 2018a). At each site, from an area of approximately 20 m², equal numbers (300) of mussels were collected: 150 uninfected (Fig. 1a–c) and 150 *Coccomyxa*-infected (Fig. 1d–j). They were categorized by measuring the maximum shell anterior-posterior dimension, APD (Fig. 1f), as 100 small (18–39 mm) and 200 large (40–76 mm). The tissues were removed from shells and examined with a binocular microscope at low magnification (×5, ×10). The shells were washed carefully with filtered water and dried at room temperature. The infection with *Coccomyxa* was confirmed after observation of algal colonies (green spots) on the surface of the soft tissue (Fig. 1d); some specimens were “heavily infected” because green spots covered half or more of the mantle surface. Green spots were also clearly visible on the surface of other organs of the soft body and inside of the posterior adductor muscle. Shells of infected mussels are characterized by various degrees of L-shaped shell deformity (Fig. 1g) that is absent in uninfected mussels (Fig. 1b).

The six shells figured in present paper are deposited in the National Museum of Nature and Science, Tokyo (Japan), under registration numbers of molluscan collection NSMT-Mo 79281 - NSMT-Mo 79286.

2.2 Endoliths and shells bioerosion

To confirm endolithic infestation, selected shell fragments were coated with gold-palladium and studied with a scanning electron microscope (SEM JEOL JSM-6460 LV; an acceleration voltage of 20 kV) at Laurentian University (Canada).

Fig. 1 Wild mytilid mussels (*Mytilus* spp.) from the Lower St. Lawrence Estuary. Individuals uninfected with *Coccomyxa* algae – a–c; infected with *Coccomyxa* algae – d–j. Soft tissues – a, d; shells – b, c, e–h, j. Bioerosion of the external shell surface induced by endolithic cyanobacteria – c, f, h, j. Surface with holes of shell-boring polychaetes – h. Epibionts: brown algae (*Br. al*) – c, e, h, i; pink algae (*P. al*) – f, barnacle (*Bn*) – e. APD – maximal anterior-posterior dimension, MGA - maximum growth axis, LE - linear length of the eroded zone, “d” – shell parameter that is available only in *Coccomyxa*-infected mussels, Pe – periostracum, Prm – prismatic layer, N – nacreous layer. Scale bars 5 mm. Shells, collection number: b, c (NSMT-Mo 79281), e (NSMT-Mo 79282), f, g (NSMT-Mo 79283), h (NSMT-Mo 79284)



In the study of bioerosion by endoliths 600 mussels (1200 valves) were examined for: (1) presence or absence of bioerosion; (2) number of badly eroded shells, i.e., where at least one valve has completely lost its periostracum and actually all prismatic layer; (3) linear length of the eroded zone (LE) was measured with a digital caliper (Fig. 1c, f); (4) bioerosion degree (in percent) was calculated for both valves as the difference between APD and LE; if both valves (in one shell) were infested with endoliths, only the valve with a maximal LE value was taken into account for the calculations.

2.3 Epibionts

Epibionts were identified by Drs. W. Adey (Smithsonian Institution, Washington DC, USA) and M. Cusson (Université du Québec à Chicoutimi, Chicoutimi, Québec, Canada) based on photos. Presence/absence of epibionts per shell was counted.

2.4 Thickness measurement (non-eroded shells)

Careful examination of 1200 valves revealed that only eight shells from uninfected mussels and eight from *Coccomyxa*-infected ones (each set consists of four small and four large individuals) collected at Métis-sur-Mer site were characterised by unaltered shell (Fig. 1e). The latter were therefore used for

the measuring shell thickness. The central part of one valve (for each of 16 shells) was embedded in epoxy resin to avoid crushing during cutting by a diamond saw. After curing of the resin, shells were sliced longitudinally as shown in Fig. 1e; thin slices were glued with double-sided tape to a glass plate and were progressively polished with silicon carbide paper and diamond paste. Images of polished sections (Fig. 2h, i) were acquired with a scanner (Epson GT-X980) and shell thickness was measured using the CorelDraw program.

The shell periostracum was not considered here but we determined the thickness of two mineral layers, prismatic and nacreous, at three different points Fig. 2h, i. Both layers were measured in umbonal area where the shell has a maximal thickness (point 1), and at the midpoint (point 2) along the shell cross-section. The prismatic layer (point 3) thickness was examined at a distance of 2 to 3 mm from the posterior edge. In order to carry out equal measurement in shells with L-shaped shell deformity, the shell thickness at point 3 was determined using the length of the cathet “b” in the right triangle inscribed in the space of the thickened shell edge (Fig. 2i).

2.5 Statistical analysis

The data shown in Fig. 2e, j, were statistically analyzed using SPSS 19.0 software. Shapiro-Wilk’s test and Levene’s F-test

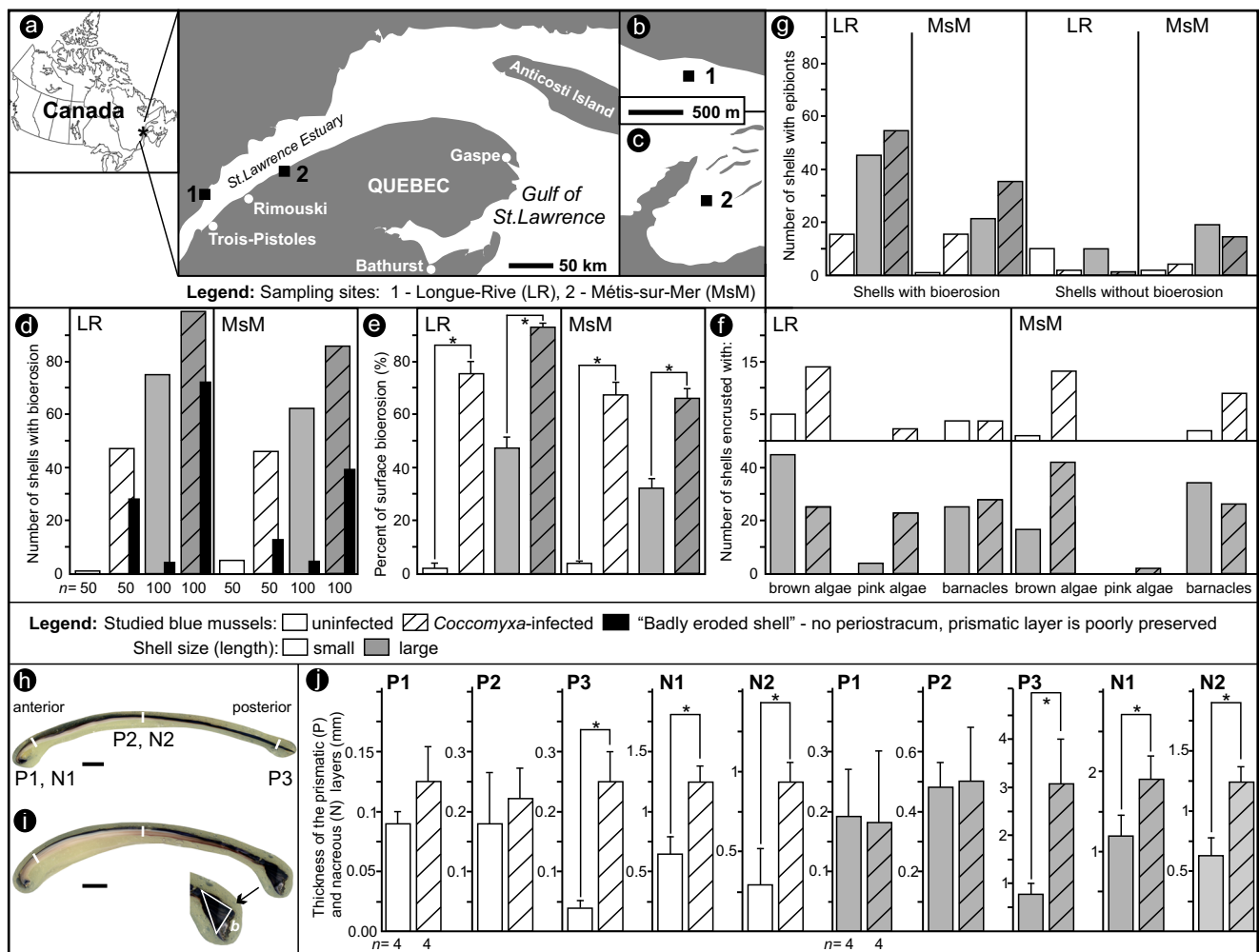


Fig. 2 Characteristics of shells of wild uninfected and *Coccomyxa*-infected mytilid mussels (*Mytilus* spp.) collected in Longue-Rive, LR (48°34'19"N, 69°11'36"W) and Métis-sur-Mer, MsM (48°40'46"N, 68°2'2"W) in the Lower St. Lawrence Estuary. Sampling sites – a–c; Number of shells with bioerosion – d; Mean (\pm SE) percent of surface bioerosion – e; Number of shells encrusted with different epibionts – f; Number of shells with/without bioerosion encrusted with epibionts – g; Shells (in section; maximum growth axis) of uninfected – h, and

Coccomyxa-infected mussels – i (b - cathet in the right triangle was considered as the thickness of prismatic layer in point 3), points where thickness of prismatic (P1, P2, P3) and nacreous (N1, N2) shell layers were measured; Mean (\pm SE) thickness of the prismatic and nacreous layers in different sets of shells – j. Asterisk denotes a significant difference between uninfected and *Coccomyxa*-infected mussels. Scale bars (for d, e) 5 mm. Shells, collection number: h (NSMT-Mo 79285), i (NSMT-Mo 79286)

were respectively applied to test if the assumption of normality and heteroscedasticity are respected. One-way analysis of variance (ANOVA) was applied subsequently to examine whether the percent of surface bioerosion and thickness of the prismatic and nacreous layers differ significantly between uninfected and *Coccomyxa*-infected mussels. Statistically significant differences were set at p -values less than 0.05.

3 Results

3.1 Shells: bioerosion and encrustation

The eroded surface was matt, and its rough tactile is indicated in the SEM image of Fig. 1j. Bioerosion and/or epibionts were

found on 60% (Métis-sur-Mer site) and 65% (Longue-Rive site) shells obtained from uninfected mussels, whereas for shells of *Coccomyxa*-infected mussels the figure is 100%. The number of mussels with bioeroded external shell surface, the number of mussels with badly eroded shell, and the bioerosion degree were also always higher among *Coccomyxa*-infected individuals (Fig. 2d, e). While all three characteristics showed a similar trend between sampling sites, absolute values vary slightly and were always higher for mussels collected from the open coast (Longue-Rive site) rather than from a small bay (Métis-sur-Mer site).

The studied mussels' shells carried three epibionts. The proportion of encrusted mussels varied (from low to high; Fig. 2f) and was as follows: pink calcareous algae (*Clathromorphum circumscriptum* (Strömfelt) Foslie) (Fig.

1f), barnacles (*Balanus* sp.) (Fig. 1e), and the crustose brown algae (*Ralfsia fungiformis* (Gunnerus) Setchell and N.L. Gardner) (Fig. 1c, h, i). They do not all occur together on the surface of one shell, but often a combination of two of them are present. Epibionts favour encrusting shells with a bioeroded surface (Fig. 2g). The epibiont load was generally low. The pink algae cover varied from small sized zones (5 × 5 mm) up to half of shell surface (Fig. 1f); brown algae completely covered the surface in one or even in both valves in fourteen shells, but commonly they only cover up to half a valve. Usually, one to three barnacles of small sizes are found per shell, but a few shells have up to 10 barnacles.

The surface of four large shells (two from Longue-Rive site and Métis-sur-Mer site) exhibited holes resulting from shell-boring polychaetes (Fig. 1h) but while the excavations were visible and significant, the internal shell surface was not penetrated.

3.2 Shell thickness

The prismatic layer revealed insignificant differences in thickness in umbonal and central shell parts (points 1, 2) for all studied sets ($p > 0.05$) (Fig. 2j). However, the thickness of both the prismatic layer on the posterior edge (point 3) and the nacreous layer (in the two studied points), was significantly higher for *Coccomyxa*-infected mussels ($p < 0.05$).

In contrast, bio-eroded shells were usually two times thinner and lighter than non-eroded shells of the same size. Many shells were “badly eroded” (Fig. 2d) and, therefore, they were very fragile and looked translucent. Fragile zones are built with eroded nacre, and, as they lack a periostracum and prismatic layer, have a greyish coloration (Fig. 1c,f). Shell thickness in fragile zones has the appearance of an eggshell, and can be even thinner, so it is possible to break the bivalve shell by a gentle finger push. These, “soft shell” zones, are especially frequent above the area where the adductor muscle attaches to the shell.

4 Discussion

The possible three-way symbiosis considered in this paper has not been examined before but the possibility was suggested after reading a paper by Mortensen et al. (2005). They made observations on *Coccomyxa*-infected mussels from southern Norway. They concluded that “mussels with the most eroded shells had the most severe deformities and the highest infection rate.”

Our results clearly provide evidence of the close association of three organisms, alga *Coccomyxa* sp., blue mussel *Mytilus* spp., and endolithic cyanobacteria. The mussel provides protected space for the *Coccomyxa* alga and access to metabolic carbon for photosynthesis (Zhao et al. 2019). The alga stimulates shell thickening (light enhanced calcification) made mussels protected against abiotic and biotic stressors.

This is very relevant nowadays, because of global warming and ocean acidification can possibly impact negatively the bivalves’ ability to produce shell without reducing its strength (structural integrity) and thickness (Fitzer et al. 2015). Endolithic cyanobacteria (bioerosion) remove black-colored periostracum providing the mussels and algae with an increased ability to survive during hot periods (sunny days at low tide). This advantage was suggested by recent experimental work by Gehman and Harley (2019), who showed that the blue mussel–endolithic cyanobacteria relationship provided a tolerance for intertidal mussels to high environmental temperature. Furthermore, within this presumed host-parasite pair, global warming is likely to promote a shift from a parasite to a mutualistic association. We agree with Mortensen et al. (2005), that the thin bioeroded shells that become more translucent to light will provide the ability for enhanced photosynthesis in the *Coccomyxa* cells growing within the mussel.

It is well known that the activity of endolithic cyanobacteria can lead to mussel mortality, as the thinner shells become more susceptible to breakage. In some cases, such bioerosion can be responsible for the death of up to 50% of the large-sized mussels in a population (Kaehler and McQuaid 1999). Usually, the shell surface of *Coccomyxa*-infected mussels display significant bioerosion (see ref. in Zuykov et al. 2018b). We hypothesise, that the bioerosion needs to be considered as the prime reason for the death of *Coccomyxa*-infected mussels at our studied sites. Therefore, the high level of infestation with endolithic cyanobacteria may be one factor that limits the spread of this pathogen worldwide. Free-living marine representatives of the genus *Coccomyxa* have not yet been described (Darienko et al. 2015). We propose that this alga migrates inside of already infected mussels that are temporarily associated with ships or fishing equipment. Transmission of *Coccomyxa* between mussels, upon arrival of infected individuals transported by boats or gear to a new site, seems to be promoted by the water pollution with various xenobiotics in estuarine and coastal waters (Zuykov et al. 2021).

In contrast to endoliths, epibionts do not occur as frequently on studied shells, and, thus, we do not consider them to be part of this triple way symbiosis. The relationship between shell colonisation (bioerosion and encrustation), the host mussel shell and its colonizers (and between colonizers) is very complex. It depends on numerous abiotic and biotic factors and requires further research especially with respect to *Coccomyxa*-infected mussels. It cannot be excluded that the periostracum in mussels at the open coast (Longue-Rive site) relative to those at the more protected small bay (Métis-sur-Mer site) could be characterised by greater microdamage (abrasion). This may be, due to the more powerful hydrodynamic conditions and sandy sediment which is more abrasive, than muddy sediment and that this is favorable for settlement of epizoic organisms (Fig. 2d, g) (Zardi et al. 2009).

5 Conclusion

In this study, we explored the existence of a three-way symbiosis (alga-mussel-endolith). This association seems to promote mussel and alga tolerance to the abiotic and biotic stresses. Understanding the potential consequences of this for the host and symbionts in terms of previously unrealized and therefore novel capabilities is a potentially rewarding research avenue. The evolutionarily significance of such symbiotic events merits further exploration. In particular, it would be interesting to examine the metabolic interactions between mussels and *Coccomyxa* and between the endosymbiotic cyanobacteria and the mussel host. This three-way symbiosis promotes rapid endolithic growth (shell colonization). There appears to be a competition between the kinetics of the two processes, (1) calcification rate (light enhanced calcification), and (2) shell destruction rate (by the endolith community) and the erosion usually dominates. Therefore, the three-way symbiosis may provide a mechanism that limits the dispersal and distribution of the invasive *Coccomyxa* as a result of the earlier death of infected host mussels.

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Authors' contributions MZ, JA, MG and PA contributed to the study conception and design, and performed the field research; MZ wrote the first version of the manuscript; GK, LZ and KS analyzed the data and worked with the literature; MG, PA and MS administrated the project. All the authors edited the manuscript.

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Data availability All data generated or analyzed in this study are included in the article.

Declarations

Conflict of interest The authors report no conflicts of interest.

Consent to participate Not applicable.

Consent for publication Not applicable.

Code availability Not applicable.

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