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Variation of parasite and fungi infection between farmed and wild mussels (*Mytilus galloprovincialis* Lamarck, 1819) from the Adriatic Sea

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Investigation of parasites and diseases affecting molluscs of ecological and economic interest is critical for the management of native stocks and aquaculture. In recent years, much attention has been devoted to investigating the World Organisation for Animal Health listed infectious diseases, so that communities can be prepared to attend public health emergencies and avoid severe income losses. In this context, the health status of Mytilus galloprovincialis *Lamarck, 1819 was analysed in two aquaculture sites (Strunjan Bay and Piran Bay, Slovenia), and in four natural mussel beds (Adriatic Croatia International Marinas Pula and Rovinj and St Catherine, Croatia; Marina Koper, Slovenia) along the coast of the northern Adriatic Sea. The mussels were sampled in February and April 2014, and processed for histological examination of several endosymbionts and pathogens that frequently occur in mytilid mussels. Endosymbionts and pathogens were not detected in farmed mussels. Prokaryotic inclusion bodies, the protozoan* Nematopsis, Ancistrocoma*-like ciliates, haplosporidian-like plasmodia, turbellarian* Urastoma cyprinae*, and basophilic inclusion bodies were observed in digestive gland cryosections of wild mussels from the coastal region of the northern Adriatic. Fungal spores of* Psilocybe *sp.,* Ulocladium *sp. and* Alternaria *sp. were detected between the digestive tubules based on their morphology. Diagnostic PCR did not reveal infection with* Marteilia refringens *during the studied period, neither in wild nor farmed mussels. We confirmed the site effects on prevalence of infected mussels. Thus, we can conclude that wild areas are more exposed to endobionts and parasites than aquaculture sites.*

Keywords: endobionts, fungi, parasites, mussel, *Marteilia refringens*, *Mytilus galloprovincialis*, Adriatic Sea

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INTRODUCTION

Bivalves constitute a prominent commodity in aquaculture but are also crucial to preserve the complexity and function of ecosystems (Zannella *et al.*, 2017). Mussels are important suspension filter feeders and thus represent an essential component in the ecology of coastal waters. Mussels are favoured commercially because many species can tolerate and accumulate xenobiotics in their tissues at levels higher than those of the aquatic environment (Torre *et al.*, 2013b; Faggio *et al.*, 2016; Pagano *et al.*, 2016, 2017; Capillo *et al.*, 2018). Moreover, since mussels are edible, nutritious, and sessile species, they have been harvested and cultured worldwide for human consumption. Bivalve species of the genus *Mytilus* are dominant mussels living in the intertidal and subtidal zones, withstanding either more impacted or pristine sites (Roginsky & Lissi, 2005; Hamer *et al.*, 2012). The Mediterranean mussel *Mytilus galloprovincialis* Lamarck, 1819 is an important commercial species in Slovenia and Croatia, in the northern Adriatic Sea, and thus it has been extensively maricultured (Gombač, 2010; Pavičić-Hamer *et al.*, 2016).

The study of parasites and diseases affecting molluscs with ecological and economic interests is critical for the management of natural stocks and aquaculture (Boehs *et al.*, 2010). The most severe mussel diseases are caused by viruses, bacteria and protistans, which can be responsible for mortality outbreaks, thus posing a substantial commercial impact (Tuntiwaranuruk *et al.*, 2008; Ceuta & Boehs, 2012; Mladineo *et al.*, 2012). To a lesser extent, other diseases are caused by ciliates, turbellarians (Cova *et al.*, 2015), trematodes (Özer & Güneydağ, 2015) and fungi (Santos *et al.*, 2017). The spread of fungal strains may be facilitated by a combination of environmental conditions, such as wind or human-conveyed propagules (Sallenave-Namont *et al.*, 2000). Fungi can also be present in the marine environment in latent forms on host organisms or potential symptomless carriers (Ein-Gil *et al.*, 2009).

According to the World Organisation for Animal Health (OIE), notifiable diseases are those with socioeconomic and/or public health importance within countries or that are significant for the international trade of aquatic animals and their products (Aranguren & Figueras, 2016). The current

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list of notifiable diseases of bivalves and the causative pathogens includes infection with *Marteilia refringens*, *Bonamia* sp., *Xenohaliotis californiensis* and *Perkinsus* sp. (Carnegie *et al.*, 2016).

The increased use of coastal areas and the sea due to expansion in urbanization, population growth and tourism associated with the intense traffic of tourist vessels and maritime transport have detrimental effects on the health status of bivalves (Burgos-Aceves and Faggio, 2017; Savorelli *et al.*, 2017; Burgos-Aceves *et al.*, 2018; Faggio *et al.*, 2018). Studies have shown that environmental factors including poor water quality and the presence of parasites can impact the health status of mussel stocks in the UK (Bignell *et al.*, 2008; Lynch *et al.*, 2014). A decrease in water quality can affect the immunological response in aquatic organisms thus making them more susceptible to parasitic infection and increasing parasite prevalence (Khan, 1991). It becomes evident that particular attention coupled with protective measures are needed both for native and cultured mussel stocks.

The study of pathogens from different mussel populations in response to regional differences is a subject of major interest, particularly in aquaculture, where mussels originate from various sources (Bratoš *et al.*, 2004; Pavičić-Hamer *et al.*, 2016). The objectives of this study are (a) to investigate the health status of mussels on the northern Adriatic coast (Croatia and Slovenia) concerning effects of on-site water quality; (b) to identify the range of parasites and fungi present in aquaculture and wild areas and the possible route of exposure; and (c) to evaluate the health status of mussels and their likely high infection risks due to detrimental effects of pollutants.

MATERIALS AND METHODS

Collection of mussels and description of sampling sites

Mussels were collected from six sampling stations (N = 10 per station) along the coast of the northern Adriatic, in Slovenia and Croatia (Figure 1), during February and April 2014. Strunjan Bay (45°16′24″N 13°34′57″′E) and Piran Bay (45°07′50″N 13°44′10″E) harbour the most important Slovenian aquaculture sites, which are located 11.4 km and 20.6 km away from Koper, respectively. The mussel farm in Strunjan Bay (site code 0024) is inside a natural reserve (Krajinski park Strunjan), and the mussel farm near to Seča (site code 0035) is located in the inner part of Piran Bay. The third sampling site was close to the Marina Koper in Slovenia (site code 00TM), where mussels were collected from a natural bed in the vicinity. The two other impacted sites are Adriatic Croatia International Marinas (ACI), the ACI Marina Pula (44°87′55″N 13°84′67″E) and ACI Marina Rovinj (45°04′32″N 13°38′08″E). They are located near a sewage outflow and boat processing area characterized by elevated pollutant concentration in biota and sediment (Center for Marine Research, 2014). St Catherine Island in Croatia (45°07′67″N 13°62′96″E) is located ~5 km away from the urban and sewage outflow area and is considered a non-impacted environment (Bihari *et al.*, 2004; Center for Marine Research, 2014). Environmental parameters were measured with an MSS 90 multiparameter probe (Sea & Sun Technology), at 2 m depth at the time of sampling using standard procedures (ARSO, 2014; Center for Marine Research, 2014) (Table 1).

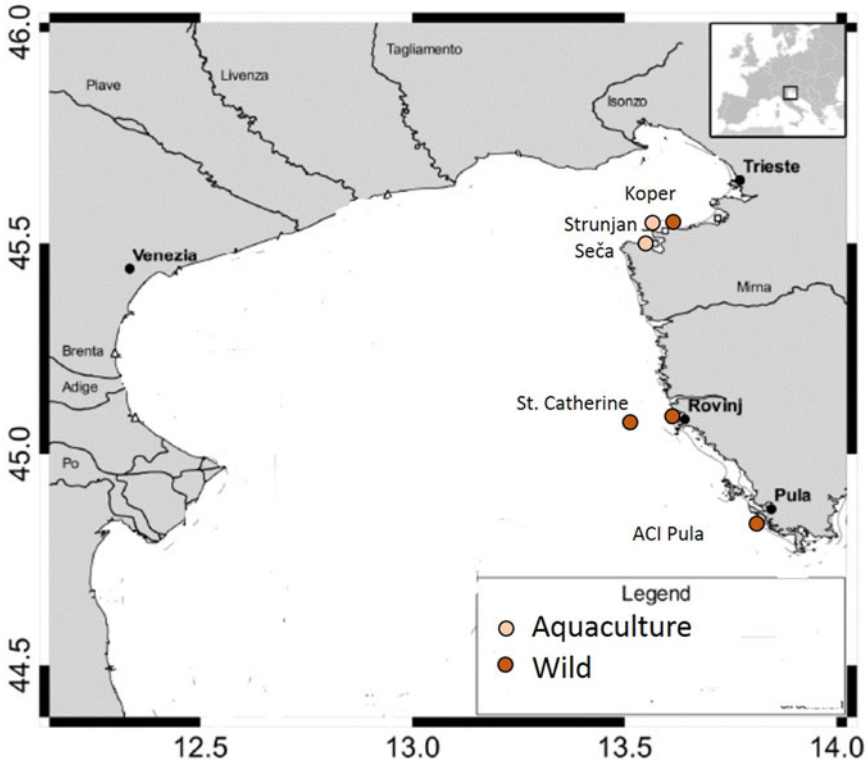

Fig. 1. Geographic location of the sampling areas and source of mussels in the northern Adriatic Sea (Slovenia and Croatia).

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Table 1. Environmental parameters estimated for the sampling sites during February and April in 2014, measured at 2 m depth.

Parameter/time	Temperature of seawater (°C)		Salinity (ppt)		O_2 concentration (mg L^{-1})		Chlorophyll *a* (μg L^{-1})	
Site	Feb	Apr	Feb	April	Feb	Apr	Feb	Apr
00TM	11.40	14.30	36.16	36.67	9.17	8.49	1.38	0.73
0024	11.48	15.43	36.76	34.97	8.99	8.38	2.11	0.49
0035	11.67	15.38	37.05	36.07	8.72	8.18	1.70	0.62
ACI Pula	10.60	13.71	28.83	31.70	5.45	5.72	0.50	0.59
ACI Rovinj	10.30	13.10	36.70	37.93	5.71	5.24	0.51	0.63
St Catherine	9.90	12.30	37.40	37.08	5.96	5.30	0.72	0.64

Histopathology

Ten mussels from each station per month were sampled and immediately transported to the laboratory where digestive glands were removed, fixed with n-hexane, and cooled in liquid nitrogen. The digestive glands were placed in a straight row on aluminium cryostat chucks (five per chuck). The chucks were stored at −80°C until analysis. Before cryosectioning, the samples were embedded in O.C.T.™ compound (Microm Inc. GmbH, Germany) and cut into 10 μm sections with a cryostat (Zeiss Hyrax C 50, Microm GmbH, Germany). Sections were stained with haematoxylin and eosin (Sigma-Aldrich, USA). Parasite morphology was studied using a light microscope (Nikon, UK). Authorities for parasite and endobionts identification included Vázquez & Cremonte (2017) for prokaryotic and basophilic inclusion bodies; Francisco *et al.* (2010), Cova *et al.* (2015) and Tuntiwaranuruk *et al.* (2008) for *Nematopsis* sp., *Ancistrocoma*-like ciliates, and turbellarian *Urastoma cyprinae* von Graff, 1882; Bignell *et al.* (2008) for haplosporidian-like plasmodia; and Zhang *et al.* (2009) for fungi. All micrographs were captured using an Ikegami ICD-803P digital video camera and the Lim Screen Measurement™ Lucia G image capture system (Nikon, UK).

Following Saffo (1992), we used the term 'infection' in referring to all organisms parasitically and endobiotically associated with their hosts. The prevalence and intensity of infection of each parasite were calculated according to Bush *et al.* (1997), as follows: the prevalence is the number of individuals infected divided by the total number of individuals in a sample, expressed as a percentage; the intensity is the number of parasites found in an infected mussel.

Diagnostic detection of *M. refringens* by PCR

Infection by *M. refringens* was surveyed by polymerase chain reaction (PCR). For this, a small piece of the mussel digestive gland of each collected individual was preserved in 95% ethanol. DNA was extracted using the DNeasy Blood & Tissue Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. After isolation, DNA samples were amplified in a SureCycler 8800 Thermal Cycler (Agilent Technologies, Santa Clara, California, USA) using standard primers for the *M. refringens* DNA, M2A (5′- CCGCACACGTTCTTCACTCC-3′); M3AS (5′-CTCGA GTTTCGACAGACG-3′), according to the protocol by Le Roux *et al.* (2001). The presence of PCR products was verified by agarose gel (1.5% w/v) electrophoresis.

Statistical analysis

The relationship between prevalence and sampling site and time was evaluated by a Chi-square test. The relationship between the intensity of infection and sampling site and time was assessed by a Kruskal–Wallis test. All tests were performed with Statistica 9.0. software at significance $P < 0.05$.

RESULTS

Along the coast of the northern Adriatic Sea, mussels were sampled from six sites in Croatia and Slovenia that were previously evaluated and classified as aquaculture sites (Strunjan Bay and Piran Bay, Slovenia), non-impacted sites (St Catherine, Croatia), and impacted sites (Marina Koper, Slovenia; ACI Pula and ACI Rovinj, Croatia).

No significant infections, i.e. parasites that cause significant mortalities or heavy infestations, were observed *in situ* during the study. Histological and molecular analyses did not show any evidence of the presence of *M. refringens*, an OIE-listed pathogen. The most common pathogens found in mussels from the impacted and non-impacted sites were haplosporidian-like organisms, prokaryotic inclusion bodies, *Ancistrocoma*-like ciliates, *Nematopsis* sp. and the turbellarian *U. cyprinae* (Figure 2). Filamentous fungi of *Ulocladium* sp., *Psylocibe* sp. and *Alternaria* sp. were detected in mussels from St Catherine, ACI Pula, ACI Rovinj (Croatia) and Marina Koper (Slovenia), but not from aquaculture sites (Figure 3). The number of parasite groups per site was higher in impacted sites (N = 9) compared with non-impacted (N = 1) and cultivation (N = 0) sites.

Specimens of a haplosporidian-like organism were detected in mussels from Koper (10% prevalence of infection). Plasmodia of haplosporidian-like organisms were spread into the epithelium of digestive tubules with more than 100 plasmodia per section (at 100× magnification).

Prokaryotic inclusion bodies that were spherical to oval shaped with basophilic structures 5–10 μm in size were found in the digestive tubules and between them (Figure 2a). In some cases, spherical inclusions were eosinophilic stained (Figure 2b). Their prevalence ranged from 10% (ACI Pula, February 2014) to 40% (ACI Pula, April 2014). A 30% prevalence was detected in all the other infected mussels (Table 2). The intensity of infection ranged between one and 12 prokaryotic inclusion bodies per section (Table 2). The site influenced both prevalence ($\chi^2 = 6.66$,

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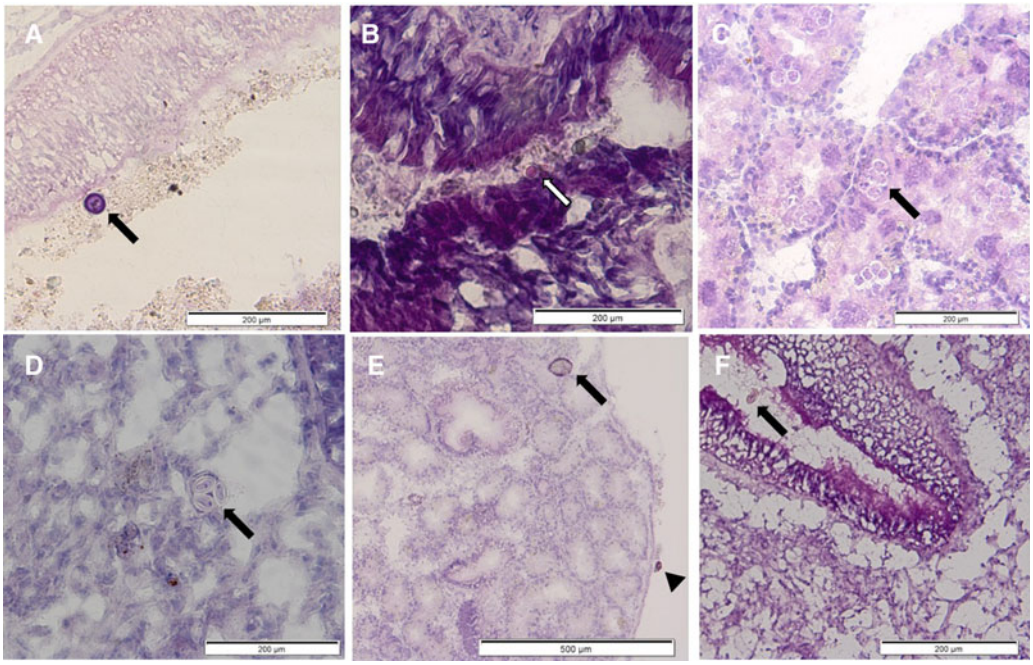

Fig. 2. Light microscopy of transverse cryosections through the mussel digestive gland of *Mytilus galloprovincialis* containing: (a) basophilic prokaryote-like organisms (arrow) in the lumen of the digestive gland; (b) eosinophilic prokaryote-like organisms (arrow) in the lumen of the digestive gland; (c) plasmodia (arrow) of a haplosporidian-like organism in the digestive tubule epithelium; (d) host phagocyte containing three *Nematopsis* sp. oocysts in the connective tissue between the digestive tubules; (e) *Urastoma cyprinae* (arrow) and *Ancistrocoma*-like ciliate (arrowhead) at the edge of the digestive gland and digestive tubules; (f) *Ancistrocoma*-like ciliate (arrow) in the lumen of digestive tubule.

df = 2, $P < 0.05$) and intensity of infection (Kruskal–Wallis test, $H = 6.32$, $P < 0.05$, Table 4).

Specimens of *Nematopsis* sp. with ungrouped and grouped oocysts were detected in the connective tissue between digestive tubules of mussels from non-impacted and impacted sites (Figure 2c). A single thickened membrane was bounding oocysts with a basophilic vermiform sporozoite inside. In infected mussels sampled in April 2014, the prevalence ranged from 20% (St Catherine and ACI Pula) to 80% (ACI Rovinj) (Table 2). In February 2014, ACI Rovinj had the highest prevalence of infection with *Nematopsis* sp. (80% prevalence). The intensity of infection was variable, with < 15 oocysts per section in most of the cases (maximum 47 oocysts/mussel). Prevalence ($\chi^2 = 6.00$, df = 2, $P < 0.05$) and intensity of infection (Kruskal–Wallis test, $H = 9.18$, $P < 0.05$) were significantly different between sampling sites (Table 4).

Specimens of the turbellarian *U. cyprinae* (10% prevalence) were observed at the edge of the digestive gland from mussels sampled in ACI Pula (Figure 2e).

In mussels from the same sampling site, oval-shaped *Ancistrocoma*-like ciliates, characterized by a ciliary fringe and a basophilic horse-shoe shaped nucleus, were observed (Figure 2f). Prevalence of *Ancistrocoma*-like ciliates in wild mussels ranged from 10% (Marina Koper, ACI Pula, ACI Rovinj, February 2014) to 20% (ACI Pula, April 2014) (Table 1), with an intensity of infection from 1 to 2. Site influenced both prevalence ($\chi^2 = 8.57$, df = 2, $P < 0.05$) and intensity of infection (Kruskal–Wallis test, $H = 7.50$, $P < 0.05$).

The fungi were observed in digestive glands of wild mussels. *Alternaria* sp. individuals (10% prevalence) were found within the connective tissue in mussel digestive gland from mussels sampled in Marina Koper (Figure 3a). Spores of *Psilocybe* sp. were recorded in the epithelial cells of the digestive tubules in the mussel digestive gland from mussels of impacted sites. Prevalence ranged from 10% (ACI Rovinj, April 2014) to 30% (Marina Koper, February 2014). We observed specimens of *Ulocladium* sp. (10% prevalence)

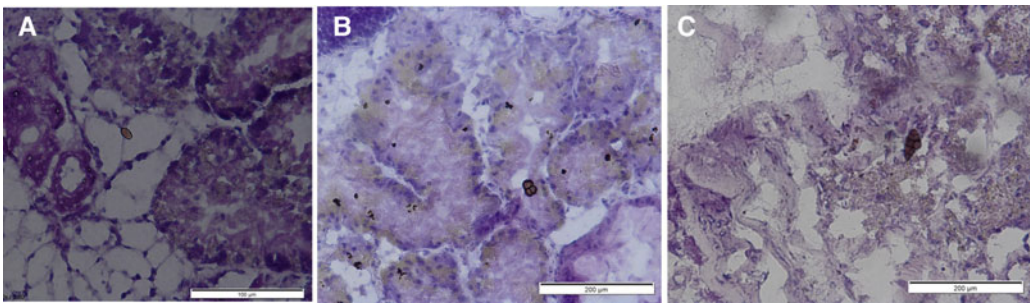

Fig. 3. Light microscopy of the transverse cryosections through the mussel digestive gland of *Mytilus galloprovincialis* containing spores of (a) *Psilocybe* sp.; (b) *Ulocladium* sp.; and (c) *Alternaria* sp. attached to the digestive tubules.

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Table 2. Prevalence (P) and intensity of infection (I) with the range in parentheses of prokaryotic inclusion bodies, *Nematopsis* sp., and *Ancistrocoma*-like ciliates from *Mytilus galloprovincialis* mussels collected in the northern Adriatic Sea.

Infection	Prokaryotic inclusion body P (%) [I (range)]		*Nematopsis* sp.P (%) [I (range)]		*Ancistrocoma*-like ciliate P (%) [I (range)]	
Site	Feb	Apr	Feb	Apr	Feb	Apr
ooTM	30 [2(1–6)]	0	70 [7 (2–16)]	10 [2]	10 [2]	0
0024	0	0	0	0	0	0
0035	0	0	0	0	0	0
St Catherine	30 [1]	30 [1(1–3)]	0	10 [1]	0	0
ACI Rovinj	30 [2(1–4)]	30 [4(1–11)]	70 [15(1–32)]	80 [11(2–47)]	10 [1]	10 [1]
ACI Pula	10 [1(1–12)]	40 [3 (1–3)]	50 [8 (1–12)]	10 [6]	10 [1]	20 [1]

Table 3. Prevalence (P) and intensity of infection (I) with the range in parentheses of the fungi *Psilocybe* sp. and *Ulocladium* sp. from *Mytilus galloprovincialis* mussels collected in the northern Adriatic Sea.

Infection	*Psilocybe* sp. P (%) [I (range)]		*Ulocladium* sp. P (%) [I (range)]	
Site	Feb	Apr	Feb	Apr
ooTM	30 [1 (1–2)]	0	10 [4]	0
0024	0	0	0	0
0035	0	0	0	0
St Catherine	0	10 [1]	10 [5]	10 [1]
ACI Rovinj	0	0	10 [1]	0
ACI Pula	0	20 [1]	0	0

Table 4. Effect of sampling time and site on the prevalence and level of infection of parasites and fungi in mussels from the northern Adriatic Sea.

		Prevalence			Level of infection	
Infection	Variable	χ^2	df	*P*	H	*P*
Prokaryotic inclusion body	Time	0.00	1	1.00	0.24	0.61
	Site	6.66	2	0.03	6.32	0.01
Nematopsis sp.	Time	1.50	1	0.22	0.27	0.86
	Site	6.00	2	0.04	9.18	0.01
Ancistrocoma-like ciliate	Time	0.34	1	0.55	0.53	0.46
	Site	8.57	2	0.01	7.50	0.02
Psilocybe sp.	Time	0.44	1	0.50	0.17	0.67
	Site	2.22	2	0.32	1.88	0.38
Ulocladium sp.	Time	1.5	1	0.22	0.73	0.39
	Site	6.0	2	0.04	6.73	0.03

Significant difference (*P*) of χ^2 test for the prevalence and Kruskal–Wallis test (H) for the intensity of infection.

inside the digestive tubules of mussels from impacted (Marina Koper and ACI Rovinj in February) and non-impacted (St Catherine) sites in both months. The intensity of infection was low (up to five spores per section). No significant difference in the prevalence of fungi ($P > 0.05$) was observed between cultured and wild mussels.

DISCUSSION

A diversity of endobionts and parasites was observed in cryosections of wild mussels from the northern Adriatic Sea, including *Ancistroma* sp., *Nematopsis* sp., *U. cyprinae*, and fungi *Ulocladium* sp., *Psylocibe* sp. and *Alternaria* sp. One of the most devastating pathologies in mussels, infection with *M. refringens*, an OIE-listed disease, was not detected by diagnostic PCR in mussels from the northern Adriatic during this survey. There was a significant difference between sites (impacted, non-impacted and aquaculture sites) concerning parasite infection. Histological examination of paraffin-embedded tissue sections is considered to be the standard screening diagnostic method. We identified parasites and endobionts in cryosections since this method preserves the tissue as close as possible to its natural state (Kovačić & Pustijanac, 2017). Nowadays, for detection of parasites causing OIE-listed diseases, more specific and sensitive molecular diagnostic techniques have been used (Aranguren & Figueras, 2016). For a presumptive diagnosis of a disease, the standard method is histopathology, but for confirmatory diagnosis, the standard methods are PCR and DNA sequencing. As the result of adopting European legislation for monitoring diseases according to OIE on shellfish farms in Slovenia, analyses of *M. refringens* in Mediterranean mussels was established from 2004 (Gombač, 2010). *Marteilia refringens* ciliates were detected in mussels from Slovenian aquaculture sites in recent years, at intensities that would cause significant pathologies (Gombač *et al.*, 2014). At the time of our study, cultured Mediterranean mussels were free from *M. refringens*. This protozoan is widespread from the Atlantic Ocean to the Persian Gulf (Bower *et al.*, 1994) and has also been detected in the northern Adriatic Sea (Zrnčić *et al.*, 2001; Gombač *et al.*, 2014). A haplosporidian-like organism was found in *M. galloprovincialis* from only one site (ACI Pula, Croatia). Multinucleated plasmodia were observed in *Mytilus edulis*, *M. galloprovincialis* and their hybrids from Southampton Water, Hampshire, and the Exe River, Devon in the UK (Bignell *et al.*, 2008). Besides, a haplosporidian infection in *Mytilus* sp. from the Exe River estuary and Southampton Water was described in 2004 and 2005 (Bignell *et al.*, 2008). However, in the northern Adriatic Sea, this is the first record of a haplosporidian-like organism in mussels.

In our study, prokaryotic inclusion bodies were found infecting epithelial cells or the lumen of the digestive tubule in mussels from the Northern Adriatic, similarly to their detection in several bivalve species (Cova *et al.*, 2015; Vázquez & Cremonte, 2017). They occurred as small rounded intracellular basophilic colonies, evoking the lysis of infected cells of the digestive tubule. In some cases, colonies caused the lysis of the infected cells and were released from the

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lumen of the tubule into organs of bivalves (Vázquez & Cremonte, 2017), as we detected in this study. In the host tissue, the parasites can be associated with an entry mechanism via food particle capture (Queiroga *et al.*, 2015). In these cases, granulocytes can cross the digestive epithelium reaching the lumen of the gut where they capture and digest food particles, and cross back transporting nutrients to bivalve tissues (Torre *et al.*, 2013a, b; Matozzo *et al.*, 2016; Sehonova *et al.*, 2018). Moreover, some inclusions detected in our study were coated by an eosinophilic fibrous cover, which is interpreted as an encapsulation response by the host (Gulka & Chang, 1985). Despite their intracellularity being unclear, these inclusions were reported as *Rickettsia*-like (RLOs) and *Chlamydia*-like organisms (Vázquez & Cremonte, 2017).

In our study, *Ancistrocoma*-like ciliates occurred on the surface of the digestive gland and in the lumen of the tubule. *Ancistrocoma*-like ciliates are presumed to be ubiquitous in many species of bivalves (Adlard *et al.*, 2003; Rayyan *et al.*, 2006). Most of them are extracellular and found in the lumen of the digestive gland tubules in the intestine. Some ciliates may be attached to or located near the gills, mantle and labial palps (*Sphenophrya*-like, *Trichodina* sp., *Ancistrum*-like). Although ciliates are mostly harmless and commensals, the intracellular ciliates that are *Sphenophrya*-like and *Rhynchodid*-like can disrupt the epithelia of the digestive tubule of *M. edulis* (Adlard *et al.*, 2003).

Our histological examination revealed *Nematopsis* sp. in the form of a single or numerous dense oocysts. Mladineo (2008) also found a high prevalence of *Nematopsis* sp. in the horse-bearded mussel *Modiolus barbatus* Linnaeus, 1758 in Mali Ston (Adriatic Sea, Croatia). *Urastoma cyprinae* turbellarians were found in low prevalence in our study, as was similarly observed in *M. galloprovincialis* from Baja California, north-west Mexico (Caceres-Martinez *et al.*, 1998) and from the Black Sea coast at Sinop, Turkey (Özer & Güneydağ, 2015). Moreover, low prevalence of *U. cyprinae* was found in the mangrove oyster *Crassostrea rhizophorae* (Guilding, 1828) in the estuary of the Graciosa River in Taperoá, Bahia State, north-east Brazil (Cova *et al.*, 2015). Specimens of *U. cyprinae* are usually found among the gill filaments. However, we found them at the edge of the mussel digestive gland, like in a previous study (Kovačić & Pustijanac, 2017).

The results obtained in our study include the first record of fungal spores of *Ulocladium* sp., *Psylocibe* sp. and *Alternaria* sp. in *M. galloprovincialis* in the northern Adriatic Sea (Croatia). Epibiotic and endobiotic fungi live on the surface and in the inner tissues of many invertebrates and algae (Zhang *et al.*, 2009). Filamentous fungi were found to be associated with several wild and farmed bivalves and are potentially toxic (Zvereva *et al.*, 2012; Borzykh & Zvereva, 2015; Santos *et al.*, 2017). Besides, fungal spores were abundant in salts from the Atlantic and Pacific Oceans (Biango-Daniels & Hodge, 2018).

In this study, the sampling site influenced the diversity and abundance of parasites present in mussels. Mussels from impacted sites (marinas and port) were more infected in comparison to mussels from the non-impacted and aquaculture sites. An association of parasite diversity and their abundance with impacted polluted sites has been observed in many studies (Aarab *et al.*, 2008; Bignell *et al.*, 2008; Morley, 2010; Bignell *et al.*, 2011), because mussels are under physiological stress and thus are susceptible to infection. Several pathogens affect cell morphology and tissue architecture, leading to altered physiological functions especially during massive infections. This is especially pronounced in the mussel digestive gland, where infection with parasites leads to considerable displacement of the digestive epithelium with individual parasites occupying much of the cytoplasm of the digestive cells, thus increasing sensitivity to the impact of pollutants (Bignell *et al.*, 2008). Moreover, pollution is often associated with a decrease in dissolved oxygen, which creates a favourable environment for bacteria and viruses, while inert suspended solids can damage the tissue epithelium and make individuals more susceptible to infection with fungi (Svobodová *et al.*, 1993; Lynch *et al.*, 2014). The dissolved oxygen together with the pollution could be the parameters that most influenced parasite and fungi diversity at sampling sites in Croatia. All the sampling sites (two aquaculture and four wild) along the Slovenian and Croatian coastlines were included in biomonitoring programmes in previous years. The site Marina Koper is near to Port Koper and is characterized by elevated pollutant concentrations in biota and sediment compared with mussel farms (Ramšak *et al.*, 2012; Tsangaris *et al.*, 2016). Likewise, ACI Pula and ACI Rovinj were characterized by elevated pollutant concentration in biota and sediment compared with mussel farms in Croatia (Kanduč *et al.*, 2018). Seasonal changes in metal concentration (Se, Cu, Pb, Cd, As and Zn) in mussels and metallothionein content did not reveal significant differences between sites in Slovenia (Ramšak *et al.*, 2012; Tsangaris *et al.*, 2016). On the other hand, significant differences in metal concentrations (Mn, Co, Ni, Cu, Zn, Se, Cd and Pb) were found in mussels from polluted sites along the Croatian coastline (Kanduč *et al.*, 2018). Values of bioconcentration factors (BCF) for metals were below the maximum values recommended by the International Atomic Energy Agency (IAEA, 2004), and no differences were found when investigating the genotoxicity of seawater by micronuclei frequency on Slovenian sites (Kristan *et al.*, 2014). No signs of organic pollution from sewage were confirmed in the mussels from investigated sites in Slovenia (Kristan *et al.*, 2014) and Croatia (Kanduč *et al.*, 2018), measured as isotopic composition of carbon ($\delta^{13}C$) *vs* nitrogen ($\delta^{15}N$) in mussel tissue. Moreover, biochemical biomarkers (catalase, GST activity and AChE) confirmed stress in mussels from sampled sites in Slovenia (Tsangaris *et al.*, 2016). In contrast to the wild sites, no significant parasites and/or pathogens were found in mussels from Slovenian aquaculture sites in the northern Adriatic, in this study. Natural characteristics of the northern Adriatic Sea in addition to aquaculture activities such as collecting mussel seeds from surrounding wild areas and/or Italian areas could pose risks of spreading parasites in such small, protected coastal regions as the Strunjan Bay and Piran Bay (Gombač *et al.*, 2014). Mussel seeds are collected from native beds or collector ropes and transferred to aquaculture areas for on-growing (FAO, 2015). Occasionally, small quantities of mussel seeds are bought in Italy (Gombač, 2010). Moreover, because water currents generally flow from the south along with the Istrian coast and turn west along the Slovenian coast (Bricelj & Rejec Brancelj, 2009), they can transport parasites preferentially northwards (Kovačić *et al.*, 2016) to the aquaculture areas in the northern Adriatic, particularly during winter and early spring. Moreover, the tides in the Gulf of Trieste are very high for the Mediterranean Sea and the difference between low and high tides can reach more

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than 180 centimetres, representing the largest tidal range in the Adriatic Sea (Bricelj & Rejec Brancelj, 2009). Tides could transfer the parasites with indirect life cycles from hosts living at the bottom to their intermediate hosts, such as bivalves (Boehs *et al.*, 2010). Since the transmission route of parasite infection can vary (Cable *et al.*, 2017), we decided to perform a study in which farmed and wild Mediterranean mussels were collected in late winter and early spring.

During this study, farmed mussels from two protected areas in Slovenia were free of endobionts and parasites. This may indicate that aquaculture areas are somehow protected against infection, at the same time that they are protected from transfer of endobionts and parasites by natural water currents or translocation of mussels, at least for now. Although wild mussels from Croatian and Slovenian coasts were infected with a high diversity of parasites and fungi, they did not present infection with *M. refringens*, an OIE-listed disease pathogen.

Finally, a variety of stressors in these coastal communities coupled with different modes of species translocation (e.g. ballast water, wind dispersion) and climate change may further impact parasite dynamics. These relationships need to be further evaluated, particularly regarding the OIE-listed diseases.

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