Detection of undeterminated *Haplosporidia* DNA in mussels (*Mytilus galloprovincialis*) after mass mortality event in Adriatic Sea

I. G. Zupičić, D. Oraić, I. Arzul, L. Canier, M. Noyer, B. Chollet and S. Zrnčić*

Abstract

We investigated a mass mortality event of Mediterranean mussels (*Mytilus galloprovincialis*) in the Novigrad Sea, Croatia during winter 2022/2023. The mortality rate was up to 70% and was initially linked to an influx of freshwater from the Zrmanja River. Despite mitigation measures that included placing mussels deeper to avoid the influence of freshwater, mortalities continued. Subsequent analysis of 64 samples using cytology, histology and real-time PCR to exclude listed diseases, yielded negative results. PCR testing for *Haplosporidium pinnae* revealed the presence of a haplosporidian-like DNA resembling *Minchinia mytili*. In the study of predominant bacteria, *Psychrobacter* sp., *Colwellia* sp., and *Vibrio splendidus* were detected. Histological examination showed no haplosporidium structures, and *in situ* hybridisation with probes for detection of *Haplosporiidae* did not confirmed the presence of *M. mytili* in mussel tissue. However, our study reports the first detection of *M. mytili* DNA in the Adriatic Sea, emphasising the need for extensive research and further analysis to determine the exact cause of these mass mortality events and the origin of the haplosporidium DNA.

Key words: *Minchinia mytili*; *Mytilus galloprovincialis*; *Croatian Adriatic coast*

Introduction

Mussel production represents an important aquaculture commodity. Globally, mussel production reached 2.2 million tonnes in 2020, primarily through aquaculture, accounting for 97% of the total. China was the leading producer, contributing to 43% of global mussel production in 2020, followed by the EU-27 and Chile, with 20% and 19%, respectively. Spain, known for its Mediterranean mussel (*Mytilus galloprovincialis*) production, dominated the EU market in 2020, accounting for 47% of the EU production, followed by France (14%), Italy (12%),

Ivana Giovanna ZUPIČIĆ*, PhD, Croatian Veterinary Institute, Zagreb, Croatia, (Corresponding author, e-mail: zupicic@veinst.hr); Dražen ORAIĆ, PhD, Scientific Advisor with Tenure, Croatian Veterinary Institute, Zagreb, Croatia; Isabelle ARZUL, PhD, Researcher, Lydie CANIER, PhD, Researcher, Mathilde NOYER, Expert Associate, Bruno CHOLLET, Expert Associate, French Institute for Ocean Science, La Tremblade, France; Snježana ZRNČIĆ, PhD, Scientific Advisor with Tenure, Croatian Veterinary Institute, Zagreb, Croatia

Netherlands (8%), Denmark (7%), Greece (4%), and Ireland (3%) (EUMOFA, 2022). In Croatia, the annual production of *M. galloprovincialis* in 2022 reached 1020 tons (Ministry of Agriculture, 2023).

The phylum *Haplosporidia* comprises 36 recognised species distributed among four genera: *Urosporidium*, *Minchinia*, *Haplosporidium*, and *Bonamia*. Some haplosporidian species have been implicated in causing severe marine disease outbreaks, particularly in shellfish species (Carnegie et al., 2016). In recent years, more than ten newly discovered haplosporidian species have been added to this phylum, including species in the *Bonamia* and *Minchinia* lineages (Arzul and Carnegie, 2015). A significant challenge in detecting novel haplosporidian taxa is the use of broadly targeted molecular probes, which are unsuitable for the highly divergent genes characteristic of these parasites (Hartikainen et al., 2014).

Parasites belonging to the phylum *Haplosporidia* have been reported in various bivalve hosts across Europe. Notable examples include *Haplosporidium edule*, *Minchinia tapetis*, and *Minchinia mercenariae* infecting *Cerastoderma edule* (Longshaw and Malham, 2013; Ramilo et al., 2017), as well as *Haplosporidium sp*. in *Mytilus edulis* (Figueras and Jardon, 1991), a haplosporidian-like parasite described in *M. edulis* (Stephenson and McGladdery, 2002), and *M. mytili* in *M. edulis* (Ward et al., 2019).

Lynch et al. (2014) carried out a health assessment of *Mytilus spp.* along the coasts of Ireland and Wales and identified a previously undescribed haplosporidian (Haplosporidia sp. SAL-2014) belonging to the *Minchinia* clade in a single *M. edulis* from Wales (Lynch et al., 2014). Later, Ward et al. (2019) first described *M. mytili* of the blue mussel *M. edulis* during screening sampling in England and Scotland.

The life cycles of haplosporidian parasites remain largely unknown. The presence of diverse haplosporidian sequence types in water columns and sediment suggests the potential for either free-living transmissible stages or the involvement of planktonic metazoans in haplosporidian life cycles (Hartikainen

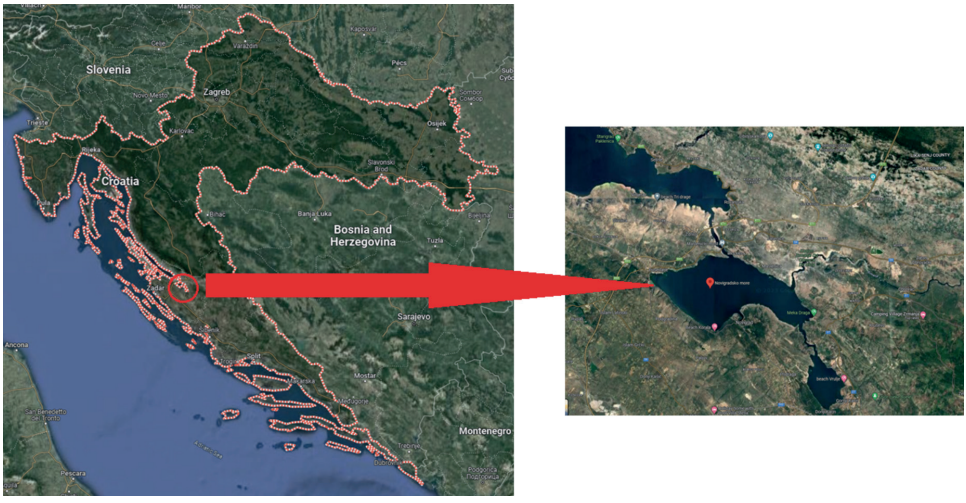

Figure 1. Map of the Croatian Adriatic coast showing the sampling site

et al., 2014). While haplosporidians have been observed in *Mytilus spp.* mussels on multiple occasions, only three have been officially described: *Haplosporidium tumefacientis*, *Haplosporidium mytilovum* (Sprague et al., 1972), and *M. mytili* (Ward et al., 2019).

The objective of this study was to evaluate putative involvement of detected *Haplosporidia* DNA in Mediterranean mussels (*Mytilus galloprovincialis*) at specific sites in Croatia following a mass mortality event.

Material and methods

The first increased mortality event was reported from the Novigrad Sea (Figure 1) in early November 2022, with a sea temperature of 18°C. At that time, farmers submitted the first sample for diagnosis the cause of mortalities. The second sample was submitted in mid-December, coinciding with a sea temperature of around 15°C. Finally, the third sample was submitted in mid-January (Table 1).

All submitted samples were tested for the presence of listed diseases using cytological and molecular methods and efforts were made to determine the predominant bacteria using bacteriology and molecular tools for bacteria identification. Additionally, histology and *in situ* hybridisation were implemented aiming to visualise possible parasites in mussel tissues (Table 1).

Cytological and histological examination

For diagnosis of *M. refringens* by cytology, a piece of the digestive gland of each mussel was sampled and briefly dried on absorbent paper. A series of digestive gland imprints were prepared on a glass slide. Imprints were stained with Hemacolor® staining kit according to manufacturer instructions (Merck, Darmstadt, Germany).

For histological examination, transversal sections of soft tissue of each mussel were placed in histo-cassettes and immersed in Davidson's alcohol, formalin, acetic acid (AFA) fixative. Davidson-fixed tissues were dehydrated through a graded ethanol series, succeeded by xylene, and embedded in paraffin, sectioned at 3 µm, and mounted on Microme EC 350-2 slides (Thermo Scientific, Waltham, MA, USA). Mounted slides were heated to 60°C, deparaffinised, and rehydrated in xylene, a graded series of alcohol, and finally, water, followed by staining with haematoxylin and eosin (H&E). Stained tissue impressions and histological sections were examined on a Zeiss Axiskop 2 binocular microscope (Carl Zeiss, Jena, Germany) at 400 and 1000 magnifications with immersion oil.

Isolation and molecular identification of predominant bacteria

We analysed individual samples of five mussels according to the EURL for Molluscs Diseases (2015). A sample weighing approximately 50 mg was taken from each animal, consisting of gills, mantle and posterior adductor muscle tissues. Each organ mixture was homogenised in 200 µL sterile seawater using a mortar and pestle with sterile sand. Homogenates were centrifuged at 2000 g for 10 min, supernatants were collected and serial dilutions were made with sterile seawater up to 10^{-4}. Then, 50 µL amounts of 10^{-2} and 10^{-4} dilutions were plated on Marine agar (Condalab, Spain) and incubated at 20°C for 48 h. After incubation, each plate was visually checked for individual bacterial colonies, and morphological differences were evaluated. A predominant colony was defined as a colony

Table 1. List of submitted samples and performed analyses

Sampling period	Sampling site	No. of individuals in samples	Analysis	No. of analysed samples	Observation or detection of positive PCR product of parasites
November 2022	Novigrad Sea	35	Cytological and histological examination	35	–
			PCR for detection of *M. refringens*, *B. ostreae*, *B. exitiosa* and *H. pinnae*	35	–
			PCR for detection of *M. mytili*	35	+ 4/35
December 2022	Novigrad Sea	19	Cytological and histological examination	19	–
			PCR for detection of *M. refringens*, *B. ostreae*, *B. exitiosa* and *H. pinnae*	19	–
			PCR for detection of *M. mytili*	19	+ 17/19
			In situ hybridisation	6	–
January 2023	Outside of Novigrad Sea	10	Cytological and histological examination	10	–
			Identification of predominant bacteria	5	+
			PCR for detection of *M. refringens*, *B. ostreae*, *B. exitiosa* and *H. pinnae*	10	–
			PCR for detection of *M. mytili*	10	+ 9/10
			In situ hybridisation	5	–

observed on the two dilutions (10^{-2} and 10^{-4}) and present in at least at 5.10^4 CFU/mL. Predominant bacteria were subcultivated on TCBS (BD, Germany), Chromogenic (Condalab, Spain) and Marine (Condalab, Spain) agar plates at 20°C for 24 h.

DNA was extracted from the predominant colonies identified in the morphological evaluation and abundance. Extraction was performed using the NucleoSpin Microbial DNA kit (Macherey-Nagel, Germany) according to manufacturer instructions.

For molecular identification of predominant bacterial colonies, the 16S rRNA gene was amplified and sequenced. We used universal bacterial 16S primers 16S_27FYM (5'AGA GTT TGA TYMTGG CTC AG3') (Frank et al. 2008) and 16S_1492YR (5'TAC GGY TAC CTT GTT ACG ACT T3') (Newby et al. 2004). PCR was performed on the ProFlex PCR System (Applied Biosystems, USA) using GoTaq G2 Hot Start Colorless Master Mix (Promega, USA). All reactions were done in a final volume of 20 µL, with 0.5 µM primers and between 40 and 120 ng of DNA as measured on a DS-11 Series Spectrophotometer (DeNovix, USA). The temperature profile used was 95°C, 2 min for enzyme activation, followed by 35 cycles at 94°C, 1 min denaturation at 49°C, 30 s annealing and 72°C, 2 min elongation, finishing with the final elongation step at 72°C for 5 min. PCR products obtained by amplification were submitted for sequencing to Macrogen Europe (Amsterdam, Netherlands). The sequences obtained were identified using BLAST (Altschul et al., 1990).

DNA Extraction from tissue

DNA was extracted from approximately 25 mg of the digestive gland, using innuPREP AniPath DNA/RNA Kit—IPC16 (Analytik Jena, Germany) on InnuPure C16 touch (Analytik Jena, Jena, Germany) according to the manufacturer's instructions. The extracted DNA was stored at 20°C until analysis.

Real-time PCR for detection of Marteilia refringens type M / type O

For the detection of *M. refringens*, specific primers described in the EURL for Molluscs Diseases (2023) were used. We used 5 µL of extracted DNA (5 ng/µL) and 10 µM primers TaqMar F (5' GTGTTCGGCACGGGTAGT 3'), TaqMar R (5' TGATCTGATATTATTCAGCTGTTCG 3') and TaqProb M HEX (5' GCGCTTGCCCTACGGCCGTGC 3') and TaqProb O FAM (5' GCCCTTTCCCCGACGGCCG 3'). PCR reactions were performed using the GoTaq G2 Hot Start Colorless Master Mix (Promega, Madison, WI, USA) on a ProFlex PCR System (Applied Biosystems, USA) with a final volume of 20 µL. The temperature protocol involved initial denaturation at 95°C for 3 min, followed by 40 cycles at 95°C for 30 s, and 60°C for 1 min. Ct cut-off values were set-up at 38.

Real-time PCR for detection of Bonamia ostreae and Bonamia exitiosa

For the detection of *B. ostreae*, specific primers described in the EURL for Molluscs Diseases (2023) were used. We used 5 µL of extracted DNA (5 ng/µL) and 10 µM primers BO2_F (5' AAATGGCCTCTTCCCAATCT 3') and BO2_R (5' CCGATCAAACTAGGCTGGAA 3') and BO2_probe (5' TGACGATCGGGAATGAACGC 3'). For the detection of *B. exitiosa*, specific primers described by the EURO for Molluscs Diseases (2023) were used. We used 5 µL of extracted DNA (5 ng/µL) and 10 µM primers BEa_F (5' GACTTTGACCATCGGAAACG 3'), BEa_R (5' ATCGAGTCGTACGCGAGTCT 3') and BEa_probe (5' GGCAGCGAATCGATGGGAAT 3'). PCR reactions were performed using the GoTaq G2 Hot Start Colorless Master Mix (Promega, Madison, WI, USA) on a ProFlex PCR System (Applied Biosystems, USA) with a final volume of 20 µL. The temperature protocol involved initial denaturation at 95°C for 3 min followed by 40 cycles at 95°C for 15 s, and 60°C for 20 s. Ct cut-off values were set-up at 38.

PCR for detection of Haplosporidium pinnae

For the detection of *Haplosporidium pinnae*, specific primers described by Ca-

tanese et al. (2018) were used. We used 2 µL of extracted DNA and 0.5 µM of the primers HPN-F3 (5′ CATTAGCATGGAATAATAAAACACGAC 3′) and HPN-R3 (5′ GCGACGGCTATTTAGATGGCTGA 3′). PCR reactions were performed using the GoTaq G2 Hot Start Colorless Master Mix (Promega, Madison, WI, USA) on a ProFlex PCR System (Applied Biosystems, USA) with a final volume of 20 µL. The temperature protocol involved enzyme activation at 95°C for 2 min, followed by 40 cycles of denaturation at 94°C for 30 s, primer annealing at 48°C for 30 s, and elongation at 72°C for 60 s, and the process was completed with a final elongation step at 72°C for 5 min. The results of the PCR were checked through electrophoresis on a QIAxcel system (Qiagen, Hilden, Germany).

PCR for detection of M. mytili

For the detection of *M. mytili*, specific primers described by Ward et al. (2019) were used. We used 2 µL of extracted DNA and Hap-M258f (5′ AAC TTT TAG CGT CCA GCC CA 3′) and Hap-M412r (5′ CGA GGT TGC CAA GTT CTT TCG 3′) primers. PCR reactions were performed using the GoTaq G2 Hot Start Colorless Master Mix (Promega, Madison, WI, USA) on a ProFlex PCR System (Applied Biosystems, USA) with a final volume of 20 µL. The temperature protocol involved 2 min denaturation at 95°C, followed by 40 cycles of 94°C for 1 min, annealing at 55°C for 1 min and extension at 72 °C for 1 min. Amplicons were extended by final incubation at 72°C for 7 min and stored at 4°C. The results of the PCR were checked through electrophoresis on a QIAxcel system (Qiagen, Hilden, Germany).

Sequencing

All samples testing positive for *H. pinnae* were sequenced using Sanger sequencing provided by Macrogen Europe (Amsterdam, The Netherlands). The obtained sequences were then subjected to identification using BLAST (Altschuletal et al., 1990).

Characterisation by in situ hybridisation

In situ hybridisation was carried out on the deparaffinized histo-slides of animals testing positive for *Minchinia mytili* aiming to confirm the presence of the haplosporidian parasites in the tissue. For hybridisation, the probe using primers BO (5′ CAT TTA ATT GGT CGG GCC GC 3′) and BoAS (5′ CTG ATC GTC TTC GAT CCC CC 3′) according to Cochennec et al. (2000) with addition of Dig labelled dUTP (Roche) in the PCR Mix was used on 11 individual samples using the protocol described by EURL for Molluscs Diseases (2000). This probe is considered to recognise members of the *Haplosporidiidae* family (Helmer et al., 2019).

Results

Macroscopically, watery appearance of soft tissues in affected mussels was observed in all the samples analysed in this study (Figure 2).

Cytological, histological and in situ hybridisation examination

Throughout the study period, all samples consistently tested negative for the presence of *M. refringens* when analysing digestive gland imprints (Table 1).

Histological slides gave no findings of parasites from the *Minchinia* and *Haplosporidium* genera. However, upon closer examination, the histological analysis did uncover several significant findings: there was evidence of varying degrees of necrosis in the digestive gland, gills, and mantle, ranging from low to high levels. In some individuals examined, hypersecretion in the cytoplasm of epithelial cells

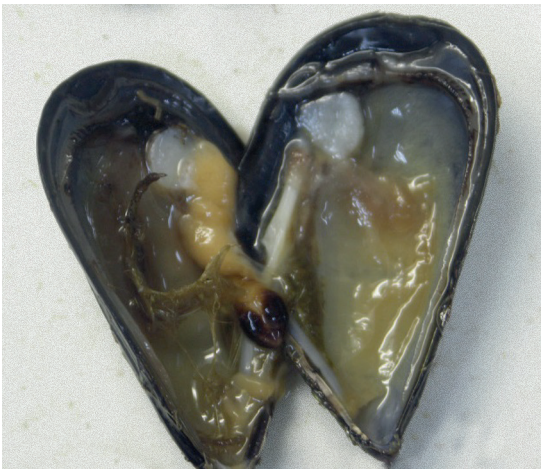

Figure 2. Watery appearance of soft tissue observed during macroscopic examination

of the digestive diverticula was observed. In a single sample, a high quantity of unidentified bacteria was noted in both the mantle and gills.

Further investigation involved *in situ* hybridisation using probes specific for the *Haplosporiidae* family on 11 individuals. However, no labelling or positive signal was observed with this probe in the analysed individuals (Table 1).

Identification of predominant bacteria

The third sample submitted to the laboratory in January was analysed for predominant bacteria (Table 1). The identification of isolated predominant bacterial colonies was accomplished through 16S rRNA sequencing, revealing the presence of *Psychrobacter* sp., *Colwellia* sp., and *Vibrio splendidus*.

Subsequently, upon subcultivation of these established bacteria on various agar media, it was observed that *Psychrobacter sp.* and *Colwellia sp.* exhibited growth on Marine agar, forming distinctive yellow colonies. On the other hand, *V. splendidus* displayed growth on Marine, TCBS, and Chromogenic agar, giving rise to colonies of varying colour, including yellow, green, and purple, depending on the specific agar plate employed (Table 2).

Table 2. Characterisation of the growth patterns of predominant bacteria on different agar plates

Bacterium / Agar	*Psychrobacter* sp.	*Colwellia* sp.	*V. splendidus*
Marine	Yellow colonies	Yellow colonies	Yellow colonies
TCBS	–	–	Green colonies
Chromogenic	–	–	Purple colonies

Results of molecular analyses

Real-time PCR for the detection of *M. refringens* (n=64), *B. ostreae* (n=64) and *B. exitiosa* (n=64) tested negative (Table 1). Following the implementation of conventional PCR for the detection of *H. pinnae* (n=64), electrophoresis unveiled a 591 bp observable band in a total of 30 samples from the three samplings. Notably, the size of the positive control band was 551 bp. Analysis of obtained sequences using BLAST indicated that all sequences showed over 98% sequence similarity with most *M. mytili* sequences available in the database.

Detection of DNA from an unknown Haplosporida

Our analysis showed that 47% (30/64) of the samples tested positive when employing the conventional PCR method (Table 1) using specific primers designed for the identification of *M. mytili*. However, it is important to note that there was no amplification observed for the positive *H. pinnae* control when using PCR with *M. mytili* specific primers. The tested PCR products had a size of 180 bp, which unfortunately proved too small for sequencing.

Discussion

The study presented here aimed to elucidate a cause of increased mortality of mussels occurring during late autumn and winter. The initial peak of mortality occurred in the Novigrad Sea (Figure 1) in early November 2022, with a sea temperature of 18°C. Farmers observed mortalities of up to 30%. Usually, in this region, an influx of freshwater from the Zrmanja River is common during the autumn months. To mitigate the effects of the freshwater influence, farmers reposition mussel ropes deeper. However, despite these efforts, mussel mortality in the deeper layers persisted, reaching up to 70% of affected farms over the following three months. In mid-December, the second set of samples was sent to our laboratory when the sea temperature was around 15°C, since the mortalities had not stopped. By mid-January, the first occurrence of mortalities was reported in the outer part of Novigrad Sea. According to information provided by farmers, mortalities ceased in some areas with higher salinity while in other area they suffered very high losses.

Throughout the study, a total of 64 samples were analysed. Since our first concern was that mortalities were being caused by marteiliosis, we performed real-time PCR analysis (EURL for Molluscs Diseases, 2023) to exclude the listed disease *M. refringens* that affects *M. galloprovincialis*, and the test results were negative. We also performed tests for the detection of *B. ostreae* and *B. exitiosa*, despite no previous reports that these pathogens infect mussels (Figueras Huerta and Robledo, 1994; Carrasco et al., 2012). Considering the presence of *H. pinnae*, associated with the mortality of *Pinna nobilis* in recent years in Croatia (Mihaljević et al., 2021) and the Mediterranean region (Catanese et al., 2018; Carella et al., 2020), we examined the samples for this parasite. The PCR examination indicated the presence of *H. pinnae*-like amplification products, though sequencing revealed that the amplified product more closely resembled *M mytili*. All positive samples were further tested using specific primers for *M. mytili*, and almost all yielded positive results. However, sequencing of the five obtained amplicons did not give the *M. mytili* sequence, but rather an undetermined member of the Haplosporida order.

However, histological analyses did not enable visualisation of parasites and no haplosporidium structures were observed. *In situ* hybridisation using BO-BOAS probes, which is capable of recognising members of the *Haplosporiidae* family (Cochennec et al., 2000), was also performed and yielded negative results.

M. mytili is a parasite first described by Ward et al. (2019) in digestive gland tissue of blue mussels (*M. edulis*) using PCR and histology. Due to the obtained results in this study, it is difficult to connect the increased mortalities with the detected parasite DNA. Still, our study presents the first detection of *M. mytili* similar DNA or undetermined Haplosporida in the Adriatic Sea, and its first occurrence in Mediterranean mussels. Further studies are required to explain the presence and role of an undetermined Haplosporida organism in mussel tissue. Hypothetically, it could be there because of the filtration behaviour of mussels.

Additionally, the assessment of predominant bacteria revealed the presence of *Psychrobacter* sp., *Colwellia* sp., and *V. splendidus*. Marine bivalves can harbour a diverse range of both indigenous and non-indigenous bacteria in their tissues, which can have implications for bivalve health and consequentially for public health. Commercially valuable edible bivalves, including mussels, clams, and oysters, are routinely monitored for microbial content, including the detection of *Escherichia coli* as an indicator of faecal contamination, and members of the genera *Vibrio* or *Aeromonas*, which can be pathogenic to humans and animals (Richiardi et al., 2023). *Psychrobacter* is known for its wide environmental distribution and adaptation to cold temperatures (Welter et al., 2021). Gerpe et al. (2021) reported the presence of *Psychrobacter* in the tissue of mantle, gills, and gonads in grooved carpet-shell (*Ruditapes decussatus*) and manila clams (*Ruditapes philippinarum*) during colder months. The presence of this bacterium may be attributed to its role in the microbiome. Bacteria from the genus *Colwellia* are typically associated with permanently cold marine environments (Xu et al., 2017) while *V. splendidus* is known to induce severe vibriosis, occasionally leading to episodes of mass mortality in bivalves (Frizzo et al., 2021). It is considered a pathogenic agent, as evidenced by its association with oyster mortality during summer events and its demonstrated virulence in laboratory trials with oysters and clams (Le Roux et al., 2009). However, there are no description of mortalities in mussels caused by *V. splendidus*. The presence of these bacteria in the studied mussels could also contribute to the mortalities, not as a primary cause, though it is likely that the bacteria were propagated due to unfavourable environmental conditions for host.

To definitively ascertain the cause of the mass mortality events during the late autumn and winter months, comprehensive future research, expanded sample collection, and analysis are a must.

Conclusion

This study highlights the complexity of marine health concerns and underscores the necessity of molecular and histological analyses, as well as bacteriology for future research, and surveillance to safeguard mussel populations and coastal environments. The detection of the DNA of a haplosporidian parasite resembling *M. mytili* in Mediterranean mussels in the Adriatic Sea raises concerns about its potential impact, highlighting the importance of monitoring parasites

and microbial content, due to the detection of *V. splendidus* in marine bivalves. Despite efforts to mitigate the impact of environmental factors such as freshwater influx and sea temperature changes, high mussel mortality rates persisted, necessitating further investigation to fully understand the causes of these events in the Novigrad Sea.

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Nalaz DNA neidentificiranog haplosporidija u dagnji (*Mytilus galloprovincialis*) iz Jadranskog mora nakon masovnog uginuća

Dr. sc. Ivana Giovanna ZUPIČIĆ, dr. sc. Dražen ORAIĆ, znanstveni savjetnik u trajnom zvanju, dr. sc. Isabelle ARZUL, znanstvenica,; dr. sc. Lydie CANIER, znanstvenica; Mathilde NOYER, stručna suradnica, Bruno CHOLLET, stručni suradnik, French Institute for Ocean Science, La Tremblade, Francuska; dr. sc. Snježana ZRNČIĆ, znanstvena savjetnica u trajnom zvanju, Hrvatski veterinarski institut, Zagreb, Hrvatska

Ovo istraživanje opisuje masovno uginuće dagnji u Novigradskom moru tijekom zimskog razdoblja. Kod izbijanja bolesti zabilježeno je ugibanje do 70 % populacije i isprva je bilo povezano s dotokom slatke vode iz rijeke Zrmanje. Međutim, čak i uz mjere ublažavanja, ugibanje dagnji se nastavilo. Za potrebe istraživanja je pretraženo je ukupno 64 uzoraka pojedinačnih dagnji, korištena je metoda PCR u stvarnom vremenu da bi se isključile poznate bolesti dagnji, a to je dalo negativne rezultate. Daljnjim istraživanjem, otkrivena je prisutnost DNK nametnika *Minchinia mytili* iz porodice *Haplosporidiidae*. Analiziranjem dominantnih bakterija otkriveno je da prevladavaju *Psychrobacter* sp., *Colwellia* sp. i *Vibrio splendidus*. Histološkim pregledom nije utvrđena prisutnost struktura haplosporidija, a *in situ* hibridizacija je isto tako dala negativne rezultate. Ovo istraživanje označava prvi opis DNK *M. mytili* u Jadranskom moru na mediteranskoj dagnji (*Mytilus galloprovincialis*), naglašavajući potrebu za opsežnijim istraživanjem i daljnjom analizom da bi se utvrdio točan uzrok masovnog ugibanja dagnji u Novigradskom moru tijekom zimskog razdoblja.

Ključne riječi: *Minchinia mytili, Mytilus galloprovincialis, hrvatska obala jadrana*