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Mitja Gombač, M. Sc., DVM

PROTOZOAN INFESTATION DYNAMICS AND OCCURRENCE OF NEOPLASIAS IN DIGESTIVE GLAND OF MEDITERRANEAN MUSSELS *(Mytilus galloprovincialis)* IN SLOVENE SEA IN CORRELATION WITH SEA TEMPERATURE, SALINITY AND OXYGENATION

Doctoral Thesis

DINAMIKA PROTOZOARNIH INFESTACIJ PREBAVNIH ŽLEZ IN NOVOTVORB V UŽITNIH KLAPAVICAH (*Mytilus galloprovincialis*) V SLOVENSKEM MORJU V POVEZAVI S TEMPERATURO, SLANOSTJO IN OKSIGENACIJO MORJA

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mag. Mitja Gombač, DVM

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The study was performed on:

Institute of Pathology, Forensic and Administrative Veterinary Medicine,

Veterinary Faculty of Ljubljana

Head of Institute: Prof. Dr. Vojteh Cestnik, DVM

Institute for Breeding and Health Care of Game, Fishes and Bees,

Veterinary Faculty of Ljubljana

Head of Institute: Prof. Dr. Vlasta Jenčič, DVM

Institute of Microbiology and Parasitology, Veterinary Faculty of Ljubljana

Head of Institute: Dr. Brane Krt, DVM

The public presentation was held _____.

Mentor: Prof. Dr. Vlasta Jenčič, DVM

The expert comitee members:

Prof. Dr. Milan Pogačnik., president and member, VF

Prof. Dr. Tamara Lah Turnšek, member, BF

Assist. Prof. Dr. Isabelle Arzul, member, IFREMER, France

Assist. Prof. Dr. Matjaž Ocepek, member, VF

STATEMENT ABOUT THE STUDY

Doctoral thesis is a result of my own research work.

Mitja Gombač

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ABSTRACT

PROTOZOAN INFESTATION DYNAMICS AND OCCURRENCE OF NEOPLASIAS IN DIGESTIVE GLAND OF MEDITERRANEAN MUSSELS *(Mytilus galloprovincialis)* IN SLOVENE SEA IN CORRELATION WITH SEA TEMPERATURE, SALINITY AND OXYGENATION

Key words:
Bivalvia – pathology; *Mytilus* – parasitology; digestive system – pathology; neoplasms; haemocytes – pathology; Eukaryota – classification – pathogenicity; polymerase chain reaction – methods; seasons; Slovenia

1280 adult Mediterranean mussels (*Mytilus galloprovincialis*), 960 from shellfish farms and 320 from natural beds, were monthly collected over a one-year period from three sampling sites (two in shellfish farms and one in natural beds) in the Slovene sea. Water temperature, oxygenation and salinity were measured at each sampling and shellfish farms were checked monthly for eventual mortality. All 1280 mussels were macroscopically inspected, measured and weighted to calculate the condition index. One slide per mussel containing digestive gland was stained with haematoxylin and eosin and microscopically examined for the presence of protozoa and neoplasia.
No mortality occurred during our sampling.
Marteilia spp. was detected in 4 mussels (0.3% prevalence), intracellular ciliates of mussels in 293 mussels (22.9% prevalence) and haemocytic neoplasia of mussels in 14 mussels (1.1% prevalence). Performing PCR-RFLP, *Marteilia refringens* type M was determined in all four infected mussels.
M. refringens was detected only in cultured mussels in both shellfish farms with the same prevalence. Neither cross-infection with intracellular ciliates of mussels nor haemocytic neoplasia of mussels were seen in the infected mussels. All stages of *M. refringens* life cycle severely infected digestive gland ducts and tubules and free spores were noticed in the digestive tubules lumina in all infected mussels. Sporadic disruption

of epithelial cells of digestive tubules and focal destruction of digestive tubules were observed in all infected mussels, whereas diffuse haemocytic infiltration and focal granulocytomas were noticed in two mussels. *M. refringens* was more frequently detected in winter, when the sea temperature was equal to or below 10 ^{o}C, the oxygenation above 10 mg/l and the salinity equal to or above 37‰. The average condition index in *M. refringens* infected mussels was lower than in uninfected ones. Reliable statistical evaluation of differences between infected and non-infected mussels was not possible due to the low number of *M. refringens* positive mussels.

The prevalence of infection with intracellular ciliates of mussels was higher in cultured mussels, but the differences between cultured and wild mussels were not statistically relevant. Intracellular ciliates were found inside the digestive tubule epithelia or were free in lumens of digestive tubules, the infection was predominantly mild. A slight enlargement of epithelial cells that carried ciliates of large size was the only alteration of digestive glands' tubules. A mild diffuse haemocytic infiltration in digestive gland connective tissue and haemocytic neoplasia of mussels was diagnosed in some infected mussels. The highest prevalence of infection was detected in spring when the average sea temperature was 15.2 ^{o}C, the average oxygenation 9.3mg/l and the average salinity 29.6‰ and the lowest prevalence was found in summer (average sea temperature 24.1 ^{o}C, average oxygenation 7.6mg/l and average salinity 38.1‰). We determined that only salinity had an impact on infection with intracellular ciliates - the higher the salinity, the lower the infection. The average condition index of infected mussels was slightly higher than of the healthy ones, but the differences between infected and healthy mussels were not statistically significant.

The prevalence of haemocytic neoplasia was higher in cultured mussels. Neoplastic cells infiltrate connective tissue singularly, in small foci or diffusely. Necrosis and multifocal atrophy of digestive tubules were noticed in mussels with diffuse neoplasia whereas severe haemocytic infiltration of connective tissue was seen in mussels with single neoplastic cells. The haemocytic neoplasia was more frequently observed in spring and in autumn, when the sea temperature was between 11 ^{o}C and 20.3 ^{o}C, the oxygenation below 7.1 mg/l and the salinity between 26‰ and 39‰. The average condition index of mussel with haemocytic neoplasia was slightly higher than in healthy

ones. The number of mussels with haemocytic neoplasia was too low to enable reliable statistical testing.

IZVLEČEK

DINAMIKA PROTOZOARNIH INFESTACIJ PREBAVNIH ŽLEZ IN NOVOTVORB V UŽITNIH KLAPAVICAH (*Mytilus galloprovincialis*) V SLOVENSKEM MORJU V POVEZAVI S TEMPERATURO, SLANOSTJO IN OKSIGENACIJO MORJA

Ključne besede:
Školjke - patologija, *Mytilus* – parazitologija; prebavni system – patologija; neoplazme; hemociti – patologija; Eukaryota – klasifikacija – patogenost; verižna reakcija s polmerazo – metoda; letni časi; Slovenija

V našo raziskavo smo vključili 1280 odraslih mediteranskih klapavic (*Mytilus galloprovincialis*), 960 iz školjčišč in 320 iz naravnih rastišč. Mediteranske klapavice smo enkrat mesečno v enoletnem obdobju odvzeli iz treh rastišč v slovenskem morju: dveh školjčišč (Seča in Strunjan) in iz enega naravnega rastišča. Ob vsakem odvzemu smo v vseh rastiščih izmerili temperaturo, oksigenacijo in slanost morja ter v školjčiščih preverili odstotek smrtnosti školjk. Vseh 1280 klapavic smo makroskopsko pregledali, jim izmerili dolžino in jih stehtali ter na podlagi rezultatov tehtanja izračunali njihov kondicijski indeks. Vsaki školjki smo odvzeli del prebavne žleze, iz nje izdelali tkivno rezino, jo obarvali s hematoksilinom in z eozinom ter s svetlobnim mikroskopom ugotavljali praživali in novotvorbe.

Med vzorčenjem v školjčiščih nismo ugotovili smrtnosti školjk.

Praživali iz rodu *Marteilia* smo ugotovili v štirih klapavicah (0,3-odstotna prevalenca), znotrajcelične migetalkarje klapavic v 293 klapavicah (22,9-odstotna prevalenca) in novotvorbo hemocitov v 14 klapavicah (1,1-odstotna prevalenca). Z molekularno metodo PCR-RFLP smo marteilije v vseh štirih klapavicah vrstno določili za *Marteilio refringens* M-tip.

M. refringens smo ugotovili le pri gojenih klapavicah; prevalenca je bila v obeh školjčiščih enaka. V klapavicah, invadiranih z *M. refringens*, nismo ugotovili niti znotrajceličnih migetalkarjev niti novotvorbe hemocitov. V vseh invadiranih klapavicah

smo ugotovili vse razvojne stadije marteilij, ki so v velikem številu naseljevali vode in kanalčke prebavnih žlez, v svetlinah prebavnih tubulov vseh invadiranih klapavic pa so bile tudi proste spore. Propad posameznih epitelnih celic prebavnih kanalčkov in nekrozo posameznih prebavnih kanalčkov smo opazili v vseh invadiranih klapavicah, difuzno infiltracijo hemocitov in posamezne granulocitome pa v dveh klapavicah. *M. refringens* smo pogosteje ugotovili pozimi, ko je bila temperatura morja enaka ali nižja od 10 ^{o} C, oksigenacija okoli 10 mg/l, slanost pa enaka ali višja od 37 ‰. Povprečni kondicijski indeks invadiranih klapavic je bil nižji kot pri zdravih. Zaradi premajhnega števila klapavic, invadiranih z *M. refringens*, statistično sklepanje ni bilo mogoče.

Prevalenca invazije z znotrajceličnimi migetalkarji klapavic je bila višja v gojenih klapavicah, vendar razlika med gojenimi in divjimi statistično ni bila značilna. Znotrajcelični migetalkarji so naseljevali epitelij prebavnih kanalčkov, v manjšem številu pa smo jih opazili tudi v njihovih lumnih. Prevladovala je blaga stopnja invazije. Blaga razširitev epitelnih celic, invadiranih z velikimi migetalkarji, je bila edina ugotovljena patološka sprememba v kanalčkih prebavnih žlez. V vezivu prebavnih žlez majhnega števila invadiranih klapavic smo ugotovili blag difuzen infiltrat hemocitov in novotvorbo hemocitov klapavic. Najvišja prevalenca invazije z znotrajceličnimi migetalkarji je bila ugotovljena spomladi, ko je bila povprečna temperatura morja 15,2 ^{o} C, povprečna oksigenacija 9,3 mg/l in povprečna slanost 29,6 ‰, najnižja pa poleti, ko je bila povprečna temperatura morja 24,1 ^{o} C, povprečna oksigenacija 7,6 mg/l in povprečna slanost 38,1 ‰. S statistično analizo smo dokazali, da višja slanost morja zavira invazijo z znotrajceličnimi migetalkarji klapavic. Povprečni kondicijski indeks z migetalkarji invadiranih klapavic je bil rahlo višji kot pri zdravih, vendar razlika med skupinama ni bila statistično značilna.

Prevalenca novotvorbe hemocitov klapavic je bila višja pri gojenih klapavicah. Neoplastične celice so infiltrirale vezivo prebavnih žlez posamično, v majhnih skupinah ali difuzno. Nekrozo in žariščno atrofijo prebavnih kanalčkov smo ugotovili pri klapavicah z difuzno obliko novotvorbe, močno difuzno infiltracijo hemocitov pa smo opisali pri klapavicah s posameznimi neoplastičnimi celicami. Novotvorbo hemocitov smo pogosteje ugotovili spomladi in jeseni, ko je bila temperatura morja med 11 in 20,3 ^{o} C, oksigenacija pod 7,1 mg/l, slanost pa med 26 ‰ in 39 ‰. Zaradi premajhnega števila klapavic z novotvorbo hemocitov klapavic statistično sklepanje ni bilo mogoče.

1 GENERAL INTRODUCTION

Two species of protozoa invade the digestive gland of mussels in Europe, i. e. *Marteilia* spp., which causes the marteiliosis or marteiliasis of mussels, and intracellular ciliates of mussels or digestive gland ciliates - Rhynchodid-like ciliates (Ceschia, 2008; Bower and McGladdery, 2009). Haemocytic neoplasia of mussels is the only neoplasia discovered in mussels to date (Bower, 2006).

Although much is known about the subject, there are still many questions regarding the epidemiology of protozoan infections and the epidemiology and aetiology of haemocytic neoplasia in mussels.

Marteiliosis of mussels

Marteiliosis has been reported in numerous species of oysters (*Ostreidae*), i.e. *Ostrea edulis, O. angasi, O. puelchana, O. denselamellosa, O. chilensis, Crassostrea gigas, C. virginica, Saccostrea cucullata,* cockles (*Cardiidae*), i.e. *Cardium edule,* scallops, clams (*Pectinidae, Solenidae* (Ceschia et al., 2001)), i.e. *Ruditapes decussates, R. philippinarum, Tapes rhomboides, T. Pullastra, Enis minor, E. siliqua, Solen marginatus, Scrobicularia piperata* and mussels (*Mytilidae*), i.e. *Mytilus galloprovincialis* and *M. edulis,* caused by protistan parasites of the genus *Marteilia* (Auffret and Poder, 1987; Berthe et al., 2004). Marteiliosis of the Mediterranean mussel (*Mytilus galloprovincialis*) and blue mussel (*Mytilus edulis*) is caused by two different species of genus *Marteilia*: *Marteilia refringens* and *Marteilia maurini* (Bower and McGladdery, 2007) or two types of a single species *Marteilia refringens* type M and type O (Berthe et al., 2004). So far, marteiliosis of mussels has been observed in the Atlantic from the North West of France to Portugal (Comps et al., 1980; Comps et al., 1982; Auffret and Poder, 1983; Villalba et al., 1993a; Robledo and Figueras, 1995; Villalba et al., 1997; Longshaw et al., 2001; Fuentes et al., 2002; Novoa et al., 2005; Bower and McGladdery, 2007), in the Mediterranean, including the northern Adriatic (Ceschia et al., 1992; Zrnčić et al., 2001) and Albania (Pëllumb et al., 2006), in the Persian Gulf (Bower and McGladdery, 2007) and in the Gulf of Thermaikos in northern Greece (Virvilis et al., 2003; Rayyan et al., 2006). The prevalence of the infection ranges from 1.67% (Carrasco et al., 2008) to 70% (Auffret and Poder, 1983).

Many authors report that the infection occurs mostly in spring and summer (Ceschia et al., 1992; Photis et al., 1992; Carrasco et al., 2008), when the sea temperature is up to 17 ^{o}C (Audemard et al., 2002; Carrasco et al., 2008). In spite of this, the infestation in experimentally introduced healthy Mediterranean mussels persisted throughout the year (Villalba et al., 1993a; Robledo and Figueras, 1995; Villalba et al., 1997). The infection was most frequently observed in the shallow sea near the coast (Robledo and Figueras, 1995) and was higher in polluted sea areas (Rayyan et al., 2006). High salinity inhibits the infection (Ceschia et al., 1992).

The transmission route of the infection is still unknown. Direct transmission of *M. refringens* between flat oysters by co-habitation, injection and feeding of spore suspension (Comps and Joly, 1980; Perkins, 1988; Berthe et al., 1998), sediment (Berthe et al., 1998) by feeding of fish with large number of spores (Grizel, 1985) and feeding of *M. refringens* spores to small crustaceans *Carcinus maenas*, *Carcinus crangon* and *Marinogammarus marinus* (Van Banning, 1979) has been shown to be unsuccessful. Using PCR and in situ hybridisation, *Marteilia* spp. was discovered in copepod *Paracartia* (*Acartia*) *grani*, which was nominated as a potential host for *M. refringens* (Audemard et al., 2001; Audemard et al., 2002) and later also in *Acartia latisetosa*, the cyclopoida *Oithona* sp., an indeterminate harpaticoida species (Carrasco et al., 2008), *Acartia discaudata*, *A. clausi*, *A. italica*, harpaticoida *Euterpina acutifrons* and larval stages of decapods crustaceans, probably *Portumnus* sp. (Carrasco et al., 2007b).

M. refringens and *M. maurini* are potentially lethal pathogens (Villalba et al., 1993b). In mussels, they cause the haemocytic infiltration of epithelial cells and connective tissue of the digestive gland (Villalba et al., 1997). Heavy infection results in the massive destruction of digestive gland - which is most extensive during the release of spores from sporangia (Figueras et al., 1991; Villalba et al., 1997; Rayyan et al., 2006) - the inhibition of gonadal development and tissue storage and subsequently the loss of body condition (Figueras et al., 1991). Mortality increases with the intensity of the infection (Villalba et al., 1993a). 8.3% to 29.6% mortality was noted in infected Mediterranean mussels (Robledo and Figueras 1995).

Diagnostic methods used for the detection of marteiliosis of mussels involve the histological and cytological examination of the digestive gland, electronic microscopy and PCR (Bower and McGladdery, 2007).

Intracellular ciliates of mussels

Ciliates are protozoa belonging to the phylum *Ciliophora*. They are ubiquitous and have been reported in many species of mollusc (Chollet et al., 2003). Many are extracellular, although some intracellular ciliates have also been reported (*Sphenophrya*-like and *Rhynchodid*-like) (Chollet et al., 2003). They are mostly harmless commensals, but intracellular ciliates can cause xenoma (*Sphenophrya*-like) (Chollet et al., 2003) of the gills (McGladdery and Bower, 2002) and disruption of the digestive tubule epithelia (*Rhynchodid*-like) (McGladdery and Bower, 2002).

Mediterranean mussels are often invaded by *Rhychodid*-like ciliates (Bower et al., 1994), which inhabit the epithelium of the digestive gland (Ceschia, 2008) and are named intracellular ciliates of mussels or digestive gland ciliates (McGladdery and Bower, 2002). Although they may disrupt digestive tubule epithelia, no mortalities have been linked with the infection. The reported prevalence was 2.67% in Mediterranean mussels in the French sea (Carrasco et al., 2008), 21.4% in cultured Mediterranean mussels in the Slovene sea (Gombač et al., 2008) and up to 90% in cultured Mediterranean mussels in Spain (Villalba et al., 1997).

Reported epidemiological properties of intracellular ciliates of mussels in Mediterranean mussels are scarce. No data on the time of infection, the manner of transmission and eventual influence of sea temperature, salinity and oxygenation on the infection were available.

Intracellular ciliates of mussels are diagnosed by a histological examination of the digestive gland (McGladdery in Bower, 2002).

Haemocytic neoplasia of mussels

Disseminated neoplasia, also known as leukaemia, haematopoietic or haemic or haemocytic neoplasia (Bower, 2006), leukocytic neoplasia or sacomatous neoplasia (Elston, 1990) has so far been diagnosed in 15 species of bivalves including oysters

(*Ostreidae*), cockles (*Cardiidae*), clams (*Tellinidae*) and mussels (*Mytilidae*) (Ciokan and Sunila, 2005). In mussels, it has been term the haemocytic neoplasia of mussels (Bower, 2006). Haemocytic neoplasia of mussels is a ubiquitous disease (Elston, 1990; Bower, 2006), which has been diagnosed in blue (*Mytilus edulis*), Mediterranean (*Mytilus galloprovincialis*) and pacific (*Mytilus trossulus*) mussels (Bower, 2006), with unknown aetiology and poorly known epidemiology.

The disease is characterised by the proliferation of large, anaplastic, hypertrophied cells with large, hyperchromatic and often pleomorphic nucleus (Barber, 2004) and high mitotic activity in the connective tissue, blood vessels and sinuses of the visceral mass, muscle and mantle tissue (Elston et al., 1992).

In the early stages of the disease, only single abnormal cells or small foci of neoplastic cells are observed in the circulatory system (Barber, 2004). Later on, neoplastic cells progressively replace normal haemocytes (Elston et al., 1992). Subsequently the fibrosis, displacement, compression of gills, gonad and connective tissue and general degeneration and necrosis of tissues occur (Barber, 2004). The haemocytes lose their defence capabilities and the capabilities of digestion, absorption and food transportation, which leads to starvation (Barber, 2004). The condition index in diseased clams was significantly lower than in healthy ones (Leavitt et al., 1990). The disease is normally lethal, but remission can also occur (Elston et al., 1988a; Elston et al., 1988b).

In Mediterranean mussels only sporadic cases have been recorded to date: one case in California (Hillman, 1990), two cases in Italy in March (Zizzo et al., 1991) and four in July (Tiscar et al., 1990), one case in Spain in 1991 (Figueras et al., 1991) and five cases six years later (Villalba et al., 1997) and one case in Romanian Black Sea (Ciocan and Sunila, 2005).

The occurrence of haemocytic neoplasia of blue mussels is higher in late autumn and in winter, from October to March (Elston, 1990) or from January to March (Barber, 2004). Older mussels are more frequently affected (Elston, 1990). The possible influence of sea temperature, salinity and oxygenation is unknown.

The aetiology of the disease is unknown. The transfer by inoculation of neoplastic cells and healthy haemocytes of diseased mussels to healthy mussels was successful (Elston et al., 1988b). Some authors assume that the causative agent is a virus (Rasmussen, 1986; Elston et al., 1988b; Kent et al., 1991), but other possible factors are also marine

pollution (Hillman, 1993; Usheva and Frolova, 2000) and biotoxins (Landsberg, 1996). The viral hypothesis is supported by the finding of virus-like particles in neoplastic cells nuclei in blue mussels using an electron microscope (Rasmussen, 1986), although Elston et al. (1992) interpreted these structures to be nuclear pores. The fact that the disease transmission between different species of shellfish is unsuccessful suggests the possibility of species specificity of potential infective agents (Kent et al., 1991).
Haemocytic neoplasia can generally not be recognized by gross examination of the sick mussel, because tissue emaciation, pale digestive gland and mantle recession can be encountered in many other diseases (Barber, 2004). The usual diagnostic techniques are histological examination and hemocytology (Elston et al., 1992).

The health status of cultured and wild Mediterranean mussels in the Slovene sea has so far been almost unknown. As the result of joing the European Union (EU) and adopting European legislation, a Slovene National Reference Laboratory for shellfish diseases was established in 2004 and was required to implement system of monitoring shellfish farms, including monitoring of marteiliosis in Mediterranean mussels, the only cultured shelfish species in the Slovene Sea. Two sampling sites in the Slovene sea, i.e. Seča and Strunjan were designated for this purpose. 150 Mediterranean mussels were collected annually in late spring and summer from each, until the renewed Annex IV 2006/88/EC Directive was adopted. To date, all officially collected samples resulted negative for the presence of *Marteilia* spp., but intracellular ciliates of mussels were observed and described in 2004 (Gombač et al., 2008).
Although the Slovene sea, due to its position, shallowness and many other factors, highly influencing the temperature, oxygenation and salinity fluctuations, represents a very specific habitat (Richter, 2005; Lipej, 2004), it was quite surprising that cultured Mediterranean mussels were free of *Marteilia* spp., knowing that theie protozoa are widespread from the Atlantic to the Persian Gulf (Bower and McGladdery, 2009) and have also been detected in the northern Adriatic (Ceschia et al., 1992; Zrnčić et al., 2001). Because of this fact we performed a study in which monthly collected cultured and wild Mediterranean mussels were histopathologicaly inspected for the presence of *Marteilia* spp. and intracellular ciliates of mussels and the species of any *Marteilia* spp. detected was determined using PCR and PCR-RFLP sequencing. Sea temperature,

oxygenation and salinity were measured at each sampling to determine the influence of these parameters on the infection. Finally the condition index of sampled Mediterranean mussels was calculated in order to determine the influence of infection on mussel's condition. Later on, also haemocytic neoplasia of mussels was diagnosed in some samples and included in the study. The influence of the sea temperature, oxygenation and salinity on the occurrence of haemocytic neoplasia of mussels was evaluated and the influence of haemocytic neoplasia on the mussel's condition was also determined.

This investigation of protozoan infection in digestive gland and neoplasia in cultured and wild Mediterranean mussels and of its dynamic relationship to sea temperature, oxygenation and salinity is the first study of this kind in the Slovene sea, which represents a specific ecosystem.

UVOD

Dve vrsti praživali naseljujeta prebavne žleze evropskih klapavic: *Marteilia* spp., ki povzroča marteiliozo klapavic, in znotrajcelični migetalkarji klapavic – migetalkarji, podobni Rynhchodidom (Ceschia, 2008; Bower in McGladdery, 2009). Novotvorba hemocitov klapavic je edina do danes opisana novotvorba klapavic (Bower, 2006).
Čeprav so navedene bolezni splošno znane, je prav v epidemiologiji invazij praživali in epidemiologiji in etiologiji novotvorbe hemocitov klapavic še veliko neznank.

Marteilioza klapavic

Marteilioza je bolezen številnih vrst ostrig (*Ostreidae*), srčank (*Cardiidae*), pokrovač (*Pectinidae*) in klapavic (*Mytilidae*), ki jo povzročajo praživali iz rodu *Marteilia* (Berthe in sod., 2004). Marteiliozo klapavic pri mediteranskih klapavicah (*Mytilus galloprovincialis*) in navadnih klapavicah (*Mytilus edulis*) povzročata dve vrsti iz rodu *Marteilia*, in sicer *Marteilia refringens* in *Marteilia maurini* (Bower in McGladdery, 2007), ali dva tipa iste vrste *Marteilia refringens*: M-tip in O-tip, vendar taksonomska sorodnost in vrstna determinacija še vedno povzročata dvom (Berthe in sod., 2004). Marteiliozo klapavic so do danes diagnosticirali pri klapavicah v Atlantiku od severa Francije do Portugalske (Comps in sod., 1980; Comps in sod., 1982; Auffret in Poder, 1983; Villalba in sod., 1993a; Robledo in Figueras, 1995; Villalba in sod., 1997; Longshaw in sod., 2001; Fuentes in sod., 2002; Novoa in sod., 2005; Bower in McGladdery, 2007), Sredozemskem morju – tudi v severnem Jadranu (Ceschia in sod., 1992; Zrnčić in sod., 2001) in albanskem morju (Pëllumb in sod., 2006), Perzijskem zalivu (Bower in McGladdery, 2007) in zalivu Thermaikos v severni Grčiji (Virvilis in sod., 2003; Rayyan in sod., 2006). Prevalenca invazije niha med 1,67 % (Carrasco in sod., 2008) in 70 % (Auffret in Poder, 1983).
Avtorji opisujejo, da sta invazija klapavic in prevalenca najpogostejši spomladi in poleti (Ceschia in sod., 1992; Photis in sod., 1992; Carrasco in sod., 2008), ko je temperatura morja višja od 17 ^{o}C (Audemard in sod., 2002; Carrasco in sod., 2008). Kljub temu so marteiliozo pri eksperimentalno vnesenih zdravih mediteranskih klapavicah ugotavljali vse leto (Villalba in sod., 1993a; Robledo in Figueras, 1995; Villalba in sod., 1997). Marteilioza je pogostejša v plitvem morju, blizu obale (Robledo in Figueras, 1995) in v

onesnaženih delih morja (Rayyan in sod., 2006). Visoka slanost zavira invazijo (Ceschia in sod., 1992).

Način prenosa marteilioze je še vedno neznan. Direkten prenos *M. refringens* med navadnimi ostrigami pri sobivanju, injiciranju in hranjenju s suspenzijo spor (Comps in Joly, 1980; Perkins, 1988; Berthe in sod., 1998), s sedimentom (Berthe in sod., 1998) in s hranjenjem rib (Grizel, 1985) in majhnih morskih rakcev vrst *Carcinus maenas*, *Carcinus crangon* in *Marinogammarus marinus* z veliko količino spor *M. refringens* (Van Banning, 1979) je bil neuspešen. Z molekularno metodo PCR so marteilije najprej ugotovili v kopepodu *Paracartia* (*Acartia*) *grani* in ga predlagali kot potencialnega vmesnega gostitelja *M. refringens* (Audemard in sod., 2001; Audemard in sod., 2002), pozneje pa tudi v kopepodu *Acartia latisetosa*, ciklopoidu *Oithona* sp., in nedeterminiranih vrstah harpatikoid (Carrasco et al., 2008) in *Acartia discaudata*, *A. clausi*, *A. italica*, harpatikoidu *Euterpina acutifrons* in ličinkah dekapodov, najverjetneje *Portumnus* sp. (Carrasco et al., 2007b).

M. refringens in *M. maurini* sta potencialno letalno patogeni (Villalba in sod., 1993b). Pri klapavicah izzoveta hemocitno infiltracijo epitela in intersticija prebavne žleze (Villalba in sod., 1993a), močna invazija pa povzroči obširno destrukcijo prebavne žleze, ki je najizrazitejša ob sproščanju spor iz sporangijev (Figueras in sod., 1991; Villalba in sod., 1997; Rayyan in sod., 2006), zavira razvoj gonad in preprečuje ustvarjanje tkivnih zalog, zaradi česar školjke hirajo (Figueras in sod., 1991). Smrtnost narašča Ali lahko napišeš se povečuje, da se ne ponovi narašča z naraščanjem? z naraščanjem stopnje invazije (Villalba in sod., 1993a). Robledo in Figueras (1995) sta pri mediteranskih klapavicah opisala 8,3 do 29,6-odstotno smrtnost.

Diagnostične metode, s katerimi ugotavljajo marteiliozo klapavic, so histološki in citološki pregled prebavne žleze, elektronska mikroskopija in molekularna metoda PCR (Bower in McGladdery, 2007).

Znotrajcelični migetalkarji klapavic

Migetalkarji so najverjetneje ubikvitarne praživali, ki pripadajo deblu *Ciliophora*. Invadirajo vse vrste mehkužcev (Chollet in sod., 2003). Najpogosteje živijo zunajcelično, migetalkarji, podobni Sphenophryjam (ang. Sphenophrya-like ciliates), in migetalkarji, podobni Rhynchodidom (ang. Rhynchodid-like ciliates), pa znotrajcelično

(Chollet in sod., 2003). Običajno so neškodljivi komenzali, migetalkarji, podobni Sphenophryjam, pa lahko povzročajo ksenome (Chollet in sod., 2003) v škrgah užitnih klapavic (McGladdery in Bower, 2002), migetalkarji, podobni Rhynchodidom, pa propad epitelija prebavne žleze (McGladdery in Bower, 2002).

Mediteranske klapavice so pogosto invadirane z migetalkarji, podobnimi Rhynchodidom (Bower in sod., 1994), ki naseljujejo epitel prebavne žleze (Ceschia, 2008), zato so jih poimenovali znotrajcelični migetalkarji klapavic (McGladdery in Bower, 2002). Čeprav lahko povzročijo propad prebavne žleze, pa njihova prisotnost ne povzroča smrtnosti. Prevalenca znotrajceličnih migetalkarjev klapavic je 2,67-odstotna pri mediteranskih klapavicah v francoskem morju (Carrasco in sod., 2008), 21,4-odstotna pri mediteranskih klapavicah v slovenskem morju (Gombač in sod., 2008), najvišjo prevalenco – 90-odstotno, pa so zaznali pri mediteranskih klapavicah v Španiji (Villalba in sod., 1997).

O epidemioloških značilnostih znotrajceličnih migetalkarjev je znanega zelo malo; čas invazije, način prenosa in vpliv temperature, slanosti in oksigenacije morja na invazijo so še vedno neznani.

Znotrajcelične migetalkarje klapavic ugotavljamo s histološkim pregledom prebavne žleze (McGladdery in Bower, 2002).

Novotvorba hemocitov klapavic

Diseminirano neoplazijo, imenovano tudi levkemija, hematopoetična oz. hemična oz. novotvorba hemocitov (Bower, 2006), levkocitna neoplazija ali sarkomatozna neoplazija (Elston, 1990), so opisali pri 15 vrstah školjk iz družin ostrig (*Ostreidae*), zijavk (*Myacea*), srčank (*Cardiidae*), telin (*Tellinidae*) in klapavic (*Mytilidae*) (Ciokan in Sunila, 2005). Pri slednjih so jo dokončno poimenovali novotvorba hemocitov klapavic (Bower, 2006). Bolezen je ubikvitarna, z neznano etiologijo in s slabo poznano epidemiologijo. Za novotvorbo hemocitov klapavic obolevajo užitne klapavice (*Mytilus edulis*), mediteranske klapavice (*Mytilus galloprovincialis*) in zalivske klapavice (*Mytilus trossulus*) (Bower, 2006).

Značilnost novotvorbe hemocitov klapavic je brstenje anaplastičnih, hipertrofičnih celic z velikimi, hiperkromatičnimi, pogosto pleomorfnimi jedri (Barber, 2004) in visoko mitotično aktivnostjo v vezivu, krvnih žilah in sinusih prebavil, mišic in plašča (Elston

in sod., 1992). V zgodnji fazi bolezni opazimo zgolj posamezne abnormalne celice ali majhna žarišča neoplastičnih celic v cirkulatornem sistemu (Barber, 2004), kasneje pa neoplastične celice postopoma zamenjajo normalne hemocite (Elston in sod., 1992). Spremljajoči procesi so fibroza, sprememba položaja in stisnjenost škrg, gonad in veziva ter generalizirana degeneracija in nekroza tkiv in organov (Barber, 2004). Neoplastični hemociti izgubijo obrambno sposobnost, sposobnost prebave, absorpcije in transporta hrane, kar povzroča stradanje školjke (Barber, 2004). Kondicijski indeks obolelih zijavk je signifikantno nižji od zdravih (Leavitt in sod., 1990). Bolezen je običajno smrtna, opisani pa so posamezni primeri ozdravitev (Elston in sod., 1988a; Elston in sod., 1988b).

Pri mediteranskih klapavicah so do sedaj opazili le sporadične primere bolezni: en primer v Kaliforniji (Hillman, 1990), šest v Italiji, in sicer dva marca (Zizzo in sod., 1991) in štiri julija (Tiscar in sod, 1990), šest primerov v Španiji, in sicer enega leta 1991 (Figueras in sod., 1991) in pet primerov šest let kasneje (Fuentes in sod., 2002) ter en primer v Črnem morju v Romuniji (Ciocan in Sunila, 2005).

Novotvorba hemocitov se pri navadnih klapavicah pogosteje pojavlja v pozni jeseni in pozimi, od oktobra do marca (Elston, 1990) ali od januarja do marca (Barber, 2004). Starejše školjke zbolevajo pogosteje (Elston, 1990). Vpliv temperature, slanosti in oksigenacije morja na novotvorbo hemocitov klapavic ni znan.

Etiologija bolezni ni znana. Prenos z inokulacijo neoplastičnih celic in zdravih hemocitov obolelih školjk na zdrave je bil uspešen (Elston in sod., 1988b). Nekateri avtorji menijo, da je povzročitelj virus (Rasmussen, 1986; Elston in sod., 1988b; Kent in sod., 1991), možni povzročitelji novotvorbe hemocitov pa so še onesnaženost morja (Hillman, 1993; Usheva in Frolova, 2000) in biotoksini (Landsberg, 1996). Virusno hipoteza podpira odkritje virusom podobnih delcev v jedrih neoplastičnih celic v navadnih klapavicah z elektronskim mikroskopom (Rasmussen, 1986), katere pa je Elston s sod. (1992) razglasil za jedrne pore. Neuspešen prenos bolezni med različnimi vrstami školjk pa kaže na možnost vrstne specifičnosti potencialnega infektivnega povzročitelja (Kent et al., 1991).

Novotvorbe hemocitov ne moremo prepoznati z makroskopskim pregledom bolne klapavice, saj shiranost, bledost prebavne žleze in atrofijo plašča ugotavljamo tudi pri

drugih boleznih klapavic (Barber, 2004). Diagnostični tehniki za ugotavljanje novotvorbe hemocitov sta histološka preiskava in hemocitologija (Elston in sod., 1992).

Patologija gojenih in divjih mediteranskih klapavic v slovenskem morju je zelo slabo raziskana. Z vstopom v Evropsko unijo in s spoštovanjem njene zakonodaje smo v Slovenskem nacionalnem referenčnem laboratoriju za bolezni školjk leta 2004 vzpostavili sistem nadzora nad školjčišči v slovenskem morju in razvili diagnostiko marteilioze, edine bolezni klapavic, ki jo je treba obvezno prijaviti, uvrščene v Anex A Seznama bolezni rib, mehkužcev in rakov Direktive sveta EGS (Annex A, Council Directive 91/67/EEC), pozneje pa tudi v prenovljeni Anex IV Direktive 2006/88/EC med neeksotične bolezni mehkužcev. Določili smo dve uradni mesti vzorčenja gojenih klapavic: školjčišče v Seči in školjčišče v Strunjanu, iz katerih smo vsako leto v spomladanskih in poletnih mesecih odvzeli 150 gojenih mediteranskih klapavic in jih pregledali na marteiliozo. Do sedaj so bili vsi uradno odvzeti vzorci gojenih klapavic negativni na praživali *Marteilia* spp., ugotovili pa smo znotrajcelične migetalkarje (Gombač in sod., 2008). Čeprav je slovensko morje zaradi svoje lege, plitkosti in drugih dejavnikov zelo specifično življenjsko okolje, z visokimi nihanji temperature, slanosti in oksigenacije (Lipej in sod., 2004; Richter, 2005), nas je negativen rezultat na *Marteilio* spp. presenetil, saj je znano, da je marteilioza bolezen, ki je razširjena od Atlantika do Perzijskega zaliva (Bower in McGladdery, 2009), diagnosticirali pa so jo tudi v italijanskem severnem Jadranu (Ceschia in sod., 1992) ter v hrvaškem morju (Zrnčić in sod., 2001). Zato smo se odločili za raziskavo, v kateri smo v enoletnem obdobju mesečno odvzeli gojene in divje mediteranske klapavice in s histopatološko preiskavo v njihovih prebavnih žlezah ugotavljali praživali *Marteilia* spp. in znotrajcelične migetalkarje. Ugotovljene marteilije smo vrstno določili z molekularno metodo PCR in PCR-RFLP. Ob vsakem vzorčenju smo izmerili temperaturo, slanost in oksigenacijo morja, saj smo skušali pojasniti njihov vpliv na invazijo praživali. Na koncu smo izračunali še kondicijski indeks odvzetih klapavic, da bi ugotovili vpliv praživali na kondicijo klapavic. Med histopatološkim pregledom klapavic smo ugotovili tudi novotvorbo hemocitov klapavic, jo vključili v raziskavo in ugotavljali vpliv temperature, slanosti in oksigenacije morja na njeno pojavljanje ter njen vpliv na kondicijo obolelih klapavic.

Ugotavljanje invazij praživali v prebavnih žlezah klapavic in novotvorb pri gojenih in divjih mediteranskih klapavicah v slovenskem morju ter njihova letna dinamika v povezavi s temperaturo, slanostjo in oksigenacijo morja je prva tovrstna študija v slovenskem morju.

2 INTRODUCTION

2.1 THE SLOVENE SEA

The Slovene Sea is part of the Gulf of Trieste, the northernmost end of The Adriatic Sea, where the Mediterranean pushes furthest into the European continent (Richter, 2005). It is bordered by the line between Grado and Savudrija and the coastline. The entire Slovene coast is 46.6 km long and is composed of Eocene sedimentary flysch (Lipej, 2004; Richter, 2005). The average depth of the sea is only about 17 metres (Richter, 2005) and the deepest point – the Piran Punta - is 37.25 metres deep (Rejec Brancelj, 2003).

The Adriatic current flows from the south, along the Istrian coast, turns west along the Slovene coast and finally turns southwards along the Italian coast (Lipej, 2004; Richter, 2005). The tides in the Gulf of Trieste are very high for Mediterranean sea. Due to prevailing winds and lunar phases, the difference between low and high tides can reach more than 180 centimetres and is the largest in the whole of the Adriatic. Tides are one of the main reasons for strong currents, which renew five percent of the water mass of the gulf twice a day (Richter, 2005).

The sea temperature varies considerably: during the summer the shallows can heat up to 30 ^{o}C and the coastline can even freeze during very cold winters (Richter, 2005). The average temperature is 15.8 ^{o}C (Rejec Brancelj, 2003). Pronounced stratification is one of the characteristics of our sea, which warms up from the surface to the bottom from winter onwards. Winds and different salinities form layers of different temperatures, separated by sharp transitions, which decrease the possibilities for exchanging heat, salinity, and nutritive substances between the layers. These transitions are known as thermoclines. Their number starts to diminish at the beginning of autumn, but they never disappear (Richter, 2005).

The average salinity of Slovene sea is between 37 and 38‰ (Rejec Brancelj, 2003). Many large and small rivers, groundwater and underwater springs called “brojnice” (Richter, 2005) have a strong influence on salinity, which fluctuates between 20‰ after abundant rainfall and 38‰ during the late summer and winter (Lipej, 2004; Richter, 2005).

The oxygen concentration varies depending on the sea temperature (Richter, 2005). The average oxygen concentration at the sea bottom is 6 mg/l in summer and 9 mg/l during winter (Čermelj et al., 2003).

The shallowness of the sea and a great amount of nutritive substances, borne by rivers, make it a rich home for numerous sea animals and plants (Rejec Brancelj, 2003). One hundred and six taxons of molluscs and 38 taxons of bivalves, including over 40 species, live naturally in the Slovene Sea (Lipej, 2004), but only Mediterranean mussels (*Mytilus galloprovincialis*) are farmed.

2.1.1 MUSSEL CULTURES IN SEČA AND STRUNJAN

Mussels are farmed in three locations, i.e., Seča, Strunjan and Debeli rtič in natural fisheries - shellfish farms. In 2004, the Slovene National reference laboratory for mollusc diseases established two official sampling sites, Seča and Strunjan.

The Seča and Strunjan shellfish farms are situated in semi-closed small bays, the first in Piran bay and the second in Strunjan bay. The farms are quite small and composed of 5 fields, each measuring 20 000 square meters. In each field there are 20 lines with 18 to 20 butts, which are lashed to each other with two ropes, called "ventija". Twenty clusters of mussels in nylon socks called "rešta", up to 3 m long, hang from each ventija approximately 1 m under the sea surface. The annual production of each field is from 25 to 30 tons (Fonda, 2009; personal communication).

Seeds of 1 to 1.5 cm shell length are collected manually from buoys and ropes in the shellfish farm or from buoys and ropes in neighbouring a fish farm, but often the so-called "seed traps" are used - old, worn and semi-torn ropes approximately 1.5 m in length are fixed in shellfish the farm and used for catching the seeds. The timing of the seed collection is determined by the autumn arrival of sea bream (*Sparus aurata*) – if the seeds are put in socks before their arrival, sea breams usually eat all the seeds and completely ruin the shellfish farm. Occasionally, small quantities of seeds are bought in Italy (Fonda, 2009; personal communication).

After collection, small clusters of seeds are tightly filled in the dense white cotton socks which are put in white nylon mesh sleeves or socks. These are fastened to ropes, which hang from rafts and are submerged in the water. In a few days, the

mussels attach themselves by byssus. After approximately 6 months, the cotton socks decompose, which enables the mussels to grow through the nylon socks. When they are half-grown and out-grow the nets, they are removed, cleaned and reapportioned in to bigger socks, where they reach market size (Fonda, 2009; personal communication).

During their growth, the mussels are exposed to direct sunlight once or twice for a whole day. This procedure kills all the living organisms attached to the shells. Mussels reach commercial size in approximately 18 months and at that time, they are 5 to 7 cm long. Smaller specimens, called "half-mussels" are removed and reinserted into the cotton socks where they reach market size (Fonda, 2009; personal communication).

The constant inflow of freshwater rich in minerals contributes to the good taste and very high quality of the mussels (Bojc, 2004).

2.2 MEDITERRANEAN MUSSEL (*Mytilus galloprovincialis*, Lamarck, 1819)

2.2.1 TAXONOMY

The Mediterranean mussel (*Mytilus galloprovincialis*) belongs to the phylum *Mollusca* (Linnaeus, 1758), class *Bivalvia* (Linnaeus, 1758), subclass *Metabranchia*, superorder *Fillibranchia*, order *Pteriomorpha*, superfamily *Mytiloidea*, family *Mytilidae* - mussels and genus *Mytilus*. Bivalves (*Bivalva* – the name derived from the Latin *bis*, meaning two, and *valvae* meaning *the leaves of a door***)** are marine and freshwater molluscs (Matoničkin, 1970; Buchsbaum and Milne, 1971). The class includes 7.500 species (Gosling, 2004), among them scallops, clams, oysters and mussels, of which 1.000 species live in Europe (Garms and Borm, 1981) and 43 in Slovene sea (Lipej, 2004).

2.2.2 GENERAL MORPHOLOGY

2.2.2.1 THE SHELL

The mussel's external shell is composed of two hinged, generally symmetrical triangulate (Gosling, 2004) halves or "valves", the right and the left one, separated by a mantle and designed to support the soft tissues and protect them from predators and desiccation (Mengoli, 1998). They are composed of proteins, mucopolysaharides and calcium carbonate (Mengoli, 1998; Gosling, 2004), which are mostly secreted in summer (Kadunc et al., 2006). The valves are joined by a ligament and reduced hinge teeth, and are closed, when necessary, by strong internal adductor muscles (Mengoli, 1998).

2.2.2.2 THE MANTLE

The mantle covers the visceral mass and is composed of soft tissue. It constitutes a large opening and with prolonged margins often forms two siphons: the inhalant, which also constitutes a filter that prevents entrance to bigger food particles and the exhalant (Mengoli, 1998). It secretes the shell and ligament and via numerous nerve endings in the marginal and pallial zone provides tactile sensory functions (Jabbour-Zahab et al., 2003).

2.2.2.3 THE FOOT AND BYSSUS GLAND

The foot is small, tongue-like in shape, with a groove in the ventral surface in which a byssus gland opens. The byssus gland produces a viscous secretion, which enters the groove and hardens gradually upon contact with sea water. This forms extremely tough, strong, elastic, byssus threads that secure the mussel to its substrate (Mengoli, 1998; INET). The byssus thread, which is produced during the whole of the mussel’s lifespan (Mengoli, 1998) is also sometimes used by mussels as a defensive mechanism, to tether predatory molluscs, such as dog whelks, that invade mussel beds, immobilising them and thus starving them to death (INET).

2.2.2.4 THE EXCRETORY ORGANS

U-shaped kidneys – nephridia (Mengoli, 1998) are situated on the both sides of the heart and filtrate blood. The pericardial glands lie over the auricular walls. Waste accumulates in the pericardial glands and is periodically discharged into the pericardial cavity and from there travels to the kidneys (Gosling, 2004). Nephridia principally excrete ammonium and urea. The excretion is done via the gills and kidneys. The filtrates end up in the mantle cavity and are finally expelled (Mengoli, 1998).

2.2.2.5 THE RESPIRATORY SYSTEM

Respiration takes place via the gills (Mengoli, 1998) which are engaged in blood hematosis, in the capture of food particles, in the uptake of nutrients and dissolved organic particles and in bacterial symbiosis (Mengoli, 1998; Le Pennec et al., 2003; Gosling, 2004). Mussels have gills called filibranchie (Mengoli, 1998). The gills lie between the visceral mass and the mantle on both sides of the corpus (Mengoli, 1998) in the form of two blades or laminae, i. e. internal and external (Le Pennec et al., 2003). Each lamina consists in turn of a descending leaflet or lamella opposite the foot, and an ascending lamella, located on the mantle side. The two gills form a W-shape, seen in a cross section (Mengoli, 1998; Le Pennec et al., 2003). The gill surface measures 100 to 110 square centimetres in a 6 to 7 cm long mussel (Mengoli, 1998).

2.2.2.6 THE LABIAL PALPS

The gills end in the labial palps (Gosling, 2004), which are located in the antero-dorsal part under the mantle (Le Pennec et al., 2003). They consist of four triangular lips, two external and two internal, framing the mouth. Labial palps have a role in the nutritional process in cleaning the gills to prevent their saturation and in screening and guiding the nutrients towards the digestive tract (Le Pennec et al., 2003).

2.2.2.7 THE REPRODUCTIVE ORGANS

The paired gonads encircle the digestive gland and are composed of branching tubules, which unite to form ducts that lead into larger ducts that terminate in a short gonoduct (Gosling, 2004), and of follicles within which the germ cells evolve during spermatogenesis or oogenesis (Thielley and Grizel, 2003). Their volume and external aspect vary during gametogenesis. The sexes are generally separated but in some cases a functional hermaphroditism may be present (Thielley and Grizel, 2003).

2.2.2.8 THE CARDIO-VASCULAR SYSTEM

The cardio-vascular system is an open system – the blood inundates the tissues and forms a lagoon system (Mengoli, 1998), but it also contains capillaries enabling a closed loop micro-circulation of the hemolymph (Le Pennec and Grizel, 2003). The heart, which consists of one ventricle and two lateral atria (Mengoli, 1998), also called auricula (Le Pennec and Grizel, 2003; Gosling 2004), is dorsally positioned, enclosed within a pericardium and connected to the excretive system via reno-pericardial ducts (Mengoli, 1998; Le Pennec and Grizel, 2003).

The circulation flows from the ventricle which propels the hemolymph through the anterior and posterior aortas, through arteries to arterioles. The hemolymph then flows through lacunae of variable size, before reaching the ventral sinus, located above the reno-pericardial complex. It reaches the kidneys, depurates and flows to the gills via afferent vessels. After hematosis, it returns to the heart via efferent vessels leading to the auricles (Le Pennec and Grizel, 2003; Gosling, 2004).

The hemolymph can not coagulate (Mengoli, 1998) and is composed of colourless plasma and haemocytes (Gosling, 2004). It has numerous roles, including gas exchange, osmoregulation, nutrient distribution, waste elimination, internal defence and also forms a fluid skeleton, giving temporary rigidity to labial palps, foot and the mantle edge (Gosling, 2004) Three types of haemocytes are known, although their classification is still under debate, i.-e. hyalinocytes, agranular

haemocytes and granular haemocytes (granulocytes); they are involved in the regeneration of various soft tissues as well as of the shell, in digestion, excretion and defence system owing to their ability to phagocytise foreign particles and pathogens or to form cysts and encapsulate them. They also play a significant role in the detection and destruction of external agents by secretion of lectins and enzymes or by producing free radicals. Granulocytes in particular are also involved in metal storage (Grizel at al., 2003).

2.2.2.9 THE NERVOUS SYSTEM

The nervous system is fundamentally simple, bilaterally symmetrical and consists of three pars of ganglia, i. e. cerebral, pedal and visceral ganglia (Luber and Mathieu, 2003), and several pairs of nerves (Gosling, 2004). Mussels contain various types of neurosecretory cells, which are located mostly in the cerebral ganglia (Gosling, 2004). Most sensory receptors are located in the mantle (Mengoli, 1998; Gosling, 2004), also including the ocelli, which can detect sudden changes in light intensity (Gosling, 2004).

2.2.2.10 THE DIGESTIVE SYSTEM AND DIGESTIVE PROCESSES

The digestive system is adapted to digest particulate food (Boucaud-Camou and Henry, 2003).

The anterior part is composed of the mouth with cilia (Gosling, 2004), which opens directly into a short oesophagus (Boucaud-Camou and Henry, 2003; Gosling, 2004). Neither a buccal cavity nor radula (dentate strap) is differentiated. The oesophageal epithelium is composed of tall ciliated and glandular cells, which lie on a thin layer of muscular and connective tissue. In their apical part, the glandular cells have numerous secretory granules (Boucaud-Camou and Henry, 2003).

The stomach is large and oval shaped, completely embedded in the digestive gland, which opens into it by several ducts (Gosling, 2004). It is a complex pouch, composed of several parts. The gastric shield, a chitinous sclerotinized cuticle of varying thickness, covers an epithelium consisting of tall cells with numerous

secretory granules and a nucleus located in the central part. It abuts against the crystalline style (Boucaud-Camou and Henry, 2003), a semi transparent gelatinous rod, which originates in the style sac at the posterior end of the stomach (Gosling, 2004). The underlying stomach wall contains numerous muscle fibres and haemocytes. The dorsal hood is a more or less developed pouch lying above the gastric shield. The ciliated epithelium has microvilli – some cells are secreting and contain numerous dense and homogenous secretory granules, others are not. Non-glandular cells lack microvilli. The ciliary tracts or sorting areas occupy most of the internal gastric surface. It is divided in the right (primary or posterior sorting area) and the left tracts corresponding to the right and the left opening of the digestive gland ducts, respectively. Longitudinal folds define deep parallel grooves along these tracts. Both tracts are bound by two ridges, the major and minor typhlosoles. Secretory and non-glandular cells of the ciliary tract have cilia and microvilli (Boucaud-Camou and Henry, 2003).

The polysyringian stomach has numerous openings in the digestive ducts and a large extension of the major intestinal typhlosole and intestinal groove into the floor of the stomach. This type of stomach is specialized for microphagous filter-feeding and occurs in three morphological types. Mussels have the type III stomach, which is characterized by the long slender tongue of the major typhlosole, bordered on both sides by the intestinal groove. The stomach contains numerous grooves formed by folds in the stomach epithelium. Long folds have ciliated cells of equivalent height and contain numerous dense inclusions. The epithelium covers a thick basal lamina, which lies on a dense connective tissue, forming the core of the fold. Short folds are covered by ciliated epithelial cells of different height, which lie on a thick basal lamina. The large stomach wall is composed of connective tissue and muscle fibres (Boucaud-Camou and Henry, 2003).

Three kinds of caeca may be found, but they are not always present: the duct caeca (right and left) into which the ducts of the digestive gland open, the sorting caecum, which may develop left of the stomach, and the appendix - a small caecum lying in an anterodorsal or posterodorsal position. Large food particles

that can not pass trough the digestive system remain in the appendix (Boucaud-Camou and Henry, 2003).

The stomach extends into the straight or gastric intestine corresponding to the style sac – midgut complex. The epithelium in this area is composed of tall cells containing dense inclusions and clear vacuoles. It lies on a thin basal lamina, which covers a thick layer of connective tissue containing numerous haemocytes and muscle fibres. The epithelium is composed of non-secretory ciliated cells providing for rotation of the crystalline style, which dissolves within a few hours after emersion (Boucaud-Camou and Henry, 2003).

The midgut or ascending intestine runs parallel to the style sac. It is either fully separated from the style sac or conjoined, forming an intestinal gutter prolonging the intestinal groove and communicating with the style sac trough a narrow slit delimited by the major and minor typhlosoles, which are covered with tall ciliated cells. The midgut extends into the posterior intestine (hindgut, comprising the mid intestine with an ascending and descending branch covered with ciliated epithelium and sporadic glandular cells) and the terminal intestine (rectum), passing over the posterior adductor muscle and ending with the anus in the mantle cavity. The rectum has plicated epithelium, composed by ciliated and glandular cells, and a fibro-muscular wall, which is thicker than in other parts of the intestine (Boucaud-Camou and Henry, 2003).

The digestive gland (digestive diverticula) completely surrounds the stomach and part of the intestine. It is composed of numerous blind-ending tubules, which open into non-ciliated branched secondary ducts (Boucaud-Camou and Henry, 2003), leading to a network of primary ducts ending in the stomach (Boucaud-Camou and Henry, 2003; Gosling, 2004).

The digestive tubules consist of the digestive cells characterized by numerous clear vacuoles in their apical part, and the crypts, dark areas composed of secretory cells, flagellated cells and nests of stem cells (Boucaud-Camou and Henry, 2003; Gosling, 2004). Tubules are surrounded by the peritubular tissue, composed of fibrocytes, muscle fibres, amoebocytes (granulocytes) and macrophages (Boucaud-Camou and Henry, 2003).

The digestive tubules undergo a sequence of cytological changes during the digestive cycle. In fasting animals, the digestive cells are flat and the lumen wide (type 1 tubules) – this phase is known as a holding phase. With the arrival of food, absorption and intracellular digestion begin and the digestive cells become taller and filled with digestive vacuoles. The lumen becomes narrow and the crypt cells develop cilia (type 2 tubules). In the final, disintegrated phase, the apical part of the digestive cells swells and protrudes into the lumen, releasing numerous free spherules subsequently discharged into the stomach (type 3 tubules). After this, a reconstituting phase follows, during which the crypt cells regenerate new digestive cells (type 4 tubules) (Boucaud-Camou and Henry, 2003).

The food particles, mostly phytoplankton cells (microalgae) wrapped in mucous stings, enter the stomach through the mouth and the short oesophagus with rotation, produced by the crystalline style. The initial and essential phase of digestion involves the selection of particles, sorting by size, density and digestibility and occurs in the stomach. This phase is followed by extracellular digestion. The mucous strings from the oesophagus are subjected to mechanical (grinding against the gastric shield) and chemical (enzymes from the style and stomach) decomposition. The ciliary activity sorts the particles in the stomach in the following manner: small and heavy or excess particles are immediately rejected through the intestinal groove to the midgut by the major typhlosole, small and light particles are conveyed to the opening of the digestive ducts and propelled into the digestive gland by back-flow and large and light particles are recirculated for further decomposition (Boucaud-Camou and Henry, 2003). Small particles enter the digestive gland via the brush-border of the ducts, full of enzymes, which absorb the soluble molecules. The largest particles and molecules travel directly to the digestive tubules. A second phase of extracellular digestion may occur in the lumen of tubules due to the presence of extracellular enzymes. Whereas extracellular digestion is quite strong, intracellular digestion appears to be the main digestive process occurring in the digestive gland (Boucaud-Camou and Henry, 2003). After it is finished, nutrients go from the digestive cells to the hemolymph, amoebocytes and periglandular connective tissue, while digestion

residues accumulate in residual bodies that are ultimately rejected (Boucaud-Camou and Henry, 2003; Gosling, 2004). In the final phase of digestion, the digestive cells break up and release residual bodies and lysosomes accumulated in the apical part through the lumen of tubules into the stomach (Boucaud-Camou and Henry, 2003; Gosling, 2004). Some of these enzymes can also assist in extra-cellular digestion (Gosling, 2004). Digestion and absorption continue in the intestine (Boucaud-Camou and Henry, 2003; Gosling, 2004). Rejected material from the stomach and waste from the digestive gland are formed into faecal pellets, which travel through the anus and are finally expelled through the exhalant opening into the sea (Gosling, 2004).

The digestive gland stores glycogen and lipids in the glandular cells or in the periglandular tissue (Boucaud-Camou and Henry, 2003; Gosling, 2004). These metabolic reserves are consumed during gametogenesis and periods of physiological stress (Gosling, 2004). The accumulation of pigments sometimes stain the digestive gland darkish brown or black (Boucaud-Camou and Henry, 2003; Gosling 2004).

The digestive glands also stores metal and organic contaminants. When challenged with a great quantity of contaminants, the tubules can show some degenerative changes and even inflammatory reaction, the formation of granulocytomas (Gosling, 2004).

2.2.3 BIOLOGY

2.2.3.1 DESCRIPTION

Mediterranean mussels are dark blue or brown (Pagad, 2006) to almost black-violet (Fantuzzi, 2005). The inner part of the shell is of mother-of-pearl colour (Fantuzzi, 2005). The length of adult organisms varies from 6 cm to 8 cm and can be attained in 14 months (Fantuzzi, 2005), but mussels can grow up to 15 cm (Pagad, 2006). The average life span is 4 years (Fantuzzi, 2005), but mussels can live more than 12 years (Gosling, 2004).

2.2.3.2 GEOGRAPHIC DISTRIBUTION AND HABITAT

Mediterranean mussels form large colonies on rocky coasts and sandy bottoms in the cooler waters of the northern and southern hemispheres (Gosling, 2004; Pagad, 2006). Although they are native to the Mediterranean Black and Adriatic Seas (Pagad, 2006), they can nowadays be found also in the Atlantic ocean from the United Kingdom and Ireland to Morocco (Fantuzzi, 2005) and along the south African, Japanese, Chinese, Australian and American West coasts (Gosling, 2004).

Mediterranean mussels are found predominantly in the intertidal to subtidal regions, occupying a wide range of habitats from rocky sheltered to extremely wave-exposed shores and estuaries (Gosling, 2004). They settle on various substrates that provide secure anchorage, e.g. rocks, stones, pebbles, shell, wood (Gosling, 2004) and different artificial materials (Fantuzzi, 2005), e.g. cement, ropes, chains, buoys, ships (Tušnik, 1985). They are able to survive out of water for several days (Fantuzzi, 2005).

Mussels can survive within a temperature range from -3^{o}C to 44^{o}C and salinity from 4‰ to 40‰ (Gossling, 2004) with optimum temperature for growth ranging from 8^{o}C to 25^{o}C and salinity from 27‰ to 30‰ (Fantuzzi, 2005).

2.2.3.3 FEEDING

Mussels are filter feeders. They feed on numerous suspended particles such as bacteria, phytoplankton, micro-zooplankton, and detritus and also dissolved organic material, particularly amino acids and sugars (Gosling, 2004).

2.2.3.4 REPRODUCTION

Mytilus galloprovincialis is a species without sexual dimorphism. Hermaphrodites are rare (Tušnik, 1985). Mussels can reach sexual maturity in their first year of life – in the Adriatic sea mainly at 5 to 8 months and 15 to 35 mm in length (Tušnik, 1985), and can modify their reproductive cycle according to environmental influences, in particular sea temperature and salinity, food and light (Gosling, 2004) and the time of settlement (Tušnik, 1985). They have an annual

reproductive cycle which involves a period of gametogenesis, followed by single or several spawning events which are followed by gonad reconstruction (Gosling, 2004).

During spawning, which mostly occurs from March to June (Fantuzzi, 2005), in the northern Adriatic from December to April at sea temperatures from 8 to 14^{o}C (Tušnik, 1985), red-orange coloured females produce a substance in their ovaries, which, released into the sea, stimulates white coloured male to ejaculate their sperm (Mengoli, 1998). Eggs and sperm are released into the water, where fertilization takes place (Gosling, 2004). After 4 to 5 hours, cilia appear in a fertilised egg and after 24 hours, a ciliated trochophore stage is reached and starts to produce the primary larval shell. The larva is called veliger and drifts for three weeks to six months in the surface water. After the foot appears, the larva becomes pediveliger and descents on a hard surface as a young mussel. It is capable of moving slowly to obtain a better life position and initially settles on algae and hydroids. After that, the post-larva grows up to 2 mm shell length and finally produces byssus-like threads and finds its way into the adult mussel bed (Gosling, 2004).

2.2.3.5 PREDATORS

Gastropods, especially dogwhelk (*Nucella lapillus*) are the significant predators of mussels worldwide. Predation is seasonal but whelks remain in shellfish farms also over the winter. Other important predators are starfish, crabs, seabream and birds. Among the known predators are also urchins, lobsters, flatfish, seals, walruses and turtles (Gosling, 2004).

2.2.4 PATHOLOGY - DISEASES AND PARASITES

The major disease causing agents of mussels are viruses, bacteria, fungi, protozoans, helminths and parasitic crustaceans (Gosling, 2004). The diseases affecting the world's mussel population are till date the following (Bower and McGladery, 2009):

- Virus-Like Disease of Mussels
- *Marteilia refringens*/*maurini* of Mussels

- *Steinhausia mytilovum* (Mussel Egg Disease)
- Phototrophic Endolith Invasion of Mussel Shells
- *Proctoeces maculatus* Trematode Disease of Mussels
- Mussel Gill Turbellaria
- *Mytilicola intestinalis* (Red Worm Disease) of Mussels
- Kidney Coccidia of Mussels
- Haplosporidian Infection of Mussels
- Bucephalid Trematode Diseases of Mussels
- Mytilicola orientalis (Red Worm) of Mussels
- Pea Crabs in Mussels
- Haemocytic Neoplasia of Mussels
- *Mytilicola porrecta* (Red Worm) of Mussels
- *Rickettsia*-like and *Chlamydia*-like Organisms of Mussels
- Gregarine Parasitism of Mussels
- Intracellular Ciliates of Mussels
- *Sphenophrya*-like Ciliates of Mussels
- *Ancistrum mytili* Gill Ciliate of Mussels
- Mycotic Periostracal Sloughing of Mussels
- Trematode Metacercariae of Mussels
- Parasitic copepods on Mussel Gills
- Bivalve-inhabiting hydroids of Mussels
- Shell-boring Polychaetes of Mussels
- Shell-burrowing Sponges of Mussels

2.2.4.1 PROTOZOAN DISEASE OF DIGESTIVE GLAND IN MUSSELS

The most extensive and economically the most significant epizooties in molluscs are caused by protozoa, viruses, fungi and bacteria (Renault, 1996).

In Europe two species of protozoa invade the digestive gland of mussels, i. e. *Marteilia* spp., which causes the marteiliosis or marteiliasis of mussels, and

intracellular ciliates of mussels or digestive gland ciliates - Rhynchodid-like ciliates (Ceschia, 2008; Bower in McGladdery, 2009).

2.2.4.1.1 MARTEILIOSIS OF MUSSELS

2.2.4.1.1.1 General information about the disease

Marteiliosis, due to *Marteilia refringens*, has been reported in numerous species of oysters (*Ostreidae*), cockles (*Cardiidae*), scallops and clams (*Pectinidae*, *Solenidae* (Ceschia et al., 2001) and mussels (*Mytilidae*), caused by protistan parasites of the genus *Marteilia* (Berthe et al., 2004). In Europe, marteiliosis has been described as a disease of flat oysters (*Ostrea edulis*), Mediterranean (*Mytilus galloprovincialis*) and blue (*Mytilus edulis*) mussels caused by *Marteilia refringens* (OIE, 2009).

Protists of the genus *Marteilia* (Berthe et al., 2004) belong to the phylum Paramyxea (Desportes and Perkins, 1990; Berthe et al., 2000). Paramyxea are eukaryotic microorganisms, Protozoa, in which the sporulation results from series of internal cleavages within an ameboid stem cell that germinates from spores in tissues of invertebrate marine animals. The development is characterised by the production of offspring cells that remain inside the parent cell (Desportes and Perkins, 1990).

The genus *Marteilia* encompasses several species: *Marteilia refringens*, *Marteilia maurini*, *Marteilia sydneyi*, *Marteilia christenseni* and *Marteilia lengehi* (Berthe et al., 2004). *Marteilia refringens* and *Marteilia sydneyi* have caused mass mortality in oysters (Berthe et al., 2004) and are listed as notifiable agents to the Office International des Epizooties (OIE) and the World Organisation for Animal Health (OIE, 2009).

Marteiliosis of mussels is caused in Mediterranean (*Mytilus galloprovincialis*) and blue (*Mytilus edulis*) mussels by two different species or two types of a single species (the taxonomic relationship and species determination is still under debate - Berthe et al., 2004) of genus *Marteilia*: *Marteilia refringens* and *Marteilia maurini* (Bower and McGladdery, 2007). The first descriptions of *M. refringens* were recorded in the early 1970s in flat oysters (Grizel et al., 1974).

In 1982, Comps at al. discovered on the basis of ultrastructural characteristics in Mediterranean mussels from the Venice lagoon a new species of the genus *Marteilia* - *M. maurini*. This finding was later contradicted by the fact that these two marteilia species cannot be differentiated either on the basis of ultrastructural characteristics (Longshaw et al., 2001) or sequences of the 18S rDNA genes small subunits of *Marteilia* spp., isolated from oysters and mussels (Berthe et al., 2000). As such, it was proposed to consider *M. maurini* only a junior synonym of *M. refringens* (Longshaw et al., 2001). In further studies, targeting the ITS region of the rRNA gene, two genetic types of *Marteilia*, linked to the host species, were confirmed in Europe: *M. refringens* in oysters and *M. maurini* in mussels (Le Roux et al., 2001). Finally Lopez-Flores et al. (2004) established that there is no strict correlation of *Marteilia* types to mussels or oysters and that *M. maurini* (M type) can be also found in oysters and *M. refringens* (O type) in mussels.

2.2.4.1.1.2 The development of *Marteilia* spp. in mussels

Different stages of *Marteilia* spp. vary consistently in the size, which is the consequence of continuous enlargement of the primary cell cytoplasm and increase in the number of daughter cells by internal cleavage (Berthe et al., 2004).

It is supposed that the infection by al *Marteilia* spp. begins when the primary cell or stem cell, 5 do 8 μm in diameter, invades the epithelial cells of the gut or gills develops a secondary uninucleate daughter cell in a vacuole within its cytoplasm. The daughter cell divides by binary fission to produce four daughter cells within the enlarged primary cell and within each daughter cell a uninucleate cell develops by internal cleavage. When the primary cell degenerates, it releases the daughter cells, which become new primary cells. They penetrate the basal membrane of the digestive gland tubules and become established as nurse cells, containing daughter cells, at the base of the epithelial cells. Daughter cells in the digestive gland tubules become sporangiosori called "primary cells". Sporulation occurs within the sporangiosorus via a unique

process of internal cleavages (endosporulation) and cells within cells – sporangial primordial (secondary cells) - are produced. In every primary cell, which retains its nucleus and enlarges to about 30 μm in diameter, 8 to 16 sporangial primordial, each about 12 μm in diameter at maturity, are formed within the sporangiosorus. Each secondary cell matures into a sporont, containing 2 to 4 spore primordial (tertiary cells), which matures into spores. Each spore contains 3 uninucleate large sporoplasms, with one smaller sporoplasm being enclosed within the cytoplasm of the large one (Perkins, 1976). Mature spores measure 3.5 to 4.5 μm in diameter and are surrounded with a continuous wall without operculum. As spores mature, light refractile inclusion bodies, from which the name of *M. refringens* derives, appear in the sporont cytoplasm (Bower and McGladdery, 2007). Finally, mature spores are released into the tubule lumen (Bower and McGladdery, 2007). This event is associated with the destruction of the host digestive gland epithelia (Robledo and Figueras, 1995).

2.2.4.1.1.3 The pathogenicity of *Marteilia* spp.

M. refringens and *M. maurini* are potentially lethal pathogens (Villalba et al., 1993b). In mussels they cause the haemocytic infiltration of epithelial cells and connective tissue of digestive gland (Villalba et al., 1997), connective tissue of the mantle, labial palps and gills (Garcia et al., 2009) and the occasional formation of granulocytomas, which destro both the parasite and host tissue (Villalba et al., 1993a). Heavy infection results in the massive destruction of digestive gland, which is most extensive during the release of spores from sporangia (Figueras et al., 1991; Robledo and Figueras, 1995; Villalba et al., 1997; Rayyan et al., 2006). No alterations nor host reaction have been observed in the epithelial cells of the stomach (Robledo and Figueras, 1995). Heavy infection causes also the reduced absorption of nutrients, which results in the inhibition of gonadal development and tissue storage and subsequently the loss of body condition (Figueras et al., 1991; Rayyan et al., 2006).

Infection by *M. refringens* in Mediterranean mussels may a cause significant increase of haemocytes in circulation, which can decelerate or even stop the infection (Carballal et al., 1998). Mortality increases with the intensity of the infection (Villalba et al., 1993a). 8.3% to 29.6% mortality has been observed in Mediterranean mussels (Robledo and Figueras 1995).

Up till now marteiliosis of mussels has been observed in the Atlantic from the north of France to Portugal (Comps et al., 1981; Comps et al., 1982; Auffret and Poder, 1985; Villalba et al., 1993a; Robledo and Figueras, 1995; Villalba et al., 1997; Longshaw et al., 2001; Fuentes et al., 2002; Novoa et al., 2005; Bower and McGladdery, 2007), in the Mediterranean sea including the northern Adriatic (Ceschia et al., 1992; Zrnčić et al., 2001), in the Persian Gulf (Bower and McGladdery, 2007) and in the Gulf of Thermaikos in northern Greece (Virvilis et al., 2003; Rayyan et al., 2006).

2.2.4.1.1.4 Epidemiological properties of infection with *Marteilia* spp.

The prevalence of marteiliosis in Mediterranean mussels in Delta de l'Ebre in Spain was 1.67% in 2003, from 2.23% to 12.2% in 2004 (Carrasco et al., 2008) and from 3.34% to 26.67% in 2005 (Carrasco et al., 2007), from 5.5% to 38.5% in Galicia between 1987 and 1990 (Robledo and Figueras, 1995) and even up to 65% in the case of experimentally introduced Mediterranean mussels (Villalba et al., 1993a), 5% in Croatia (Zrnčić et al., 2001), 14.6% (Rayyan, 2006) and 21.25% (Virvilis et al., 2003) in Greece, from 16.6% to 22.2% in the Adriatic sea in Italy (Ceschia et al., 1992), 19.3% in Albania (Pëllumb et al., 2006) and from 37% to 70% in northern Brittany (Auffret and Poder, 1985). In experimentally introduced Mediterranean mussels and hybrid crosses between Mediterranean and blue mussels in Galicia, the infection with *M. refringens* was equal or even higher than 50% in hybrid crosses, whereas in Mediterranean mussels it was 40% or less (Fuentes et al., 2002).

Marteilia spp. was detected in mussels throughout the year, but the prevalence varies also according to the season (Carrasco et al., 2007b): in Spain it was observed mainly from February to October (Carrasco et al., 2008), in Greece

from May till November (Photis et al., 1992), in Croatia in August (Zrnčić et al., 2001) and in Italy in April and from June to September (Ceschia et al., 1992). Audemard et al. (2001) and Carrasco et al. (2008) reported that the infection of healthy mussels and oysters occurs when the sea temperature is higher than 17 ^{o}C, Grizel (1985) on the other hand found out that the temperature minimum, necessary for the development of marteilia, is 12 ^{o}C, Carrasco et al. (2007) reported the presence of all stages of *M. refringens* even at 8 ^{o}C. Experimentally introduced healthy Mediterranean mussels became infected with *M. refringens* four months after the introduction (in June) in the infected sea (Villalba et al., 1993a). The infection persisted over the year with peaks in prevalence in warm summer months (Villalba et al., 1993a), from May till August (Robledo and Figueras, 1995) and in January (Villalba et al., 1997). The probability of becoming infected rises as mussels grow (Fuentes et al., 1998). The infection was most frequent in the shallow sea near the coast (Robledo and Figueras, 1995; Fuentes et al., 1995) and was higher in polluted sea areas (Rayyan et al., 2006). High salinity inhibits the infestation (Ceschia et al., 1992).

The transmission route of infection is still unknown. The direct transmission of *M. refringens* between flat oysters by co-habitation, injection and feeding of spore suspension (Comps and Joly, 1980; Perkins, 1988; Berthe et al., 1998) and sediment (Berthe et al., 1998) was unsuccessful. Negative results point to the existence of an intermediate host, but also the attempt to transmit the infection on healthy oysters by feeding of fish with large number of spores (Grizel, 1985) and the feeding of *M. refringens* spores to small crustaceans *Carcinus maenas*, *Carcinus crangon* and *Marinogammarus marinus* (Van Banning, 1979) turned out to be a failure. The development of PCR and in situ hybridisation enables the detection of the parasite in potential hosts. PCR was first used to screen every species sampled in the particular, quite small oyster ponds (called claires), for the eventual presence of parasites and the copepod *Paracartia* (*Acartia*) *grani* was revealed as a potential host for *M. refirigens* (Audemard et al., 2001; Audemard et al., 2002). The involvement of *P. grani* in the life cycle of *M. refringens* is apparently consistent both with the biology

and the geographic distribution of the copepod, on the one hand, and with the epidemiology of marteiliosis, on the other hand. *P. grani* is mainly observed in spring and summer and is absent in winter, which matches the seasonal cycle of *M. refringens* (Audemard et al., 2002). Sporangia primordia of *M. refringens* do not develop during winter, because only the increase of water temperature encourages their development (Berthe et al., 1998). On the other hand, Carrasco et al. (2005) found out that marteilia isolated from mussels, as distinct from those isolated from oysters, did not multiply in copepods. In spite of all these results, it can be concluded that the involvement of *P. grani* in the life cycle of *M. refringes* requires further investigation, especially because all the investigations were conducted under experimental conditions (Berhte et al., 2004). During the research in 2003 and 2004 in the natural environment of the Delta de l'Ebre, scientists established that the most numerous zooplankton species were the copepod *Acartia latisetosa*, the cyclopoida *Oithona* sp. and an indeterminate harpaticoida species (Carrasco et al., 2008). Using the PCR method *M. refringens* was diagnosed in all species (Carrasco et al., 2007a), which makes *Acartia latisetosa*, *Oithona* sp. and indeterminate harpaticoida species a probable intermediary host than *P. grani* (Carrasco et al., 2008). In a further study performed in 2004 and 2005 also in Delta de l'Ebre, six zooplankton taxa were found positive using PCR for *M. refringens*, i. e. *Acartia discaudata*, *A. clausi*, *A. italica*, *Oithona* sp., harpaticoida *Euterpina acutifrons* and larval stages of decapods crustaceans, probably *Portumnus* sp. (Carrasco et al., 2007b). *Acartia discaudata*, *A. clausi*, *A. italica* and *Portumnus* sp. are new taxa which could be proposed as intermediate hosts in the infection process of mussels by *Marteilia* (Carrasco et al., 2007).

2.2.4.1.1.5 Diagnosis of marteiliosis

Diagnostic techniques for the detection of marteiliosis of mussels are limited to histological and cytological examination, electron microscopy, polymerase chain reaction (PCR) and immunological assay (Bower in McGladdery, 2007). Detailed protocols are described in the Office International des Epizooties

(OIE) the Manual for diagnostic of aquatic animal diseases (OIE 2009; http://www.oie.int/eng/normes/fmanual/A_summry.htm).

The **histological examination** of the digestive gland represents the screening method, recommended by OIE. Tissue sections, including the digestive gland of mussels, are fixed in the appropriate fixative (Davidson's, Carson's, 10% formalin solution), embedded in paraffin, stained with hematoxylin and eosin (HE) or other staining techniques, cut and examined under the light microscope. Various stages of the parasite can be observed in the epithelial cells of the digestive gland ducts. The unique feature of internal cleavage during sporulation differentiates *Marteilia* spp. from all other protists. This method does not enable the differentiation of different *Marteilia* spp. species (Berthe et al., 2004; Bower and McGladdery, 2007).

When the increase mortality occurs, the presumptive diagnostic method - **cytological examination** can be used in addition to histology to receive a quick result (Berthe et al., 2004). Tissue sample, containing the digestive gland, is placed on blotting paper to remove excessive water, imprinted several times onto a slide and stained by Giemsa or other methods. Marteilia are observed with the light microscopy (OIE, 2000; OIE, 2009).

When the pathogen is diagnosed during the examination, **transmission electron microscopy (TEM)** may be used to differentiate *M. refringes* from *M. sydneyi*, which does not infect the shellfish of the northern hemisphere (OIE, 2000; OIE, 2009). TEM was shown to be unsuccessful for the differentiation of *M. refrigens* from *M. maurini* (Longshaw et al., 2001).

PCR primers were designed by comparing the nucleotide sequence of the small subunit ribosomal RNA gene with that of various eukaryotic organisms. **PCR** can produce sensitive and specific results and can help to identify an unknown pathogen and confirm infection in different hosts. But PCR has also some faults: a light infection can be missed and the extreme sensitivity of PCR may result in a false positive result. Therefore positive and negative controls must always be included in PCR protocols (Le Roux et al., 1999). Scientists are dividend in the use of nested PCR for *Marteilia* spp. differentiation in mussels: Le Roux et al. (2001) considered *M. refringens* and *M. maurini* to be different

species, whereas Lopez-Flores et al. (2004) and Novoa et al. (2005) suggested that they are just two different strains of the same species.

The use of **immunological assays** is questionable. Tiscar et al. (1993) prepared polyclonal antibodies to *Marteilia* spp., isolated from Mediterranean mussels, which clearly marked the parasite by direct immunoperoxidase staining in infected mussels. Anderson et al., (1994) produced very specific polyclonal antibodies against *Marteilia sydneyi* from oysters (*Saccostrea commercialis*), which had not cross reacted with *M. refringens* and *M. maurini*.

Monoclonal antibodies against *Marteilia* spp. cross reacted with *M. refringens* from Mediterranean mussels (Bower and McGladdery, 2007). These monoclonal antibodies were found to produce various results when used in different immunological tests: better results were obtained in indirect immunoflourescence (IIFA) and ELISA (Pernas et al., 2000).

2.2.4.1.1.6 Prevention and eradication of marteiliosis

The infection in mussels can be prevented by banning the transfer of mussels from infected areas and from areas with unknown epidemiologic situation. Mussels from areas where infection persists in flat oysters should be treated with similar precautions (Bower and McGladdery, 2007). The collection of mussel seeds from uninfected areas may contribute to a reduction of the number of parasites in cultured mussels (Robledo et al., 1994). Eradication is impossible due to the persistence of the disease in wild shellfish populations.

2.2.4.1.2 INTRACELULLAR CILIATES OF MUSSELS

2.2.4.1.2.1 General information about the disease

Ciliates are protozoans which belong to the phylum *Ciliophora*. Many of them are associated with marine bivalves, particularly those of the classes *Kinetofragminophorea, Oligohymenophorea* and *Polyhymenophorea*. Ciliates are presumabe to be ubiquitous and have been reported in many species of mollusc. (Chollet et al., 2003) Most of them are extracellular and are found in lumen of the digestive gland tubules, in the intestine and in the digestive gland

ducts (*Ancistrocoma*-like), others are attached to or located near the gills, mantle and labial palps (*Sphenophrya*-like, *Trichodina sp.*, *Ancistrum*-like and *Ancistrocoma*-like). Some ciliates are also intracellular (*Sphenophrya*-like and *Rhynchodid*-like). (Chollet et al., 2003) Ciliates are mostly harmless and commensals, but intracellular ciliates can cause xenoma (*Sphenophrya*-like) (Chollet et al., 2003) of the gills of *Mytilus edulis* (McGladdery and Bower, 2002) and disruption of the digestive tubule epithelia (*Rhynchodid*-like) (McGladdery and Bower, 2002).

Mussels, including *Mytilus galloprovincialis* are often invaded with *Rhynchodid*-like ciliates, known also as mussel protozoan X (MPX) or intracellular ciliates of mussels or digestive gland ciliates (Bower et al., 1994), which inhabit the epithelial cells of digestive gland ducts and tubules (Ceschia, 2008).

2.2.4.1.3 The description of intracellular ciliates of mussels

Intracellular ciliates of mussels are pear or spindle-shaped, 3.9 to 15 μm long (Villalba et al. 1997; Gombač et al., 2008) and 2.9 to 8.4 μm width (Gombač et al., 2008). They have a polymorphic oval to globular basophilic, fragmented macronucleus, stained deep blue. Intracellular ciliates of mussels colonise the digestive tubule epithelia but are seen also in the lumens of digestive tubules (Villalba et al., 1997; Gombač et al., 2008).

2.2.4.1.3.1 The pathogenicity of intracellular ciliates of mussels

Mostly they cause only a slight enlargement of epithelial cells that contained ciliates of a large size, without an inflammatory response (Villalba et al., 1997; Gombač et al. 2008) but a specific host response in infected mussels was also reported (Figueras et al. 1991). In some cases the destruction of digestive gland has been noticed, but the infection never causes mortality (McGladdery and Bower, 2002).

2.2.4.1.3.2 Epidemiological properties of infection with intracellular ciliates of mussels

Intracellular ciliates of mussels appear to be ubiquitous. The reported prevalence is lower than 1% in blue mussels on the German coast (McGladdery and Bower, 2002) and in Mediterranean mussels in Delta de l'Ebre (Carrasco et al., 2007b), 4.2% to 8.3% in wild and 0% in cultured blue mussels in Canada (Weldon, 1999) and 21.4% in Mediterranean mussels in the Slovene sea (Gombač et al., 2008). The highest prevalence of up to 40% was detected in Mediterranean mussels in Spain (Villalba et al., 1997).

The epidemiological properties have been poorly investigated. Weldon (1999) found the infection with ciliates in cultured blue mussels in every month of the year, whereas wild mussels were uninfected in November and February, with a water temperature of between 5.8 ^{o}C and -1.5 ^{o}C. The highest infection at 8.3% was detected in September with water temperature of 15.2 ^{o}C and salinity of 34.7 ppt (Weldon, 1999). Villalba et al. (1997) on the other hand diagnosed infected Mediterranean mussels in every month of the year, but the lowest infection was detected in the period from October to January. Data concerning the time of infection, manner of transmission and the possible influence of water temperature, salinity and oxygenation on infection with intracellular ciliates of mussels is still wanting.

2.2.4.1.3.3 Diagnosis of intracellular ciliates of mussels

Intracellular ciliates are diagnosed on the basis of a histological examination of digestive gland (McGladdery in Bower, 2002).

2.2.4.2 NEOPLASIAS OF MUSSELS

Hemocytic neoplasia of mussels is the only neoplasia described in mussels to date (Bower, 2006).

2.2.4.2.1 HAEMOCYTIC NEOPLASIA OF MUSSELS

2.2.4.2.1.1 General information about the disease

Disseminated neoplasia, also known as leukaemia, haematopoietic or haemic or haemocytic neoplasia (Bower, 2006), leukocytic neoplasia or sacomatous neoplasia (Elston, 1990a) has been diagnosed in 15 species of bivalves including oysters (*Ostreidae*), cockles (*Cardiidae*), clams (*Tellinidae*) and mussels (*Mytilidae*) (Ciokan and Sunila, 2005). In mussels has finally been termed the haemocytic neoplasia of mussels (Bower, 2006).

2.2.4.2.1.2 The description of haemocytic neoplasia of mussels

The disease is characterised by the proliferation of large, anaplastic cells in the connective tissue, blood vessels and sinuses of the visceral mass, muscle and mantle tissue (Elston et al., 1992). Neoplastic cells are hypertrophied and are 2 to 4 times larger than normal haemocytes. They have a large, hyperchromatic and often pleomorfic nucleus (Zizzo et al., 1991; Villalba et al., 1997; Usheva and Frolova, 2000; Barber, 2004; Ciocan and Sunila, 2005), up to 15 μm in diameter (Villalba et al., 1997), with finely dispersed chromatin (Villalba et al., 1997), containing one or more prominent nucleoli (Villalba et al., 1997; Usheva and Frolova, 2000; Barber, 2004) or are without it (Ciocan and Sunila, 2005). Some cells are even bi-nucleated (Ciocan and Sunila, 2005). A high nucleus to cytoplasm ratio and high mitotic activities, expressed with large mitotic figures and a high mitotic index from 0.9% to 1.9% (normally 0.12%) is characteristic for this disease (Usheva and Frolova, 2000), are high (Elston et al., 1992; Villalba et al., 1997; Usheva and Frolova, 2000; Barber, 2004; Ciocan and Sunila, 2005).

In the early stage of disease only single abnormal cells or small foci of neoplastic cells are noticeable in the circulatory system (Barber, 2004), which suggests, together with the morphologic similarities with haemocytes, its hemopoietic origin (Barber, 2004). Later on neoplastic cells progressively replace normal haemocytes, especially hyaline haemocytes, and are found throughout the tissues (Elston et al., 1992). The number of apoptotic

haemocytes significantly increases (Galimany and Sunila, 2008). Subsequently fibrisos, displacement, the compression of gills, gonad and connective tissue and general degeneration and necrosis of tissues occur (Barber, 2004). Haemocytic neoplasia usually become generalised and most often results in the death of mussel (Elston et al., 1988b). Death arises from the consequence of the loss of haemocytes and their defence capabilities (Kent et al., 1989; Cao et al., 2007) especially phagocytosis, which results in host immuno deficiency (Kent et al., 1989). Neoplastic haemocytes also lose the capability of digestion, absorption and food transportation which leads to starvation (Barber, 2004). The condition index in diseased clams was significantly lower than in healthy ones (Leavitt et al., 1990).

The disease is normally lethal, but disease remission can also occur (Elston et al., 1988a; Elston et al., 1988b; Elston et al., 1990b). Elston et al. (1988a) observed a 50% lethality and 20% remission rate in diseased pacific mussels. In a later study the remission rate was even as hight 37% (Elston et al., 1990b).

2.2.4.2.1.3 Epidemiological properties of the haemocytic neoplasia of mussels

Haemocytic neoplasia of mussels is a widespread disease (Elston, 1990a; Bower, 2006), having been diagnosed in blue (*Mytilus edulis*), Mediterranean (*Mytilus galloprovincialis*) and pacific (*Mytilus trossulus*) mussels (Bower, 2006). The prevalence of disease ranges from 1.5% (Galimany and Sunila, 2008) to 50% in blue mussels from United States (Elston et al., 1988a). The disease has never been diagnosed in blue mussels from the Polish Baltic (Wolowicz et al., 2006). In Mediterranean mussels only sporadic cases have been recorded till date: one case in California (Hillman, 1990), two cases in Italy in March (Zizzo et al., 1991) and four in July (Tiscar et al., 1990), one case in 1991 (Figueras et al., 1991), five cases 6 years later (Villalba et al., 1997) and two cases in 2007 in Spain (Carrasco et al., 2007b) and one case in the Romanian Black Sea (Ciocan and Sunila, 2005).

The occurrence of the haemocytic neoplasia of mussels is higher in the late autumn and in winter, from October to March (Elston, 1990a) or from January

to March (Barber, 2004). Carrasco et al. (2007b) found one case of haemocytic neoplasia in June and another one in October. Older mussels are more frequently affected (Elston, 1990a).

The haemocytic neoplasia of mussels can be transmitted (Elston, 1990a). Transfer via the inoculation of the neoplastic cell to healthy mussels has been successful, which suggests that an infective agent, presumably virus (retrovirus) may be involved (Elston et al., 1988b). This hypothesis is supported by the finding of virus-like particles in neoplastic cell nuclei in blue mussels using an electron microscope (Rasmussen, 1986), although Elston et al. (1992) interpreted these structures to be nuclear pores. The fact that the transmission of the disease between different species of shellfish is unsuccessful suggests the possibility of species specificity of a potential infective agent (Kent et al., 1991).

Among the potential aetiologic factors are also environmental contamination and biotoxins (Barber, 2004). Hillman (1993) found out that the morbidity in mussels along the both Atlantic and Pacific United States coasts was significantly higher in areas contaminated with polycyclic aromatic hydrocarbons (PHA) than in areas polluted by pesticides, chrome, mercury and cadmium. A connection between haemocytic neoplasia and pollution was established also in Japan (Usheva and Frolova, 2000). Wolowicz et al. (2006) found out that the disease breaks out only in shellfish, which live on the sea bottom and not in those hanging from ropes, beliving the cause to be heavily polluted sea sediments.

Landsberg (1996) observed that the occurrence of haemocytic neoplasia coincided with outbreaks of several species of toxic dinoflagellates, which may increase the susceptibility to neoplasia and particular to viral agents.

A possible genetic predisposition, given that certain bivalve species are more prone to the development of haemocytic neoplasia than other, should also be considered (Barber, 2004). It is also presumed various kinds of stress could have a negative impact on the host’s defence mechanisms (Barber, 2004).

The possible influence of water temperature, salinity and oxygenation on the occurrence of the haemocytic neoplasia of mussels is also completely un-investigated.

2.2.4.2.1.4 Diagnosis of haemocytic neoplasia of mussels

The haemocytic neoplasia can generally not be recognized by gross examination of sick mussels, because tissue emaciation, pale digestive gland and mantle recession can be encountered in many other diseases (Barber, 2004). The usual diagnostic techniques are **histological examination**, where abnormal haemocytes and the presence of obvious and numerous mitotic figures are noticed, and **hemocytology**, which involves the removing of 0.1 to 0.5 ml of hemolymph from the posterior adductor muscle, diluting it with filtrated sea water and placing on a glass slide (Elston et al., 1992).

2.2.4.2.1.5 Prevention and eradication of haemocytic neoplasia of mussels

The haemocytic neoplasia can be prevented by banning the transfer of diseased mussels and mussels from areas with unknown epidemiologic situation in healthy zones. Eradication is impossible due to the persistence of the disease in wild shellfish populations (Elston, 1990).

3 MATERIAL AND METHODS

3.1 SAMPLING OF MUSSELS AND MEASUREMENT

Three sampling sites for collection of Mediterranean mussels (*Mytilus galloprovincialis*) were established in Slovene sea: two in shellfish farms, i. e. Seča and Strunjan, which are also the official sampling sites of Slovene National reference laboratory for shellfish disease, and one in natural shellfish beds in Piran. The Seča and Strunjan shellfish farms are situated in semi-closed small bays, the first in Piran bay and the second in Strunjan bay. Natural beds in Piran lie near the Piran Punta, a fairly open area sea in the Gulf of Trieste.

Twelve samplings were performed in Seča and Strunjan and 11 in Piran from November 2007 to October 2008. The exact dates of sampling are shown in tables 5, 6 and 7. The mussel's health status was checked in both shellfish farms at each sampling.

Forty adult Mediterranean mussels were stripped directly from ropes at each sampling from both shellfish farms over a one year period, at a depth of approximately 3-metres. Weather conditions made it possible to collect by diving 40 adult mussels from natural beds in the open sea at a depth of approximately 3-metres only from July to November. From January to June it was only possible to collect 20 adult Mediterranean mussels monthly in the open sea at the depth of approximately 3-metres, because of bad weather conditions or the lack of adult mussels at the sampling point. In December the collection of wild mussels proved to be impossible due to the stormy sea. In total 1280 adult Mediterranean mussels comprising 960 from shellfish farms and 320 from natural beds were collected throughout the year and included in our study.

Water temperature, oxygenation and salinity were measured at each sampling in all three sampling sites at the exact point where Mediterranean mussels lived. Oxygenation measurements were not performed in March, because the oxygen sensor suddenly and unexpectedly stopped functioning. Water temperature and oxygenation were measured using a thermometer "MultiLine P4 – Oxi 320 Set" with a dissolved oxygen probe (oxygen sensor) "CellOx 325" (WTW). Water salinity was measured using a hand-held refract meter "S/Mill-E. S= 0-100‰" (ATAGO).

Live adult mussels were transported within one hour to the laboratory in a classic cooling bag and were appropriately processed for further examinations. Dirt and water organisms attached to the shell were carefully removed. The mussel shells were then washed with fresh water.

The length of the mussels was measured from the hinge to the longest part of the bill. The shell was opened with the special knife, designed for the opening of shellfish and excess water was removed. The total weight of mussel was measured and the flesh was afterwards carefully removed from the shell intact, drained on double absorbent paper and weighed. The total weight of the mussel and weight of the flesh was measured with electronic balance PM 3000 (Metzler), accurate to 0.01g.

The flesh condition index was calculated on fresh mussels by means of the formula "condition index = fresh flesh weight x 100/total weight".

3.2 MACROSCOPIC EXAMINATION AND TISSUE SAMPLING

The shell and the flesh of mussels were macroscopically inspected for eventual abnormalities or lesions.

A standard section through the visceral mass, including mantle, gill and gonads was performed after weighting, in which a single sample of approximately 5 mm thick, was taken from each mussel and put in a plastic basket with a unique identification number for further histopathological examination.

3.3 HISTOPATHOLOGICAL EXAMINATION

Samples were immediately placed in 10% formalin solution for not longer than 24 hours at the room temperature and were routinely paraffin embedded. Four μm thick sections were stained with haematoxylin and eosin (HE) and one slide per mussel containing the stomach and digestive gland was examined with a Diastar (Reichert-Jung) light microscope at 400x magnification for the presence of protozoa and neoplasias.

3.3.1 MORPHOMETRIC CHARACTERISATION OF PROTOZOA AND NEOPLASIA

Morphometric analyses were performed on tissue slide photographs, using a DS-U2 (Nikon) digital camera and Microphot FXA (Nikon) microscope.

3.3.1.1 MORPHOMETRIC CHARACTERISATION OF *Marteilia* spp. AND INTRACELLULAR CILIATES OF MUSSELS

Measurements of the protozoa were performed using the computer programme NIS-Elements BR (Nikon) follows: the length and the width of one hundred randomly chosen *Marteilia* spp. (including different stages) and one hundred ciliates were measured and the average values of measured parameters were calculated.

3.3.1.2 MORPHOMETRIC CHARACTERISATION OF NEOPLASIA

Measurements of neoplastic cells were performed using the computer programme NIS-Elements BR (Nikon) as follows: the diameter of one hundred neoplastic cells and their nuclei were measured and the average values of the parameters measured were calculated. Mitoses were counted in 10 high power fields (HPFs) and the average value was calculated. Mitotic activity was scored as low (< 5/10 HPF), intermediate (5-10/10 HPF), or high (> 10/10 HPF).

3.3.2 THE PREVALENCE OF INFECTION WITH PROTOZOA AND THE OCCURRENCE OF NEOPLASIA

The prevalence was calculated by dividing the number of mussels found infected or affected by the number of all histologically tested mussels.

3.3.3 THE INTENSITY OF INFECTION WITH PROTOZOA

3.3.3.1 INTENSITY OF THE INFECTION WITH *Marteilia* spp.

HE stained sections were rated according to the degree of the *Marteilia* spp. infection, using the Villalba's et al. (1993a) scale, based on the intensity of infection:

- uninfected: no parasites detected;
- a mild infection – the parasites were confined to the stomach epithelium;
- a moderate infection – less than 10% of the digestive tubules were infected;
- a severe infection – more than 10% of the digestive tubules were infected.

3.3.3.2 THE INTENSITY OF THE INFECTION WITH THE INTRACELLULAR CILIATES OF MUSSELS

HE stained sections were scored on our own scale based on counting the number of intracellular ciliates:

- uninfected: no parasites detected;
- a mild infection – less than fifty parasites per section;
- a moderate infection – from fifty to one hundred parasites per section;
- a severe infection – more than one hundred parasites per section.

3.3.4 THE INTENSITY OF HAEMOCYTIC NEOPLASIA OF MUSSELS

The severity of disease was guaged using the Mix's (1983) scale, designed for mussels and based on a histological examination of the HE stained sections as follows:

- existence of the disease is characterized by the presence of a small number of neoplastic cells or small foci of cells – Stage 1,
- neoplastic cells have begun to infiltrate connective tissue - Stage 2,
- neoplastic cells are present throughout the connective tissue and are present in hemolymph sinuses - Stage 3 and
- neoplastic cells are present in high numbers throughout the body - Stage 4.

3.4 M. REFRINGENS DETECTION AND CHARACTERISATION BY POLYMERASE CHAIN REACTION – RESTRICTION FRAGMENT LENGTH POLYMPRPHISM

3.4.1 SAMPLE PREPARATION (Pislak, 2000)

A microtom knife was rinsed in 10% sodium hypochlorite for 30 minutes and carefully washed with sterile distilled water before cutting the paraffin blocks. Five sections, 6 µm tick, were cut from four paraffin blocks in which *Marteilia* spp. was diagnosed by neabs if a histopathological examination and two additional histopatologically negative samples were added as negative control and put in 1.7 ml tube. Sections were deparaffined.

Deparaffining procedure

- 1200 µl xylen (Merck, USA) was added in each tube containing the tissue samples.
- Tubes were centrifuged for 5 minutes on 1400 rpm (Centrifuge 5430 (Eppendorf, Germany)) and the supernatant was afterwards decanted.
- 1.2 ml absolute ethanol was added to the pellet, mixed and centrifuged for 5 minutes at 14 000 rpm.
- The supernatant was decanted and 1.2 ml absolute ethanol was added to the sediment, mixed and centrifuged for 5 minutes on 14 000 rpm.
- The supernatant was decanted and sediment was air dried.

DNA was extracted from a piece of digestive gland using QIAamp DNA Mini Kit (QIAGEN; Germany) folowing the seguent Tissue Protocol established by Le Roux et al. (2001):

- After deparaffinisation, samples were supplemented with 180 µl of Buffer ATL.
- 20 µl of Proteinase K was added, mixed by vortexing and icubated at 56 ^{o}C until the tissue was completely lysed (overnight). The 1.5 microcentrifuge tube was briefly centrifuged to remove drops from the lid.

- 200 µl of Buffer AL was added to the sample, mixed by pulse-vortexing for 15 seconds and incubated at 70 ^{o}C for 10 minutes. The 1.5 microcentrifuge tube was briefly centrifuged to remove drops from the lid.
- 200 µl of ethanol (96-100%) was added to the sample and mixed by pulse-vortexing for 15 seconds. The 1.5 ml microcentrifuge tube was briefly centrifuged to remove drops from the lid.
- The mixture from step 4 was careffuly applied to the QIAamp Spin Column (in a 2 ml collection tube) without wetting the rim. The cap was closed and centrifugated at 10 000 rpm for 1 minute. The QIAamp Spin Column was placed in a clean 2 ml collection tube (provided in a kit) and the tube containing the filtrate was discarded.
- The QIAamp Spin Column was carefully opened and 500 µl of Buffer AW1 was added without wetting the rim. The cap was closed and centrifugated at 10 000 rpm for 1 minute. The QIAamp Spin Column was placed in a clean 2 ml collection tube (provided in a kit) and the tube containing the filtrate was discarded.
- The QIAamp Spin Column was carefully opened and 500 µl Buffer AW2 were added without wetting the rim. The cap was closed and centrifugated at full speed (14 000 rpm) for 3 minutes.
- The QIAamp Spin Column was placed in a new 2 ml collection tube (not provided in a kit) and the collection tube containing filtrate was discarded. The cap was closed and centrifugated at full speed (14 000 rpm) for 1 minute.
- The QIAamp Spin Column was placed in a clean 1.5 ml microcentrifuge tube (not provided in the kit) and the collection tube containing filtrate was discarded. The QIAamp Spin Column was carefully opened and 50 µl of distiled water were added. After one minute incubation at room temperature it was centrifugated at 10 000 rpm for one minute.
- DNA solutions were kept at 4 ^{o}C until PCR analyses were performed.

3.4.2 POLYMERASE CHAIN REACTION (PCR) (Le Roux et al., 2001)

3.4.2.1 REACTIVES

- 10 X Buffer (furnished with polymerase)
- $MgCl_2$ (furnished with polymerase) 50 mM
- Platinum Taq DNA Polymerase (Invitrogen, USA) 5 U/µl
- dNTP Mix 2,5 mM each (GeneAmp dNTP Mix with dTTP (Appled Biosystems, USA))
- H_2O (free of DNA and RNA)

3.4.2.2 PRIMERS

M2A 5' CCG CAC ACG TTC TTC ACT CC 3'

M3AS 5' CTC GCG AGT TTC GAC AGA CG 3'

3.4.2.3 PCR MIX

PCR Mix for each tube is shown in the present table:

Table 1: PCR Mix for each tube

	Volume per tube	Final concentration
Buffer (10X)	5 µl	1X
$MgCl_2$ (50 mM)	2,5 µl	2.5 mM
dNTP (2,5 mM each)	4 µl	0.2 mM
M2A (100 µM)	0.5 µl	1 µM
M3AS (100 µM)	0.5 µl	1 µM
Taq polymerase (5 U/µl)	0.5 µl	2.5 U
dH_2O	32 µl	

- 45 µl of this DNA PCR mix was dispensed in each PCR tube
- µl of extracted DNA was added to each tube

Two types of control are used:

- Negative controls consisted of dH_2O (5 µl for 45 µl of PCR Mix).
- Positive controls consisted of DNA extracted from known highly infected mussels.

3.4.2.4 AMPLIFICATION

Amplification cycles were performed in a thermal cycle apparatus Veriti® Thermal Cycler (Applied Biosystems)

- Initial denaturation: 2 minutes at 94 ^{o}C
- Amplification: 30 cycles (1 minute at 94 ^{o}C, 1 minute at 55 ^{o}C and 1 minute at 72 ^{o}C
- Final elongation: 10 minutes at 72 ^{o}C

3.4.2.5 INTERPRETATION

A positive result was an amplicon of the appropriate size (412 bp) with a negative control negative and a positive control positive.

3.4.3 RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

3.4.3.1 REACTIVES

- 10 X Buffer (furnished with the restriction enzyme)
- H_2O (free of DNA and RNA)ž
- *Hha*I enzyme 10U/ul (Promega, USA)

3.4.3.2 DIGESTION MIX

- Digestion mix for each tube was:
- 2 µl of the appropriate buffer
- 1 µl of enzyme
- 7 µl of dH_2O
- 10 µl of this digestion mix was dispensed in each tube and 10 µl of PCR products was added to each tube.

3.4.3.3 DIGESTION

Digestion was then performed by incubating samples for 1 hour at the temperature indicated by the manufacturer.

3.4.3.4 INTERPRETATION

M2A M3AS PCR products were digested differently according to the type of *Marteilia refringens*. The expected restriction profiles were for:

- *M. refringens* type M: 157bp + 156 bp + 68 bp + 31 bp
- *M. refringens* type O: 226 bp + 156 bp + 31 bp.

3.4.4 ELECTROPHORESIS

3.4.4.1 REACTIVES

AccuGENE 10×TBE Buffer (Lonza, Switzerland)

Arose gel:

- 1% for PCR products or 2% for RFLP products of agarose in 1X TAE (Tris base Acetic acid EDTA) Ethidium bromide (0.5 µg/ml) added after cooling the gel.

Loading blue dye:

- Bromophenol blue 0.25%
- Cyanol xylene FF 0.25%
- Sucrose 40%
- Keep at 4 ^{o}C and use diluted 6 times (2 µl of loading blue buffer for 10 µl of PCR products).

Molecular weight marker:

- SimplyLoad 100 bp DNA Ladder (Lonza, Switzerland): a ready-to-use molecular weight marker including 9 regularly spaced bands from 100 to 1000 bp.

3.4.4.2 AGAROSE GEL PREPARATION

1. 0.6/1.2 g of agarose was weighted, 60 ml of 1X TBE (Tris Borate EDTA) was added and heated until the mix was melted.

2. The solution was cooled and the solution was disposed into a specific mould equipped with combs (to form wholes).
3. When gel was polymerised, the combs were removed and placed in a horizontal electrophoresis system full of 1X TAE.
4. 10 µl of PCR products or 20 µl of RFLP products was mixed with 2 µl (for PCR products) or 4 µl (for RFLP products) of blue dye (6X) and placed in the wholes.
5. One whole is dedicated to the molecular weight marker (5 µl).
6. A voltage of 100 volts was applied during 70 min.
7. After electrophoresis, gel was ethidium bromide (10 µg/ml; Invitrogen, USA).
8. Results were documented using the GeneGenius bio-imaging system (Syngene, UK).
9. The gel was lastly observed under UV.

3.5 EPIDEMIOLOGICAL ANALYSES

3.5.1 INFECTION WITH *Marteilia* spp.

In mussels infected with *Marteilia* spp. the influence of the manner of growth, the location of growth, the month of the year and the influence of the sea temperature, oxygenation and salinity on the infection were evaluated.

For the manner of growth parameter infected mussels were divided into two groups, i.e. cultured and wild mussels, for the location of growth into three groups, i.e. Seča, Strunjan and Piran and for the month, into twelve groups corresponding to the twelve months of the year. For each group the prevalence of infection was calculated.

The influence of sea parameters on the infection was evaluated for each parameter, measured at the time of sampling.

Finally the influence of infection with *Marteilia* spp. on the condition index of mussels was evaluated.

3.5.2 INFECTION WITH INTRACELLULAR CILIATES OF MUSSELS

In mussels, infected with intracellular ciliates of mussels the influence of the manner of growth and the location of growth, the month of the year and the influence of the sea temperature, oxygenation and salinity on the infection and the intensity of the infection were evaluated.

For the manner of growth parameter infected mussels were divided into two groups, i.e. cultured and wild mussels, for the location of growth into three groups, i.e. Seča, Strunjan and Piran and for the month into twelve groups corresponding to the twelve months of the year. For each group the prevalence of infection and the intensity of infection were calculated.

The influence of sea parameters on the infection and the intensity of the infection were evaluated for each parameter, measured at the time of sampling.

Finally the influence of infection and the intensity of the infection with intracellular ciliates of mussels on condition index of mussels were evaluated.

3.5.3 OCCURENCE OF HAEMOCYTIC NEOPLASIA OF MUSSELS

In mussels with haemocytic neoplasia of mussel the influence of the manner of growth, the location of growth, the month of the year and the influence of the sea temperature, oxygenation and salinity on the occurrence were evaluated.

For the manner of growth parameter, affected mussels were divided into two groups, i.e. cultured and wild mussels, for the location of growth in three groups, i.e. Seča, Strunjan and Piran and for the month into twelve groups corresponding with the twelve months of the year. For each group the prevalence of occurrence was calculated.

The influence of sea parameters on haemocytic neoplasia was evaluated for each parameter, measured at the time of sampling.

The influence of haemocytic neoplasia on condition index of mussels was also evaluated.

3.6 STATISTICAL ANALYSIS

The testing of the hypotheses required a variety of statistical methods that were applied to the sample of mussels. The statistical calculations were performed using the SPSS computer software.

The selection of the statistical methods depended primarily on the types of the variables, since our data included:

- interval variables that were used to describe purely numerical features of the mussels with a potentially infinite number of possible values (e.g.: length, condition index etc.);
- ordinal variables that imply certain quantitatively expressed features of the mussels but may only have a limited number of possible values (e.g. the intensity of infection with intracellular ciliates);
- nominal variables that describe purely non-numerical categories of the mussels (e.g. location); a special case are also the dichotomous variables that simply describe the presence (value 1) or absence (value 0) of a phenomenon, e.g. the presence or absence of an infection.

When we had to compare the values of interval variables, for instance, for different locations and months, we used variance analysis in order to find out whether the differences between the group means were statistically significant. When we only had to combine the means between the *two* different categories (for example the mean condition index between infected and uninfected mussels) a t-test was used instead of variance analysis.

When we wanted to explore the relationship between interval (e.g. condition index) and ordinal variables (e.g. intensity of infection), we applied Spearman's correlation coefficient. To analyse the possible *independent* effects of (otherwise mutually significantly correlated) temperature, oxygenation and salinity to the intensity of infection we have used *partial* correlation as well.

Moreover, to analyse the possible *independent* effects of temperature, oxygenation and salinity to the presence of the infection (a dichotomous variable with only two possible values, namely 0 and 1), we used the binary logistic regression to identify the

significance of influence of temperature, oxygenation and salinity to the probability of infection.

Finally, in order to identify the relations between nominal variables we used Cramer's V chi-square based statistical coefficient.

4 RESULTS

4.1 MUSSEL DATA AND SEA DATA

1280 Mediterranean mussels were collected over a one year period and included in the present study: 960 mussels were from shellfish farms, i.e. 480 from Seča and 480 from Strunjan, and 320 from different natural beds in Piran.

No mortality was detected in shellfish farms during the one year sampling period.

The length of the cultured mussels varied from 5 cm to 9.8 cm (average 7 cm) and from 4 cm to 11.5 cm (average 7.1 cm) in wild mussels.

The total weight of cultured mussels varied from 5 g to 39.5 g (in average 15 g) and from 3 g to 51.3 g (average 17.37 g) in wild mussels. The average total weight of the mussels from Seča was 16.66 g, from Strunjan 13.34 g and from Piran 17.37 g. The weight of the flesh varied from 1.2 g to 12.2 g (average 4.15 g) in cultured and from 1 g to 12.2 g (average 4.8 g) in wild mussels.

The average condition index in mussels, collected in Seča was 27.36 and in Strunjan and Piran 28.9. The condition index varied from 11.17 to 69.33 (average 28.14) in cultured and from 16.83 to 56.32 (average 29.63) in wild mussels. Comparing different months the lowest average condition index was in December at 25.13 and the highest in February at 33.64. The condition index values were the highest in January, February, March and May. A notable decline confirmed by the analysis of variance statistical method[1] was observed in April and from June to December. The exact condition index values in each month of sampling in all three sampling sites and the average monthly and location values are shown in table 2 below.

[1] We have used the analysis of variance to test the significance of differences for months and locations whenever we compare quantitative (interval) variables.

Table 2: The condition index values for each month of sampling in all three sampling sites and the average monthly and location values

month of sampling	Seča	Strunjan	Piran	average
January	29.12	30.47	36.91	32.17
February	35.94	28.67	36.31	33.64
March	35.61	30.42	34.27	33.43
April	24.64	26.6	27.64	26.29
May	28.46	31.62	30.52	30.2
June	28.99	29.38	23.4	27.25
July	26.41	31.83	26.81	28.35
August	20.63	26.03	29.43	25.36
September	28.65	27.77	25.51	27.31
October	24.34	34.07	24.27	27.56
November	21.25	24.36	30.91	25.5
December	24.23	26.04	*	25.13
Average	**27.36**	**28.93**	**29.63**	

Table 3: Variance analysis (descriptive) of the condition index for each month of sampling

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
1	100	31,1348185	6,27875714	,62787571	29,8889768	32,3806601	21,01911	58,19672
2	100	33,1085348	5,40881865	,54088187	32,0353078	34,1817618	20,83333	46,74556
3	100	33,2728307	5,34641281	,53464128	32,2119864	34,3336750	22,59887	69,33333
4	100	26,0286557	3,51146727	,35114673	25,3319044	26,7254070	16,01942	37,01299
5	100	30,1390385	3,16225979	,31622598	29,5115775	30,7664994	23,18841	39,02439
6	100	28,0347815	5,51253860	,55125386	26,9409742	29,1285887	16,03774	54,38596
7	120	28,3520336	3,77226258	,34435888	27,6701686	29,0338985	16,98842	36,36364
8	120	25,3692387	4,73619771	,43235372	24,5131352	26,2253423	11,17021	34,54545
9	120	27,3164200	3,78615382	,34562698	26,6320441	28,0007959	16,49485	37,50000
10	120	27,5650149	5,94408374	,54261813	26,4905769	28,6394530	15,67568	40,21739
11	120	25,4315378	5,09007689	,46465832	24,5114679	26,3516076	13,40206	32,27513
12	80	25,0222046	5,57850590	,62369592	23,7807690	26,2636403	12,44444	51,40187
Total	1280	28,3263685	5,60426977	,15664410	28,0190609	28,6336761	11,17021	69,33333

Table 4: Variance analyses (multiple comparisons) of the condition index in each month of sampling

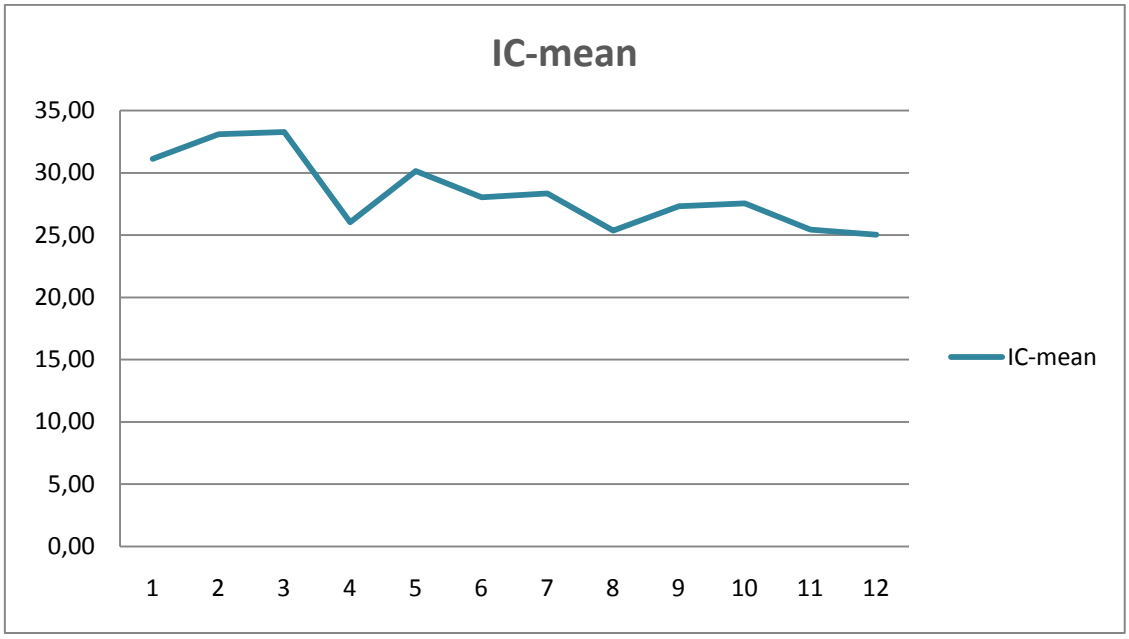
(I) MONTH	(J) MONTH	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
1	2	-1,97371633(*)	,6954426	,005	-3,3380611	-,6093716
	3	-2,13801223(*)	,6954426	,002	-3,5023570	-,7736675
	4	5,10616273(*)	,6954426	,000	3,7418180	6,4705075
	5	,99578000	,6954426	,152	-,3685648	2,3601248
	6	3,10003700(*)	,6954426	,000	1,7356922	4,4643818
	7	2,78278490(*)	,6658356	,000	1,4765242	4,0890456
	8	5,76557975(*)	,6658356	,000	4,4593191	7,0718404
	9	3,81839846(*)	,6658356	,000	2,5121378	5,1246591
	10	3,56980351(*)	,6658356	,000	2,2635428	4,8760642
	11	5,70328070(*)	,6658356	,000	4,3970200	7,0095414
	12	6,11261382(*)	,7376282	,000	4,6655077	7,5597200
2	1	1,97371633(*)	,6954426	,005	,6093716	3,3380611
	3	-,16429590	,6954426	,813	-1,5286407	1,2000489
	4	7,07987906(*)	,6954426	,000	5,7155343	8,4442238
	5	2,96949633(*)	,6954426	,000	1,6051516	4,3338411
	6	5,07375333(*)	,6954426	,000	3,7094086	6,4380981
	7	4,75650123(*)	,6658356	,000	3,4502406	6,0627619
	8	7,73929608(*)	,6658356	,000	6,4330354	9,0455567
	9	5,79211480(*)	,6658356	,000	4,4858541	7,0983755
	10	5,54351984(*)	,6658356	,000	4,2372592	6,8497805
	11	7,67699703(*)	,6658356	,000	6,3707364	8,9832577
	12	8,08633015(*)	,7376282	,000	6,6392240	9,5334363
3	1	2,13801223(*)	,6954426	,002	,7736675	3,5023570
	2	,16429590	,6954426	,813	-1,2000489	1,5286407
	4	7,24417496(*)	,6954426	,000	5,8798302	8,6085197
	5	3,13379223(*)	,6954426	,000	1,7694475	4,4981370
	6	5,23804922(*)	,6954426	,000	3,8737045	6,6023940
	7	4,92079713(*)	,6658356	,000	3,6145365	6,2270578
	8	7,90359197(*)	,6658356	,000	6,5973313	9,2098526
	9	5,95641069(*)	,6658356	,000	4,6501500	7,2626714
	10	5,70781574(*)	,6658356	,000	4,4015551	7,0140764
	11	7,84129293(*)	,6658356	,000	6,5350323	9,1475536
	12	8,25062605(*)	,7376282	,000	6,8035199	9,6977322
4	1	-5,10616273(*)	,6954426	,000	-6,4705075	-3,7418180
	2	-7,07987906(*)	,6954426	,000	-8,4442238	-5,7155343
	3	-7,24417496(*)	,6954426	,000	-8,6085197	-5,8798302
	5	-4,11038273(*)	,6954426	,000	-5,4747275	-2,7460380
	6	-2,00612573(*)	,6954426	,004	-3,3704705	-,6417810
	7	-2,32337783(*)	,6658356	,001	-3,6296385	-1,0171172
	8	,65941702	,6658356	,322	-,6468437	1,9656777
	9	-1,28776427	,6658356	,053	-2,5940249	,0184964
	10	-1,53635922(*)	,6658356	,021	-2,8426199	-,2300986
	11	,59711797	,6658356	,370	-,7091427	1,9033786
	12	1,00645109	,7376282	,173	-,4406551	2,4535573

5	1	-,99578000	,6954426	,152	-2,3601248	,3685648
	2	-2,96949633(*)	,6954426	,000	-4,3338411	-1,6051516
	3	-3,13379223(*)	,6954426	,000	-4,4981370	-1,7694475
	4	4,11038273(*)	,6954426	,000	2,7460380	5,4747275
	6	2,10425699(*)	,6954426	,003	,7399122	3,4686018
	7	1,78700490(*)	,6658356	,007	,4807442	3,0932656
	8	4,76979974(*)	,6658356	,000	3,4635391	6,0760604
	9	2,82261846(*)	,6658356	,000	1,5163578	4,1288791
	10	2,57402351(*)	,6658356	,000	1,2677628	3,8802842
	11	4,70750070(*)	,6658356	,000	3,4012400	6,0137614
	12	5,11683382(*)	,7376282	,000	3,6697277	6,5639400
6	1	-3,10003700(*)	,6954426	,000	-4,4643818	-1,7356922
	2	-5,07375333(*)	,6954426	,000	-6,4380981	-3,7094086
	3	-5,23804922(*)	,6954426	,000	-6,6023940	-3,8737045
	4	2,00612573(*)	,6954426	,004	,6417810	3,3704705
	5	-2,10425699(*)	,6954426	,003	-3,4686018	-,7399122
	7	-,31725210	,6658356	,634	-1,6235128	,9890086
	8	2,66554275(*)	,6658356	,000	1,3592821	3,9718034
	9	,71836147	,6658356	,281	-,5878992	2,0246221
	10	,46976651	,6658356	,481	-,8364942	1,7760272
	11	2,60324370(*)	,6658356	,000	1,2969830	3,9095044
	12	3,01257683(*)	,7376282	,000	1,5654707	4,4596830
7	1	-2,78278490(*)	,6658356	,000	-4,0890456	-1,4765242
	2	-4,75650123(*)	,6658356	,000	-6,0627619	-3,4502406
	3	-4,92079713(*)	,6658356	,000	-6,2270578	-3,6145365
	4	2,32337783(*)	,6658356	,001	1,0171172	3,6296385
	5	-1,78700490(*)	,6658356	,007	-3,0932656	-,4807442
	6	,31725210	,6658356	,634	-,9890086	1,6235128
	8	2,98279485(*)	,6348493	,000	1,7373242	4,2282655
	9	1,03561356	,6348493	,103	-,2098571	2,2810842
	10	,78701861	,6348493	,215	-,4584521	2,0324893
	11	2,92049580(*)	,6348493	,000	1,6750251	4,1659665
	12	3,32982892(*)	,7097831	,000	1,9373504	4,7223075
8	1	-5,76557975(*)	,6658356	,000	-7,0718404	-4,4593191
	2	-7,73929608(*)	,6658356	,000	-9,0455567	-6,4330354
	3	-7,90359197(*)	,6658356	,000	-9,2098526	-6,5973313
	4	-,65941702	,6658356	,322	-1,9656777	,6468437
	5	-4,76979974(*)	,6658356	,000	-6,0760604	-3,4635391
	6	-2,66554275(*)	,6658356	,000	-3,9718034	-1,3592821
	7	-2,98279485(*)	,6348493	,000	-4,2282655	-1,7373242
	9	-1,94718128(*)	,6348493	,002	-3,1926520	-,7017106
	10	-2,19577624(*)	,6348493	,001	-3,4412469	-,9503056
	11	-,06229904	,6348493	,922	-1,3077697	1,1831716
	12	,34703408	,7097831	,625	-1,0454445	1,7395126

9	1	-3,81839846(*)	,6658356	,000	-5,1246591	-2,5121378
	2	-5,79211480(*)	,6658356	,000	-7,0983755	-4,4858541
	3	-5,95641069(*)	,6658356	,000	-7,2626714	-4,6501500
	4	1,28776427	,6658356	,053	-,0184964	2,5940249
	5	-2,82261846(*)	,6658356	,000	-4,1288791	-1,5163578
	6	-,71836147	,6658356	,281	-2,0246221	,5878992
	7	-1,03561356	,6348493	,103	-2,2810842	,2098571
	8	1,94718128(*)	,6348493	,002	,7017106	3,1926520
	10	-,24859495	,6348493	,695	-1,4940656	,9968757
	11	1,88488224(*)	,6348493	,003	,6394116	3,1303529
	12	2,29421536(*)	,7097831	,001	,9017368	3,6866939
10	1	-3,56980351(*)	,6658356	,000	-4,8760642	-2,2635428
	2	-5,54351984(*)	,6658356	,000	-6,8497805	-4,2372592
	3	-5,70781574(*)	,6658356	,000	-7,0140764	-4,4015551
	4	1,53635922(*)	,6658356	,021	,2300986	2,8426199
	5	-2,57402351(*)	,6658356	,000	-3,8802842	-1,2677628
	6	-,46976651	,6658356	,481	-1,7760272	,8364942
	7	-,78701861	,6348493	,215	-2,0324893	,4584521
	8	2,19577624(*)	,6348493	,001	,9503056	3,4412469
	9	,24859495	,6348493	,695	-,9968757	1,4940656
	11	2,13347719(*)	,6348493	,001	,8880065	3,3789479
	12	2,54281031(*)	,7097831	,000	1,1503318	3,9352889
11	1	-5,70328070(*)	,6658356	,000	-7,0095414	-4,3970200
	2	-7,67699703(*)	,6658356	,000	-8,9832577	-6,3707364
	3	-7,84129293(*)	,6658356	,000	-9,1475536	-6,5350323
	4	-,59711797	,6658356	,370	-1,9033786	,7091427
	5	-4,70750070(*)	,6658356	,000	-6,0137614	-3,4012400
	6	-2,60324370(*)	,6658356	,000	-3,9095044	-1,2969830
	7	-2,92049580(*)	,6348493	,000	-4,1659665	-1,6750251
	8	,06229904	,6348493	,922	-1,1831716	1,3077697
	9	-1,88488224(*)	,6348493	,003	-3,1303529	-,6394116
	10	-2,13347719(*)	,6348493	,001	-3,3789479	-,8880065
	12	,40933312	,7097831	,564	-,9831454	1,8018117
12	1	-6,11261382(*)	,7376282	,000	-7,5597200	-4,6655077
	2	-8,08633015(*)	,7376282	,000	-9,5334363	-6,6392240
	3	-8,25062605(*)	,7376282	,000	-9,6977322	-6,8035199
	4	-1,00645109	,7376282	,173	-2,4535573	,4406551
	5	-5,11683382(*)	,7376282	,000	-6,5639400	-3,6697277
	6	-3,01257683(*)	,7376282	,000	-4,4596830	-1,5654707
	7	-3,32982892(*)	,7097831	,000	-4,7223075	-1,9373504
	8	-,34703408	,7097831	,625	-1,7395126	1,0454445
	9	-2,29421536(*)	,7097831	,001	-3,6866939	-,9017368
	10	-2,54281031(*)	,7097831	,000	-3,9352889	-1,1503318
	11	-,40933312	,7097831	,564	-1,8018117	,9831454

*The mean difference is significant at the .05 level.

Graph 1: Mean condition index (IC) values throughout the year (values for individual months)



The average sea temperature was 9.1 ^{o}C in winter, 15.2 ^{o}C in spring, 24.1 ^{o}C in summer and 17 ^{o}C in autumn. The average sea oxygenation was 11.6mg/l in winter, 9.3mg/l in spring, 7.6mg/l in summer and 7.7mg/l in autumn. The average sea salinity was 37.25‰ in winter, 29.6‰ in spring, 38.1‰ in summer and 37.8‰ in autumn.

The exact sea temperatures, oxygenation and salinities in each of sampling sites i. e. Seča, Strunjan and Piran at the time of sampling are presented in tables 5, 6 and 7.

Table 5: Sea temperature (T), oxygenation (O) and salinity (S) at the time of mussel sampling in Seča (cultured mussels)

date of sampling	T (^{o}C)	O (mg/l)	S (‰)
29th November 207	13.1	8.53	37
18th December 2007	9.5	10.95	35
29th January 2008	9.1	10.67	39
25th February 2008	9.2	10.05	37
31st March 2008	11.2	no data	32
28th April 2008	13.2	9.29	32
26th May 2008	20	9.06	25
30th June 2008	24.2	8.3	38
29th July 2008	24.9	7.5	36
28th August 2008	22.2	7	39
25th September 2008	20.3	6.8	39
20th October 2008	17.8	7	36

Table 6: Sea temperature (T), oxygenation (O) and salinity (S) at the time of mussel sampling in Strunjan (cultured mussels)

date of sampling	T (°C)	O (mg/l)	S (‰)
29th November 2007	12.8	9	39
18th December 2007	10.7	10.64	39
29th January 2008	9	10.3	38
25th February 2008	8.9	8,.53	35
31st March 2008	11	no data	33
28th April 2008	14.9	9.59	28
26th May 2008	20.2	8.82	28
30th June 2008	25.1	8	37
29th July 2008	25.6	7.9	38
28th August 2008	22.4	6.9	40
25th September 2008	20.3	7.1	39
20th October 2008	17.8	7.1	38

Table 7: Sea temperature (T), oxygenation (O) and salinity (S) at the time of mussel sampling in Piran (wild mussels)

date of sampling	T (°C)	O (mg/l)	S (‰)
29th November 2007	13	9.11	37
29th January 2008	8.8	10.79	38
25th February 2008	8.5	10.23	37
31st March 2008	11.7	no data	32
28th April 2008	15	9.76	30
26th May 2008	20	9.53	27
30th June 2008	25	8.,2	38
29th July 2008	25.4	7.8	37
28th August 2008	22.2	7.1	40
25th September 2008	20.3	7.1	39
20th October 2008	18.4	7.7	37

The sea in Piran was a little bit warmer than it was in Seča and Strunjan and the average oxygenation in Piran was slightly lower in comparison to Seča and Strunjan. The salinity was lower in Seča. The differences in temperature, oxygenation and salinity between sampling sites were statistically significant.

Table 8: Variance analysis (descriptive) of the sea temperatures, oxygenation and salinity for all three sampling sites

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower	Upper		
temperature	**Seča**	48	16,225	5,7607	,2629	15,708	16,741	9,1	24,9
	Strunjan	48	16,558	5,8905	,2688	16,030	17,086	8,9	25,6
	Piran	32	17,975	5,4987	,3073	17,370	18,579	8,5	25,4
	Total	128	16,787	5,7841	,1616	16,470	17,104	8,5	25,6
oxygenation	**Seča**	44	8,650	1,4233	,0678	8,516	8,783	6,8	10,9
	Strunjan	44	8,534	1,2238	,0583	8,419	8,649	6,9	10,6
	Piran	30	8,408	1,2046	,0695	8,271	8,545	7,1	10,7
	Total	118	8,545	1,2996	,0378	8,471	8,619	6,8	10,9
salinity	**Seča**	48	35,416	3,9083	,1783	35,066	35,767	25,0	39,0
	Strunjan	48	36,000	4,0249	,1837	35,639	36,361	28,0	40,0
	Piran	32	36,375	3,5032	,1958	35,989	36,760	27,0	40,0
	Total	128	35,875	3,8724	,1082	35,662	36,087	25,0	40,0

Table 9: Variance analysis (multiple comparisons) of the sea temperatures, oxygenation and salinity in all three sampling sites

Dependent Variable	(I) location	(J) location	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
temperature	Seča	Strunjan	-,33333	,37090	,369	-1,0610	,3943
		Piran*	-1,75000	,41468	,000	-2,5635	-,9365
	Strunjan	Seča	,3333	,37090	,369	-,3943	1,0610
		Piran*	-1,41667	,41468	,001	-2,2302	-,6031
	Piran	Seča*	1,75000	,41468	,000	,9365	2,5635
		Strunjan*	1,41667	,41468	,001	,6031	2,2302
oxygenation	Seča	Strunjan	,11545	,08746	,187	-,0561	,2871
		Piran	,24133	,09713	,013	,0508	,4319
	Strunjan	Seča	-,11545	,08746	,187	-,2871	,0561
		Piran	,12588	,09713	,195	-,0647	,3164
	Piran	Seča*	-,24133	,09713	,019	-,4319	-,0508
		Strunjan	-,12588	,09713	,001	-,3164	,0647
salinity	Seča	Strunjan*	-,58333	,24893	,019	-1,0717	-,0950
		Piran*	-,95833	, 27831	,001	-1,5043	-,4123
	Strunjan	Seča*	,58333	,24893	,019	,0950	1,0717
		Piran	-,37500	, 27831	,178	-,9210	,1710
	Piran	Seča*	,95833	, 27831	,001	,4123	1,5043
		Strunjan	,37500	, 27831	,178	-,1710	,9210

* The mean difference is significant at the .05 level.

4.2 MACROSCOPIC EXAMINATION

Neither macroscopic abnormalities nor lesions were detected in shells.

Emaciation with a slight yellowish coloration of flesh, which was of jelly consistence, was noticed in one mussel, in all the others there were no macroscopically visible changes.

A mussel with altered flesh was sampled in shellfish farm in Seča in August; the length of the mussel was 7.8 cm (average length of cultured mussel was 7 cm), the total weight 13.9 g (average total weight of cultured mussels was 15 g), the weight of the flesh 2 g (average weight of flesh in cultured mussels was 4.15 g) and the condition index 14.39 (the average condition index in cultured mussels was 28.1). On the basis of the weight of the flesh and the condition index we concluded that the mussel was severely emaciated.

4.3 HISTOPATHOLOGICAL EXAMINATION

A microscopic examination of digestive glands of all 1280 collected mussels revealed the presence of *Marteilia* spp. in 4 mussels, intracellular ciliates of mussels in 293 mussels and haemocytic neoplasia of mussels in 14 mussels.

Neither protozoa nor neoplasias nor inflammation were detected in the severely emaciated mussel.

4.3.1 Marteilia spp.

4.3.1.1 MORPHOMETRIC CHARACTERISTICS, LOCATION AND THE INTENSITY OF THE INFECTION

Four out of 1280 mussels were positive for protozoa of genus *Marteilia*.

In all four cases the infection was severe: in one mussel just above 10% of infected digestive tubules were noticed, in one mussel up to 90% of digestive tubules were infected and in two mussels all digestive tubules were infected.

Different stages of the life cycle of *Marteilia* spp. were seen in the epithelial cells of the digestive gland ducts and in the epithelial cells of the digestive gland tubules of all infected mussels. Early stages, prevailing in the two lesser infected

mussels, consisted of spherical to elongated multinucleated cells, up to 12 µm in length, predominately nested in the epithelial cells of digestive ducts. Numerous pseudoplasmodia (sporangiosorus or primary cells) from 13.3 µm to 21.8 µm in diameter, enclosing 8 spherical sporonts (secondary cells) from 4.7 µm to 7.2 µm in diameter, were seen in the epithelial cells of digestive gland tubules and ducts and inside their lumina in all infected mussels. Each sporont contained 2 to 4 round spores (tertiary cells) from 1 µm to 2.1 µm in diameter and light refractile inclusion bodies. Some free spores were also seen in the lumina of digestive gland tubules in all infected mussels.

4.3.1.2 PCR AND RFLP

All four histologicaly detected *Marteilia* spp. positive mussels tested positive also performing PCR - in all infected mussels *Marteilia refringens* was determined. The reaction was weakly positive in mussel with more than 10% of infected digestive tubules and in mussel with up to 90% of infected digestive tubules and strongly positive in mussels in which all digestive tubules were infected.

RFLP revealed that all *Marteilia refringens* belonged to the type M.

4.3.1.3 HISTOLOGICAL CHANGES IN INFECTED MUSSELS AND CROSS INFECTIONS

Sporadic disrupted epithelial cells of digestive tubules and single necrotic digestive tubules were observed in all infected mussels. In the less infected mussel a moderate diffuse haemocytic infiltration with focally distributed granulocytomas in digestive gland connective tissue was observed and in the mussel with up to 90% of infected digestive tubules a mild haemocytic infiltration was present. In the two mussels with all infected tubules there were no alterations in connective tissue. There was no cross infection with intracellular ciliates of mussels in any of mussel, infected with *Marteilia refringens* type M. No haemocytic neoplasia was detected in any of infected mussel.

4.3.1.4 EPIDEMIOLOGY

Protozoa *Marteilia refringens* were detected in 4 of 1280 samples, which represented a 0.3% prevalence of infection.

All four infected mussels were from shellfish farms: 2 from Seča and 2 from Strunjan, all 320 examined wild mussels from Piran were *Marteilia* spp. free. The prevalence of infection was 0.4% in cultured mussels and 0% in wild mussels respectively.

The first two *Marteilia refringens* positive mussels were detected in Strunjan in December (1.25% prevalence of infection) and in January (1% prevalence of infection), the next two in Seča in February (1% prevalence of infection) and in August (0.8% prevalence of infection). In the other months of the year all examined mussels were uninfected. More than 10% of digestive tubules were invaded by *Marteilia refringens* in December, up to 90% in February and 100% in January and August.

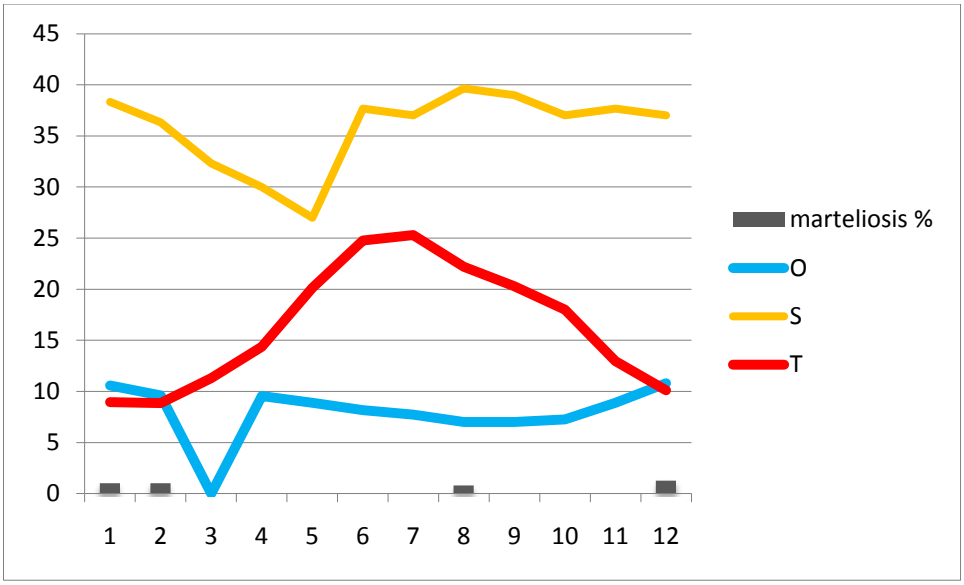
Marteilia refringens was detected three times in winter when the sea temperature was 10 ^{o}C or even lower, the oxygenation above 10 mg/l and the salinity equal to or above 37‰, and only once was noticed in the high summer, when the sea temperature was 22.2 ^{o}C, oxygenation 7 mg/l and salinity 39‰.

The precise data concerning the sea temperature, oxygenation and salinity in the months, in which the positive mussels were sampled, are shown in the table 10 below.

Table 10: Sea data, i. e. temperature (T), oxygenation (O) and salinity (S) in the months in which Marteilia refringens was detected

***Marteilia* refringens positive sample**	**Month**	**T (^{o}C)**	**O (mg/l)**	**S (‰)**
one – 100 % of infected tubules	January	9	10.3	38
one – up to 90 % of infected tubules	February	9.2	10.05	37
one – 100 % of infected tubules	August	22.2	7	39
one – above 10 % of infected tubules	December	10.7	10.64	39

Graph 2: Prevalence of marteiliosis in different months in correlation with sea temperature, salinity and oxygenation



No statistically significant differences – using chi-square method – in infection with *Marteilia refringens* between the three different locations, between cultured and wild mussels, between different months and sea temperatures, oxygenation and salinities were detected. The result was most probably the consequence of the fact, that the number of *Marteilia refringens* positive mussels was too small to enable reliable statistical evaluation.

4.3.1.5 THE CONDITION INDEX OF MUSSELS, INFECTED WITH *Marteilia refringens*

The condition index of infected mussels, sampled in January was 28.3, in February 23.71, in August 26.54 and in December 24.19. The average condition index of mussels, infected with *Marteilia refringens* was 25.68, whereas the average condition index of *Marteilia* spp. free mussels was 28.13. The average length of infected mussels was 7.1 cm (the average length of uninfected mussels was 7 cm) and the average weight was 13.47 g (the average weight of uninfected mussels was 15 g).

The average condition index in infected mussels was slightly lower than in uninfected mussels, but the number of infected mussels was too small to enable statistical testing.

4.3.2 INTRACELLULAR CILIATES OF MUSSELS

4.3.2.1 MORPHOMETRIC CHARACTERISTICS, LOCATION AND THE INTENSITY OF THE INFECTION

Two hundred ninety three mussels (293) of 1280 mussels tested positive for intracellular ciliates of mussels.

The ciliates were pear or spindle-shaped, 3.9 µm to 11.5 µm long and 2.9 µm to 8.4 µm in width. They had a polymorphic oval to globular basophilic, fragmented macronucleus, stained deep blue. They were mostly found inside the digestive tubule epithelia, only few of them were in the lumina of digestive tubules.

A mild infection was detected in 218 mussels (74.4% of infected mussels), a moderate infection in 44 (15% of infected mussels) and a severe infection in 31 mussels (10.6% of infected mussels). The intensity of the infection with intracellular ciliates in all three sampling locations and total values are presented in the table 11 below.

Table 11: Intensity of infection with intracellular ciliates in all three sampling locations and total values

sampling locations	the intensity of infection		
	mild (n)	moderate (n)	severe (n)
Seča	93	25	13
Strunjan	86	9	8
Piran	39	10	10
total (n)	**218**	**44**	**31**
total (%)	**74.4**	**15**	**10.6**

In mussels, where mild infection (<50 ciliates per section) was noticed, there was not more than one ciliate in the epithelial cell of the infected digestive tubule.

In cases of moderate infection (from 50 to 100 ciliates per section) there were mostly more than one ciliate in the infected digestive tubule and some parasites

were also noticed in the lumina of infected digestive tubules. Only one ciliate was noticed inside a single epithelial cell.

In cases of severe infection (>100 ciliates per section) there was more than one ciliate in the epithelia of the infected digestive tubule, with many also in their lumina and in some of them more than one ciliates was found in a single epithelial cell. The distribution of ciliates was quite uniform throughout the digestive gland tubules in severely infected mussels, with the exeption of some mussels where ciliates inhabited only few digestive tubules but in great number. In the most severely infected mussels more than 1000 ciliates were counted in the digestive gland and all digestive tubules were infected: up to 91 ciliates were counted in a single digestive tubule and up to 3 ciliates inhabited a single infected epithelial cell.

4.3.2.2 Histological changes in infected mussels and cross infections

A slight enlargement of epithelial cells that contained ciliates of a large size was observed. A mild diffuse haemocytic infiltration in digestive gland connective tissue was present in 6 (2%) infected mussels. In three mussels (1%) a haemocytic neoplasia of mussels was noticed.

No cross infection with *Marteilia* spp. was detected in any of mussels infected with intracellular ciliates.

4.3.2.3 Epidemiology

The infection with intracellular ciliates

Intracellular ciliates of mussels were detected in the digestive gland of 293 mussels, which represented a 22.9% prevalence of infection.

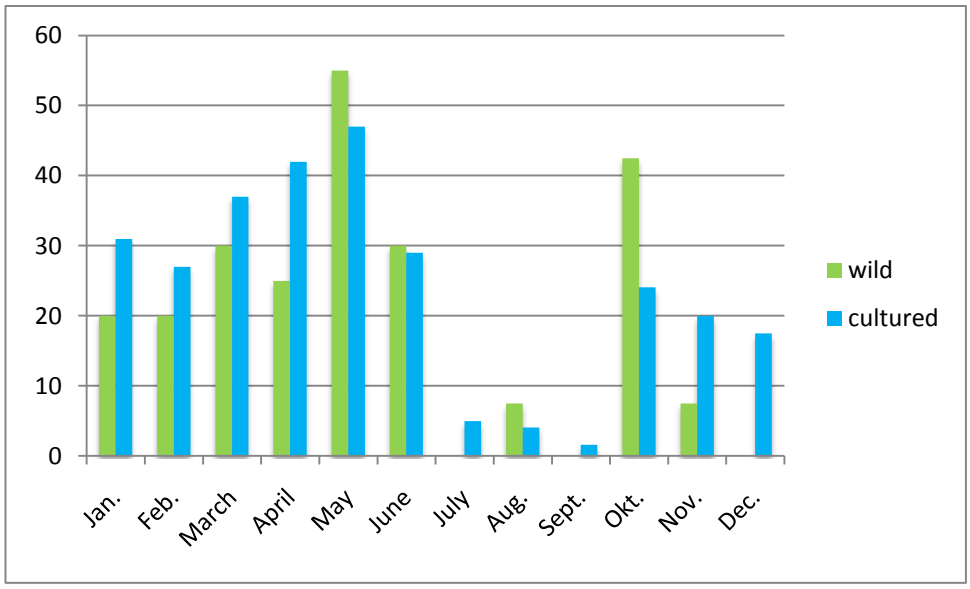
In Seča the prevalence of infection was 27.3%, in Strunjan 21.5% and in Piran 18.4%.[2] The difference between three different locations and infection with

[2] To identify relations between nominal variables we used chi-square based statistical coefficient Cramer's V. The coefficient ranges from 0 (where there is no relationship between the variables) to 1 (in the case of perfect matching between the variables). The significance value, which is also required to interpret the coefficient stands for the probability of obtaining the results that have been obtained if the null hypothesis was true. The research hypothesis that a relationship exists between the variables can be confirmed whenever the significance value is not higher than 0.05.

intracellular ciliates were statistically detectable but not relevant (Sig = 0.009; V = 0.08).

The prevalence of infection was 24.4% in cultured mussels and 18.4% in wild mussels. The differences between cultured and wild mussels and infection with intracellular ciliates was statistically detectable but not relevant (Sig = 0.029; V = 0.06).

Graph 3: Prevalence of intracellular ciliates in wild and cultured mussels by months



A pick in the infection with intracellular ciliates of mussels was detected in April and May (55%) in Seča, in January (45%) in Strunjan and in May (55%) in Piran. The lowest prevalence, detected in Seča was in September (2.5%), in Strunjan in August and in Piran in July and September, when there were no infected mussels. The average prevalence of all three locations was the highest in May (47%) and the lowest in September (1.6%).

The prevalence of infection with intracellular ciliates of mussels in different months in all three sampling locations is shown in the table 12 below.

Table 12: Prevalence of infection with intracellular ciliates by months and sampling locations

	location %			
month	**SEČA**	**STRUNJAN**	**PIRAN**	**average**
January	22.5	45	20	**31**
February	32.5	25	20	**27**
March	47.5	30	30	**37**
April	55	37.5	25	**42**
May	55	35	55	**47**
June	22.5	35	30	**29**
July	10	5	0	**5**
August	5	0	7.5	**4.1**
September	2.5	2.5	0	**1.6**
October	25	5	42.5	**24.1**
November	30	22.5	7.5	**20**
December	20	15	no sampling	**17.5**
average	**27.3**	**21.5**	**18.4**	**22.9**

Intracellular ciliates were more frequently present in spring, after that their number declined rapidly to reach their lowest prevalence in summer months. In autumn their number started to rise again and reached the half the values observed in the winter months. Statistically significant differences among different months concerning the presence of infection with intracellular ciliates were confirmed (Sig = 0.000; V = 0.342).

In one infected mussel, sampled in Seča in March and in two, sampled in Strunjan in May haemocytic neoplasia of mussels was also observed.

At Seča, at the very peak of the infection with intracellular ciliates the sea temperatures were 13.2 ^{o}C and 20 ^{o}C, the oxygenation 9.29 mg/l and 9.06 mg/l and the salinities 32‰ and 25‰. In the month of the lowest detected infection the temperature was 20 ^{o}C, the oxygenation 6.8 mg/l and the salinity 39‰. Data concerning the sea temperature, oxygenation and salinity at the time of sampling and the prevalence of infection is shown in the table 13 below.

Table 13: Sea temperature (T), oxygenation (O) and salinity (S) at sampling by months and percentage of intracellular ciliates of mussels at Seča

month	T (^{o}C)	O (mg/l)	S (‰)	ciliates
January	9.1	10.67	39	22.5
February	9.2	10.05	37	32.5
March	11.2	no data	32	47.5
April	13.2	9.29	32	55
May	20	9.06	25	55
June	24.2	8.3	38	22.5
July	24.9	7.5	36	10
August	22.2	7	39	5
September	20.3	6.8	39	2.5
October	17.8	7	36	25
November	13.1	8.53	37	30
December	9.5	10.95	35	20

The highest prevalence of infection with intracellular ciliates at Strunjan was detected when the sea temperature was 9 ^{o}C, the oxygenation 10.3 mg/l and the salinity 38‰. Uninfected mussels were detected when the temperature was 22.4 ^{o}C, the oxygenation 6.9 mg/l and the salinity 40‰. Data concerning the sea temperature, oxygenation and salinity at the time of sampling and the prevalence of infection are shown in table 14 below.

Table 14: Sea temperature (T), oxygenation (O) and salinity (S) at sampling by months and percentage of intracellular ciliates of mussels at Strunjan

month	T (^{o}C)	O (mg/l)	S (‰)	ciliates
January	9	10.3	38	45
February	8.9	8.53	35	25
March	11	no data	33	30
April	14.9	9.59	28	37.5
May	20.2	8.82	28	35
June	25.1	8	37	35
July	25.6	7.9	38	5
August	22.4	6.9	40	0
September	20.3	7.1	39	2.5
October	17.8	7.1	38	5
November	12.8	9	39	22.5
December	10.7	10.64	39	15

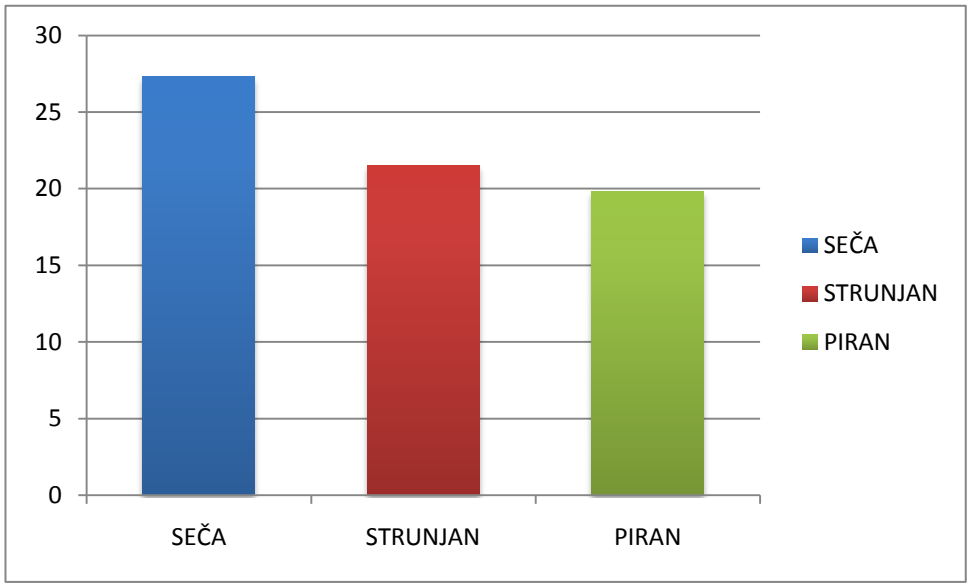
The highest prevalence of infection with intracellular ciliates at Piran was detected when the sea temperatures was 20 ^{o}C, the oxygenation 9.53 mg/l and the salinity 27‰. No infection was detected in July and September when the temperature was 25.4 ^{o}C and 20.3 ^{o}C, the oxygenation 7.8 mg/l and 7.1 mg/l and the salinity 37‰ and 39‰. Data concerning the sea temperature, oxygenation and salinity at the time of sampling and the prevalence of infection are shown in the table 15 below.

Table 15: Sea temperature (T), oxygenation (O) and salinity (S) at sampling by months and percentage of intracellular ciliates of mussels at Piran

				%
month	**T (^{o}C)**	**O (mg/l)**	**S (‰)**	**ciliates**
January	8.8	10.79	38	20
February	8.5	10.23	37	20
March	11.7	no data	32	30
April	15	9.76	30	25
May	20	9.53	27	55
June	25	8.2	38	30
July	25.4	7.8	37	0
August	22.2	7.1	40	7.5
September	20.3	7.1	39	0
October	18.4	7.7	37	42.5
November	13	9.11	37	7.5
December	no sampling			

The prevalence of intracellular ciliates was the lowest in the months with the highest sea temperatures and salinity and the lowest oxygenation.

Graph 4: Prevalence of intracellular ciliates in different sampling locations



Graph 5: Prevalence of intracellular ciliates in different sampling locations by month

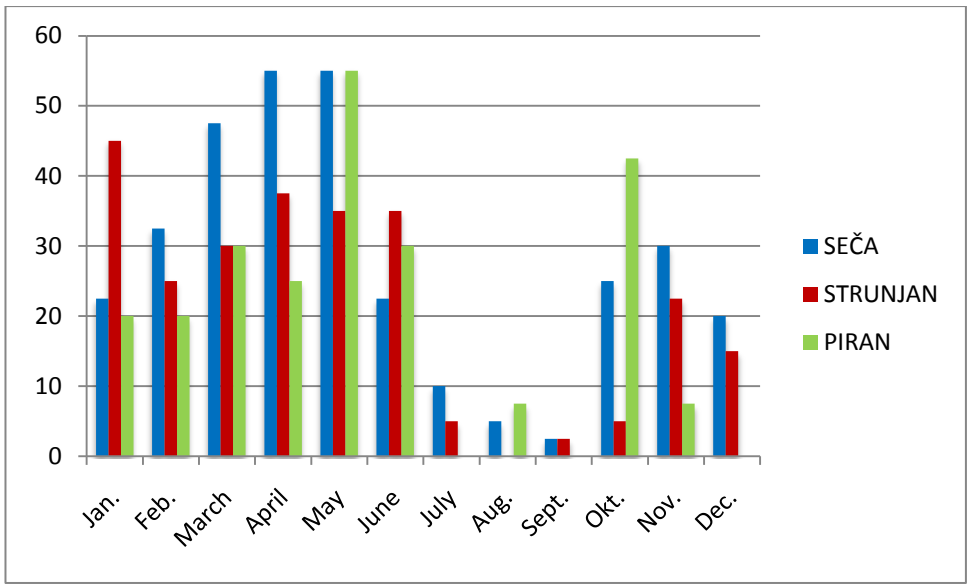


Table 16: T-test concerning the hypothetical relation between temperature, salinity and oxygenation and intracellular ciliates of mussels

Independent Samples Test										
		Levene's Test for Equality of Variances		**t-test for Equality of Means**						
		F	**Sig.**	**t**	**df**	**Sig. (2-tailed)**	**Mean Difference**	**Std. Difference**	**95% Confidence Interval of the Difference**	
									Lower	**Upper**
temperature	**Equal variances assumed**	11,140	,001	5,238	1278	,000	1,99503	,38090	1,24778	2,74228
	Equal variances not assumed			5,491	515,730	,000	1,99503	,36335	1,28120	2,70886
oxygenation	**Equal variances assumed**	37,697	,000	-7,019	1178	,000	-,63144	,08997	-,80795	-,45492
	Equal variances not assumed			-7,926	494,662	,000	-,63144	,07967	-,78796	-,47491
salinity	**Equal variances assumed**	71,241	,000	10,127	1278	,000	2,51128	,24798	2,02480	2,99777
	Equal variances not assumed			8,905	405,024	,000	2,51128	,28201	1,95689	3,06567

arrows indicate the most relevant values

In order to test the isolated (partial) effects of temperature, oxygenation and salinity on the presence of infection we performed a binary logistic regression, which enables us to determine both the existence and the intensity of the influence of temperature, oxygenation and salinity on the presence of infection. This implies the construction of a statistical model (equation) to enable predictions about the probability of the infection for certain levels of oxygenation, salinity and temperature. For the purpose of this research, however, the model is mostly applied in order to demonstrate which of the three possible factors actually contribute toward a greater probability of infection.

The logistic regression model can be summarised as follows:

Table 17: Variables in the Equation

Step 1	**B**	**S.E.**	**Wald**	**df**	**Sig.**	**Exp (B)**
T	-,030	,022	1,949	1	,163	,970
O	,181	,099	3,355	1	,067	1,199
S	-,134	,019	51,975	1	,000	,874
Constant	2,406	1,579	2,320	1	,128	11,086

Variable(s) entered on step 1: temperature (T), oxygenation (O), salinity (S).

The relationship between salinity and the infection was statistically significant (Sig = 0.000), whereas the differences in temperature (Sig = 0.163) had no significant effects on the infection. The influence of the oxygenation on the infection was questionable (Sig = 0.067). We established that only salinity had an influence on infection with intracellular ciliates - the higher the salinity, the lower the infection. The changes in salinity are normally connected to changes in temperature and oxygenation, which gives oxygenation only a spurious influence on the infection.

The intensity of the infection with intracellular ciliates

The intensity of infection with intracellular ciliates in the three sampling locations was as follows: a mild infection was detected in 71% of infected mussels at Seča, in 83.5% at Strunjan and in 66% at Piran, a moderate infection in 19% of infected mussels at Seča, in 8.7% at Strunjan and in 17% at Piran and a severe infection in 10% of infected mussels at Seča, in 7.8% at Strunjan and in 17% at Piran. The differences between three different locations and the intensity of the infection with intracellular ciliates were statistically observable but not essential (Sig = 0.005; V = 0.085).

The intensity of infection with intracellular ciliates in cultured and wild mussels was as follow: a mild infection was detected in 76.5% of infected cultured and in 66% of infected wild mussels, a moderate infection in 14.5% of infected cultured and in 17% of infected wild mussels and a severe infection in 9% of infected cultured and in 17% of infected wild mussels. The differences between cultured and wild mussels and the intensity of the infection with intracellular ciliates were not statistically significant.

The mild infection was highest in July, August and September (100%) at Seča, in June, July, September, October and December (100%) at Strunjan and in March, April and November at Piran and lowest in February (53.9%) at Seča, in March and April (66.7%) at Strunjan and in October (23.5%) at Piran. The highest incidence of moderate infection were detected in February (30.7%) at Seča, in April (20%) at Strunjan and in August (33.3%) at Piran. No moderate infection was detected in January, July, August and September at Seča, from June to

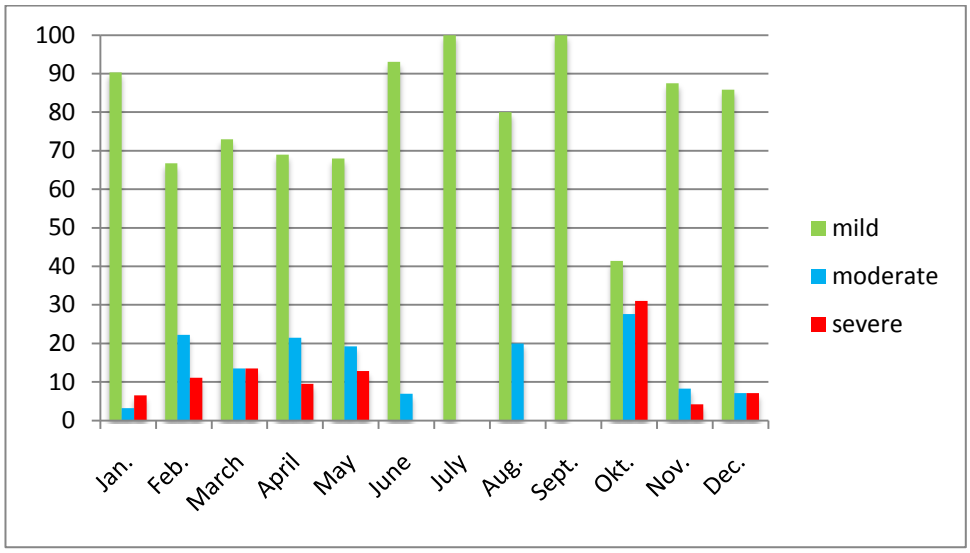
October and in December at Strunjan and in January, March, April, July, September and November at Piran. Severe infection was highest in February (15.4%) at Seča, in March (25%) at Strunjan and in October (47.1%) at Piran. No severe infection was detected from June to September at Seča, in January and from June to December at Strunjan and from February to April and from June to September and in November at Piran.

Precise values of the intensity of the infection with intracellular ciliates throughout the year are shown in the table 18 and graph 6 below.

Table 18: Intensity of the infection with intracellular ciliates by month

months	the intensity of the infection		
	Mild	moderate	severe
January	90.3	3.2	6.5
February	66.7	22.2	11.1
March	73	13.5	13.5
April	69	21.5	9.5
May	68	19.2	12.8
June	93.1	6.9	0
July	100	0	0
August	80	20	0
September	100	0	0
October	41.4	27.6	31
November	87.5	8.3	4.2
December	85.8	7.1	7.1

Graph 6: Intensity of infection with intracellular ciliates by month



A mild infection was highest in the summer months (91% on average) in all three sampling locations, when the infection with intracellular ciliates was lowest, and lowest in the spring (70% on average), when the infection was highest. Moderate and a severe infection showed the opposite pattern: highest in spring (on average 18.1% for moderate and 11.9% for severe infection) and lowest in summer (on average 8.9% for moderate and 0% for severe infection). There is a real possibility of statistically relevant differences between the intensity of the infection with intracellular ciliates and different months. Due to the limitation of the sample size, the significance cannot be established using the chi-square method.

Mild infection was highest when the average sea temperatures and salinity were highest and the oxygenation the lowest. Moderate and severe infection were highest with medium average sea temperature and oxygenation but with the lowest salinity values. A partial correlation between temperature, oxygenation and salinity and the intensity of the infection has been calculated using a partial correlation coefficient to identify the possible effects of temperature, oxygenation and salinity independently of each other. The influence of temperature on the intensity of the infection is inconsiderable (Sig = 0.031; r = 0.063) and the influence of oxygenation is not significant (Sig = 0.835; r = 0.006). The influence of salinity on the other hand is statistically significant (Sig = 0.000; r = - 0.240).

Precise values of the intensity of the infection with intracellular ciliates and sea data, obtained at the time of sampling in all three sampling locations in different months are shown in tables 19, 20 and 21.

Table 19: Intensity of the infection with intracellular ciliates and sea temperature (T), oxygenation (O) and salinity (S), obtained at the time of sampling at Seča in different months

SEČA						
	the intensity of the infection %					
month	**mild**	**moderate**	**severe**	**T (°C)**	**O (mg/l)**	**S (‰)**
January	89	0	11	9.1	10.67	39
February	53.9	30.7	15.4	9.2	10.05	37
March	68.5	21	10.5	11.2	no data	32
April	63.6	27.3	9.1	13.2	9.29	32
May	63.6	22.7	13.7	20	9.06	25
June	89	11	0	24.2	8.3	38
July	100	0	0	24.9	7.5	36
August	100	0	0	22.2	7	39
September	100	0	0	20.3	6.8	39
October	60	30	10	17.8	7	36
November	83.4	8.3	8.3	13.1	8.53	37
December	75	12.5	12.5	9.5	10.95	35
average	**71**	**19**	**10**			

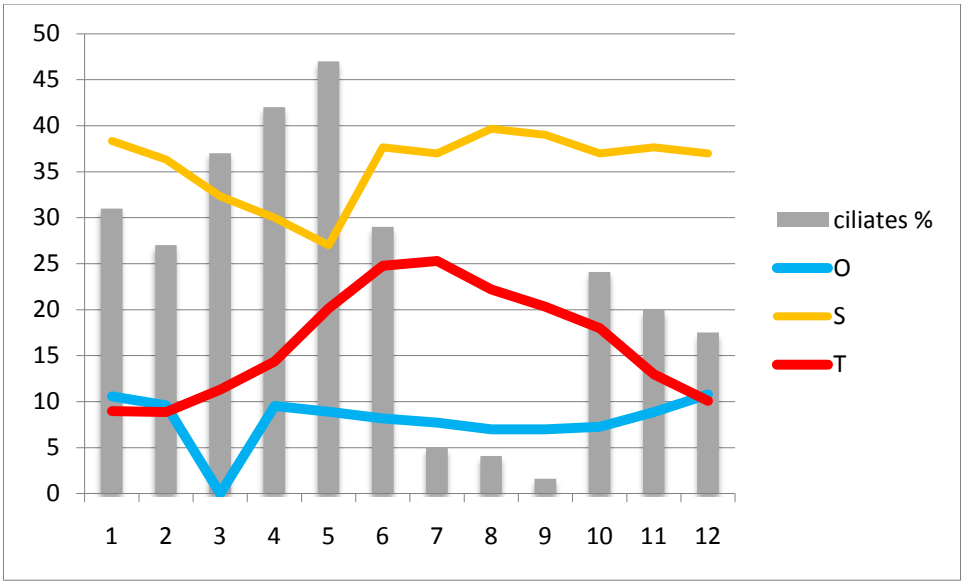
Table 20: Intensity of the infection with intracellular ciliates and sea temperature (T), oxygenation (O) and salinity (S), obtained at the time of sampling at Strunjan in different months

STRUNJAN						
	the intensity of the infection %					
month	**mild**	**moderate**	**severe**	**T (°C)**	**O (mg/l)**	**S (‰)**
January	94.4	5.6	0	9	10.3	38
February	80	10	10	8.9	8.53	35
March	66.7	8.3	25	11	no data	33
April	66.7	20	13.3	14.9	9.59	28
May	71.4	14.3	14.3	20.2	8.82	28
June	100	0	0	25.1	8	37
July	100	0	0	25.6	7.9	38
August	0	0	0	22.4	6.9	40
September	100	0	0	20.3	7.1	39
October	100	0	0	17.8	7.1	38
November	89	11	0	12.8	9	39
December	100	0	0	10.7	10.64	39
average	**83.5**	**8.7**	**7.8**			

Table 21: Intensity of the infection with intracellular ciliates and sea temperature (T), oxygenation (O) and salinity (S), obtained at the time of sampling at Piran in different months

PIRAN						
	the intensity of the infection %					
month	**mild**	**moderate**	**severe**	**T (°C)**	**O (mg/l)**	**S (‰)**
January	75	0	25	8.8	10.79	38
February	75	25	0	8.5	10.23	37
March	100	0	0	11.7	no data	32
April	100	0	0	15	9.76	30
May	72.7	18.2	9.1	20	9.53	27
June	83.3	16.7	0	25	8.2	38
July	0	0	0	25.4	7.8	37
August	66.7	33.3	0	22.2	7.1	40
September	0	0	0	20.3	7.1	39
October	23.5	29.4	47.1	18.4	7.7	37
November	100	0	0	13	9.11	37
December	no sampling					
average	**66**	**17**	**17**			

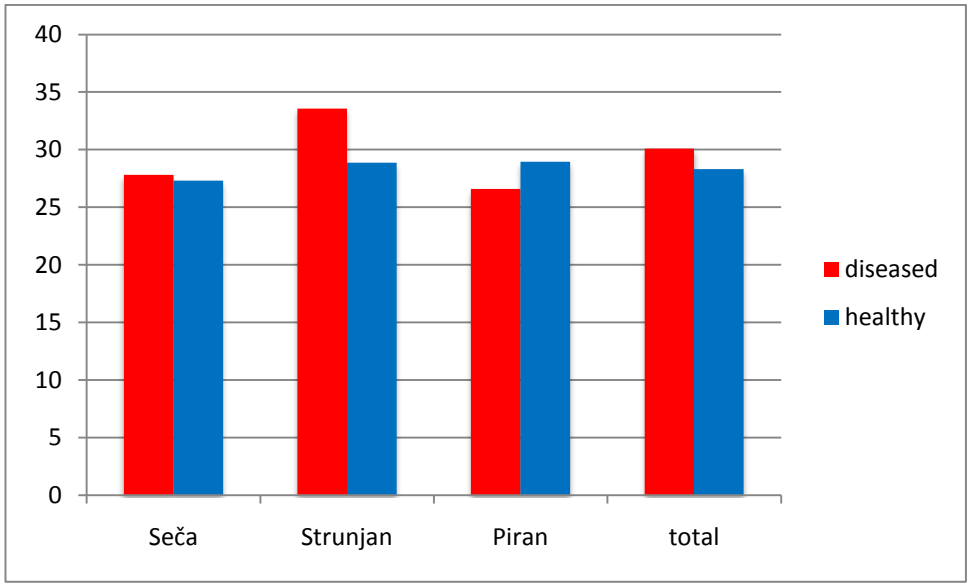
Graph 7: Prevalence of intracellular ciliates in different months in correlation with sea temperature, salinity and oxygenation



4.3.2.4 THE CONDITION INDEX OF MUSSELS INFECTED WITH INTRACELLULAR CILIATES OF MUSSELS

The average condition index of mussels, infected with intracellular ciliates was slightly higher than in healthy ones: 28.77 in infected compared to 28.28 in healthy mussels. The average condition index in infected cultured mussels was 28.63 and in healthy cultured mussels 27.96. The average condition index in infected wild mussels was 29.05 and in healthy wild mussels 28.93.

Graph 8: Condition index in healthy and in ciliated infected cultured and wild mussels



The condition indexes in infected and healthy mussels in all three sampling locations and the total condition index value are seen in table 22 below.

Table 22: Condition index (CI) of infected and healthy mussels at Seča, Strunjan and Piran

CI	health status	
location	**infected**	**healthy**
Seča	28.32	26.99
Strunjan	28.94	28.94
Piran	29.05	28.93
total	**28.77**	**28.28**

The condition index in mussels, infected with the intracellular ciliates of mussels atSeča and Piran was slightly higher than in healthy mussels. The condition index in mussels, collected at Strunjan was identical in infected and in healthy ones.
In all months, with the exception of January, May, June and August, the condition index was higher in mussels infected with intracellular ciliates of mussels. The condition indexes in infected and uninfected mussels throughout the year are presented in the table 23 below.

Table 23: Condition indexes of infected and uninfected mussels throughout the year

	condition index	
month	**infected**	**healthy**
January	30.72	32.58
February	33.91	33.54
March	33.9	33.06
April	26.29	26,29
May	30.13	30.27
June	26.12	27.6
July	28.7	28.3
August	23.74	25.4
September	28.46	27.33
October	28.10	27.27
November	25.85	25.38
December	26.15	24.79

We performed a t-test to test whether there are significant differences between infected and uninfected mussels in respect of condition index, length and weight. Intracellular ciliates of mussels did not have any effect on the condition index of infected mussels (sig = 0,250). However a slight connection was observed in relation to the infection status, length and total weight of the mussels as well as the weight of the flesh. Ciliates were most often detected in longer and heavier mussels.

Table 24: The relationship between mussels' characteristics (length, total weight, weight of the flesh and condition index) and the infection of ciliates

Group Statistics

	CILIATES	N	Mean	Std. Deviation	Std. Error Mean
length	no	987	6,969	1,1028	,0351
	yes	293	7,199	1,2709	,0742
total weight	no	987	15,36	6,523	,208
	yes	293	16,40	6,891	,403
weight of the flesh	no	987	4,242	1,7703	,0563
	yes	293	4,613	1,9730	,1153
condition index	no	987	28,2282095	5,53156839	,17607176
	yes	293	28,6570270	5,84010941	,34118283

Table 25: T-test concerning the mussels' characteristics (length, total weight, weight of the flesh and condition index) and the infection of ciliates

Independent Samples Test										
		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Difference	95% Confidence Interval of the Difference	
									Lower	Upper
Lenght	Equal variances assumed	1,565	,211	-3,019	1278	,003	-,2296	,0761	-,3789	-,0804
	Equal variances not assumed			-2,796	430,762	,005	-,2296	,0821	-,3911	-,0682
Total weight	Equal variances assumed	1,252	,263	-2,367	1278	,018	-1,041	,440	-1,903	-,178
	Equal variances not assumed			-2,297	458,361	,022	-1,041	,453	-1,931	-,150
Neto weight	Equal variances assumed	4,988	,026	-3,064	1278	,002	-,3707	,1210	-,6080	-,1333
	Equal variances not assumed			-2,889	440,806	,004	-,3707	,1283	-,6228	-,1185
Condition index	Equal variances assumed	,016	,898	-1,150	1278	,250	-,42881750	,37280075	-1,16019	,30255121
	Equal variances not assumed			-1,117	458,609	,265	-,42881750	,38393618	-1,18331	,32567476

* the most relevant values

The average condition index of mussels with a mild infection was 28.97, those with amoderate infection 28.67 and those with a severe infection 27.63. The average condition index of cultured mussels with a mild infection was 28.58 and of wild mussels 29.74, of cultured mussels with moderate infection 28.61 and of wild mussels 28.78 and of cultured mussels with severe infection 28.09 and of wild mussels 26.73. The condition index of the most severely infected mussels, which had more than 1000 ciliates in their digestive glands, was 26.63. The condition indices of mussels with different intensities of infection in three sampling sites, and the average values of condition index are presented in the table 26.

Table 26: Condition index of mussels with different intensities of infection at the three sampling sites and the average values of the condition index

CI	the intensity of infection		
location	**mild**	**moderate**	**severe**
Seča	28.14	29.23	27.14
Strunjan	29.03	28	29.04
Piran	29.74	28.78	26.73
average	**28.97**	**28.67**	**27.63**

The intensity of the infection had no relevant influence on the condition index of infected mussels. Tests were performed using Spearman's correlation coefficient.[3]

[3] Spearman coefficient ranges from -1 (complete negative correlation) to 1 (complete positive correlation), while the values close to 0 indicate the lack of correlation between the variables.

Table 27: Spearman's correlation coefficient

			The intensity of the infection
Spearman's rho	**the intensity of the infection**	**Correlation Coefficient**	1,000
		Sig. (2-tailed)	.
		N	1280
	length	**Correlation Coefficient**	,066(*)
		Sig. (2-tailed)	,018
		N	1280
	total weight	**Correlation Coefficient**	,064(*)
		Sig. (2-tailed)	,023
		N	1280
	weight of the flesh	**Correlation Coefficient**	,067(*)
		Sig. (2-tailed)	,016
		N	1280
	Condition index	**Correlation Coefficient**	,022
		Sig. (2-tailed)	,429
		N	1280

Correlation is significant at the 0.05 level (2-tailed).

** Correlation is significant at the 0.01 level (2-tailed).

The average condition index of mussels, infected with intracellular ciliates and affected with haemocytic neoplasia was 34.51 (the average condition index of mussels, infected with intracellular ciliates was 28.77 and the average condition index of mussels with haemocytic neoplasia was 30.1).

4.3.3 HAEMOCYTIC NEOPLASIA OF MUSSELS

4.3.3.1 MORPHOMETRIC CHARACTERISTIC AND THE DISTRIBUTION OF NEOPLASTIC CELLS

Haemocytic neoplasia was diagnosed in 14 of 1280 mussels.

Neoplastic cells were highly pleomorphic – spherical, oval, spindle and starry, and anysocitotic ranging from 12.3 µm to 30.1 µm in diameter. They had large, hyperchromatic and manly rounded but often also pleomorphic nucleus from 4.3 µm to 22.7 µm in diameter with finely dispersed or dense chromatin without nucleolus. Some bi- and tri-nucleated cells were noticed. The nucleus to

cytoplasm ratio was high. The number of mitoses was low - 2 mitoses per HPF were counted.

In 4 mussels neoplastic cells diffusely infiltrated the connective tissue, blood vessels and sinuses of the visceral mass and gonads (Stage 4) were observed in 2 mussels only small foci of neoplastic cells were noticed in the connective tissue of digestive gland tubules and gonads (Stage 1), whereas in 8 mussels only single neoplastic cells were observed in the vessels and connective tissue of the digestive gland (Stage 1).

4.3.3.2 HISTOLOGICAL CHANGES IN AFFECTED MUSSELS AND OTHER INFECTIONS

Necrosis and multifocal atrophy of digestive tubules were observed in mussels with diffuse neoplasia whereas severe haemocytic infiltration of connective tissue was observed in mussels with single neoplastic cells. No alterations were noticed in mussels with small foci of neoplastic cells.

In 2 mussels with single neoplastic cells and in one mussel with focuses of neoplastic cells a mild infection with intracellular ciliates was observed. In other cases of haemocytic neoplasia there were no intracellular ciliates.

4.3.3.3 EPIDEMIOLOGY

Haemocytic neoplasia of mussels was detected in 14 of 1280 mussels, which represented a 1.1% prevalence.

Six mussels with haemocytic neoplasia were sampled at Seča (1.25% prevalence), 6 at Strunjan (1.25% prevalence) and two at Piran (0.6% prevalence). The prevalence of haemocytic neoplasia was 1.25% in cultured mussels and 0.6% in wild mussels.

Two mussels from Seča were affected with haemocytic neoplasia in March (14.3% of infections), three in May from Strunjan (21.4% of infections), two in June (14.3% of infections) with one from Seča and one from Strunjan, one in July (7.15% of infections) from Strunjan, three in September (21.4% of infections) with one from Seča and two from Piran, two in October (14.3% of infections)

with one from Seča and one from Strunjan and one in December (7.15% of infection) from Seča. In the other months of the year, i.e. January, February, April, August, and November all mussels examined were uninfected. The prevalence of haemocytic neoplasia in different months in all three sampling locations is shown in table 28.

Table 28: Prevalence of haemocytic neoplasia throughout the year at Seča, Strunjan and Piran

	location			sum
month	SEČA (%)	STRUNJAN (%)	PIRAN (%)	
January	0	0	0	0
February	0	0	0	0
March	5	0	0	2
April	0	0	0	0
May	0	7.5	0	3
June	2.5	2.5	0	2
July	0	2.5	0	0.8
August	0	0	0	0
September	2.5	0	5	2.5
October	2.5	2.5	0	1.6
November	0	0	0	0
December	2.5	0	*	1.25

In one infected mussel, sampled at Seča in March and in two, sampled at Strunjan in May a mild infection with intracellular ciliates was also observed.

The haemocytic neoplasia was more frequently observed in spring (5 cases) when the sea temperature was between 11 ^{o}C and 20.2 ^{o}C, the oxygenation below 9 mg/l and the salinity between 28‰ and 32‰ and in autumn (5 cases) when the sea temperature was between 17.8 ^{o}C and 20.3 ^{o}C, the oxygenation below 7.1 mg/l and the salinity between 26‰ and 39‰. Only one affected mussel was detected in winter with the sea temperature 9.5 ^{o}C, the oxygenation 10.95 mg/l and the salinity 35‰. Precise sea data, obtained at the time of sampling in all three sampling locations throughout the year, the number of mussels, affected with the haemocytic neoplasia of mussels the distribution of neoplastic cells and a cross infection with intracellular ciliates are shown in tables 29, 30 and 31.

Table 29: Sea temperature (T), oxygenation (O) and salinity (S) at sampling in different months, the number of cases of haemocytic neoplasia of mussels, the distribution of neoplastic cells and cross infection with intracellular ciliates at Seča

SEČA					
month	**T (°C)**	**O (mg/l)**	**S (‰)**	**number of neoplasia**	**distribution of neoplastic cells and other infections**
January	9.1	10.67	39	0	
February	9.2	10.05	37	0	
March	11.2	no data	32	2	single cells (1), diffuse (1)+ciliates
April	13.2	9.29	32	0	
May	20	9.06	25	0	
June	24.2	8.3	38	1	single cells
July	24.9	7.5	36	0	
August	22.2	7	39	0	
Septembe	20.3	6.8	39	1	single cells
October	17.8	7	36	1	single cells
November	13.1	8.53	37	0	
December	9.5	10.95	35	1	diffuse

Table 30: Sea temperature (T), oxygenation (O) and salinity (S) at sampling in different months, the number of cases of haemocytic neoplasia of mussels, the distribution of neoplastic cells and cross infection with intracellular ciliates at Strunjan

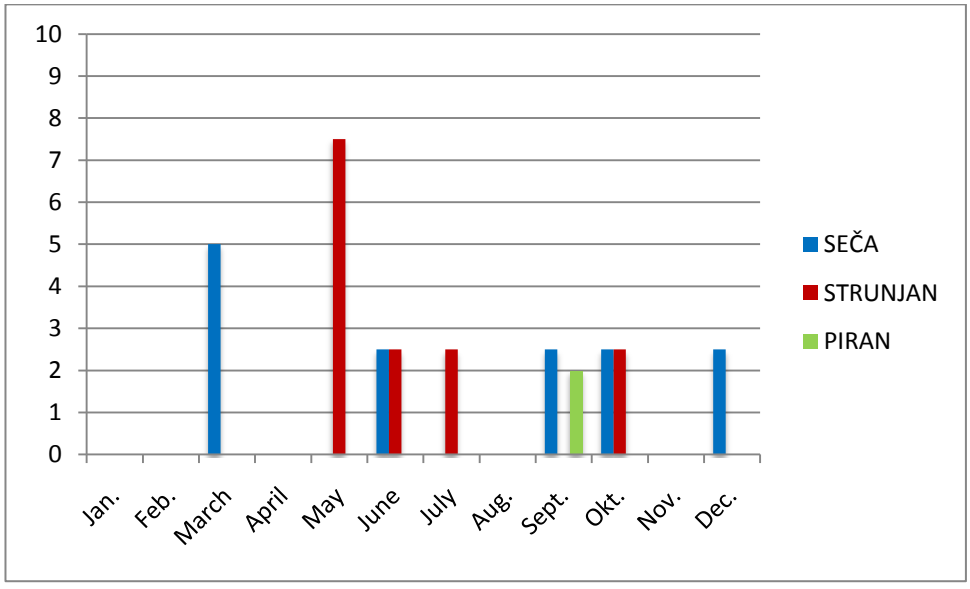
STRUNJAN					
month	**T (°C)**	**O (mg/l)**	**S (‰)**	**number of neoplasia**	**distribution of neoplastic cells and other infections**
January	9	10.3	38	0	
February	8.9	8.53	35	0	
March	11	no data	33	0	
April	14.9	9.59	28	0	
May	20.2	8.82	28	3	single cells (1), diffuse (1), multifocal (1) + ciliates
June	25.1	8	37	1	single cells
July	25.6	7.9	38	1	multifocal
August	22.4	6.9	40	0	
September	20,3	7,1	39	0	
October	17,8	7,1	38	1	diffuse
November	12,8	9	39	0	
December	10,7	10,64	39	0	

Table 31: Sea temperature (T), oxygenation (O) and salinity (S) at sampling in different months, the number of cases of haemocytic neoplasia of mussels and distribution of neoplastic cells at Piran

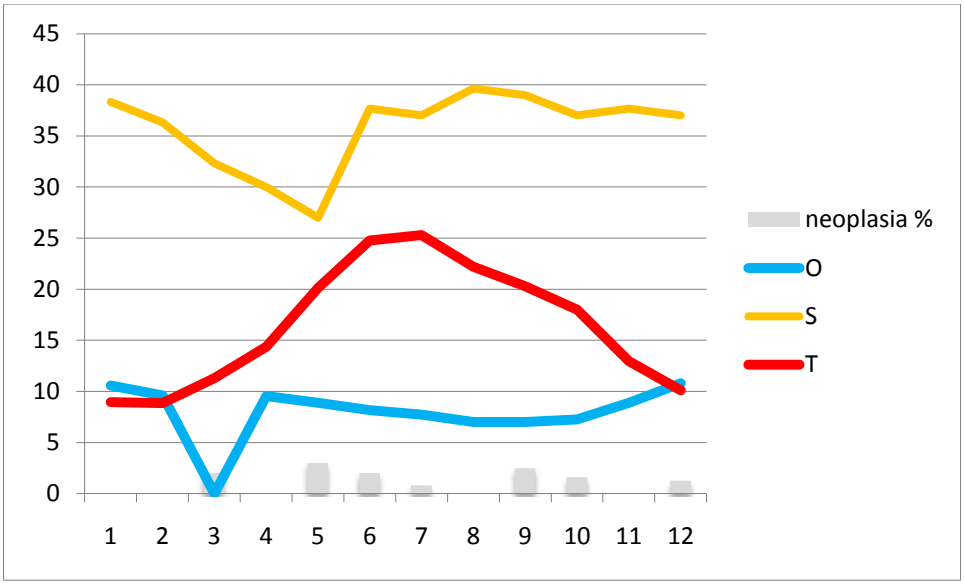
PIRAN					
month	**T (°C)**	**O (mg/l)**	**S (‰)**	**number of neoplasia**	**distribution of neoplastic cells**
January	8.8	10.79	38	0	
February	8.5	10.23	37	0	
March	11.7	no data	32	0	
April	15	9.76	30	0	
May	20	9.53	27	0	
June	25	8.2	38	0	
July	25.4	7.8	37	0	
August	22.2	7.1	40	0	
Septembe	20.3	7.1	39	2	single cells
October	18.4	7.7	37	0	
November	13	9.11	37	0	
December	no sampling				

The distribution of haemocytic neoplasia of mussels in different months in all three sampling locations is shown in the graph 9.

Graph 9: Haemocytic neoplasia in Seča, Strunjan and Piran in different months



Graph 10: Prevalence of haemocytic neoplasia in different months in correlation with sea temperature, salinity and oxygenation

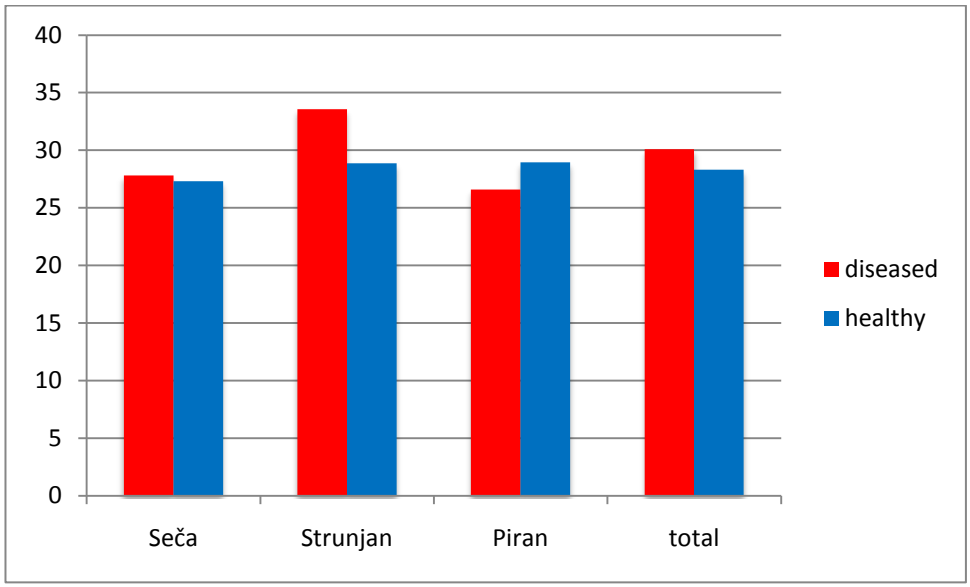


No statistically significant differences in the occurrence of haemocytic neoplasia of mussels between the three different locations, between cultured and wild mussels, between different months and among sea temperatures, oxygenation and salinities were detected. The result was most probably the consequence of the fact, that the number of mussels with haemocytic neoplasia was too small to enable reliable statistical testing.

4.3.3.4 THE CONDITION INDEX OF MUSSELS WITH HAEMOCYTIC NEOPLASIA OF MUSSELS

The average condition index of mussels with haemocytic neoplasia was higher than in healthy ones: 30.1 in diseased and 28.3 in healthy mussels. The average condition index in cultured mussels with haemocytic neoplasia was higher (30.68) than in healthy cultured mussels (28.1). The average condition index of wild mussels with haemocytic neoplasia was lower (26.59) than in healthy wild mussels (28.95).

Graph 11: Condition index of cultured and wild mussels with and without haemocytic neoplasia



The condition index of mussels with haemocytic neoplasia from Seča was just slightly higher than it was in healthy ones; the condition index of diseased mussels, collected at Strunjan was much higher than in healthy ones and the condition index in diseased mussels from Piran was lower than in healthy ones.

The comparison of condition index between mussels with haemocytic neoplasia and healthy mussels in all three sampling locations and the total value is seen in table 32.

Table 32: Comparison of condition index (CI) between infected and healthy mussels at Seča, Strunjan and Piran

CI	health status	
location	**diseased**	**healthy**
Seča	27.8	27.32
Strunjan	33.57	28.88
Piran	26.59	28.95
total	**30.1**	**28.3**

The average condition index of mussels with single neoplastic cells was 30.26, with multifocal form 33.05 and with diffuse form of haemocytic neoplasia 28.3. The lowest condition index (12.5) was detected in a mussel with a diffuse form of haemocytic neoplasia.

The average condition index of mussels with haemocytic neoplasia of mussels was slightly higher than in healthy ones, but the number of affected mussels was too small to enable the reliable confirmation of differences between affected and healthy mussels (t = -0,624; sig = 0,532).

Table 33: Relationship between mussels' characteristics (length, total weight, weight of the flesh and condition index) and haemocytic neoplasia of mussels

Group Statistics

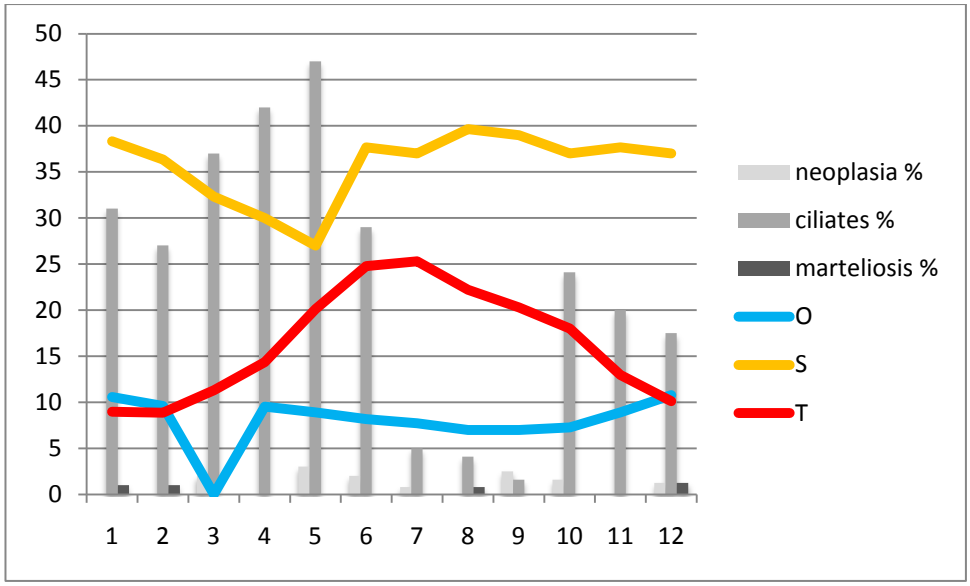
	NH	N	Mean	Std. Deviation	Std. Error Mean
length	no	1266	7,024	1,1495	,0323
	yes	14	6,764	,8975	,2399
total weight	no	1266	15,63	6,639	,187
	yes	14	12,54	3,693	,987
weight of the flesh	no	1266	4,335	1,8295	,0514
	yes	14	3,586	1,1114	,2970
condition index	no	1266	28,3160791	5,59981465	,15738263
	yes	14	29,2568283	6,14315276	1,64182664

Table 34: T-test concerning the mussels' characteristics (length, total weight, weight of the flesh and condition index) and haemocytic neoplasia of mussels

Independent Samples Test										
		Levene's Test for Equality of Variances		**t-test for Equality of Means**						
		F	**Sig.**	**t**	**df**	**Sig. (2-tailed)**	**Mean Difference**	**Std. Difference**	**95% Confidence Interval of the Difference**	
									Lower	**Upper**
Lenght	**Equal variances assumed**	,341	,559	,844	1278	,399	,2601	,3083	-,3447	,8649
	Equal variances not assumed			1,075	13,476	,301	,2601	,2420	-,2609	,7811
Total weight	**Equal variances assumed**	3,150	,076	1,737	1278	,083	3,087	1,778	-,401	6,575
	Equal variances not assumed			3,073	13,946	,008	3,087	1,004	,932	5,242
Weight of the flesh	**Equal variances assumed**	2,376	,123	1,529	1278	,127	,7493	,4901	-,2121	1,7107
	Equal variances not assumed			2,486	13,791	,026	,7493	,3014	,1018	1,3967
Condition index	**Equal variances assumed**	,001	,970	-,624	1278	,532	-,94074924	1,5064223	-3,89608	2,014583
	Equal variances not assumed			-,570	13,240	,578	-,94074924	1,6493526	-4,49740	2,615906

* the most relevant values

Graph 12: Prevalence of marteiliosis, intracellular ciliates and haemocytic neoplasia in different months in correlation with sea temperature, salinity and oxygenation



5 FIGURES

Figure 1. Mussels' farm in Strunjan, Slovenian sea

Figure 2. Mediterranean mussels (Mytilus galloprovincialis) on ropes in Strunjan

Figure 3. Mediterranean mussels (Mytilus galloprovincialis) after collection

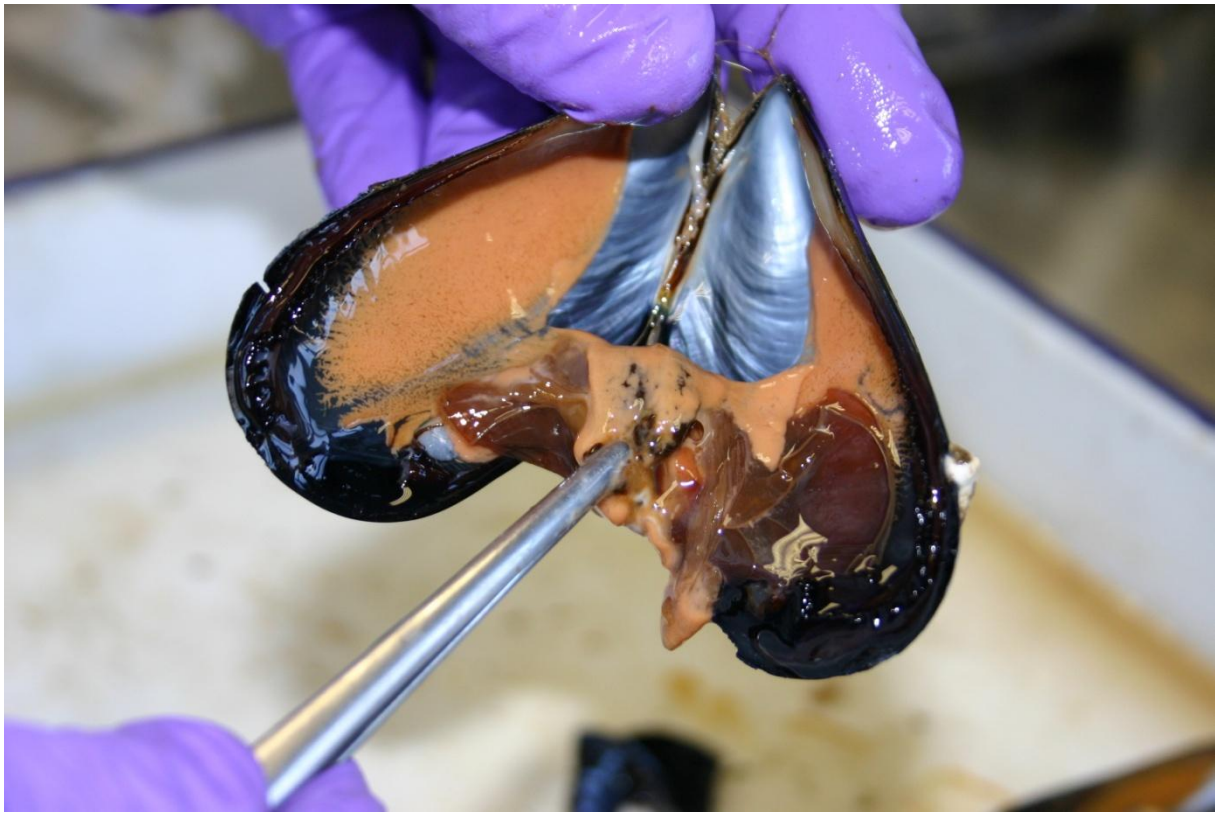


Figure 4. Removal of digestive gland for histopathological examination

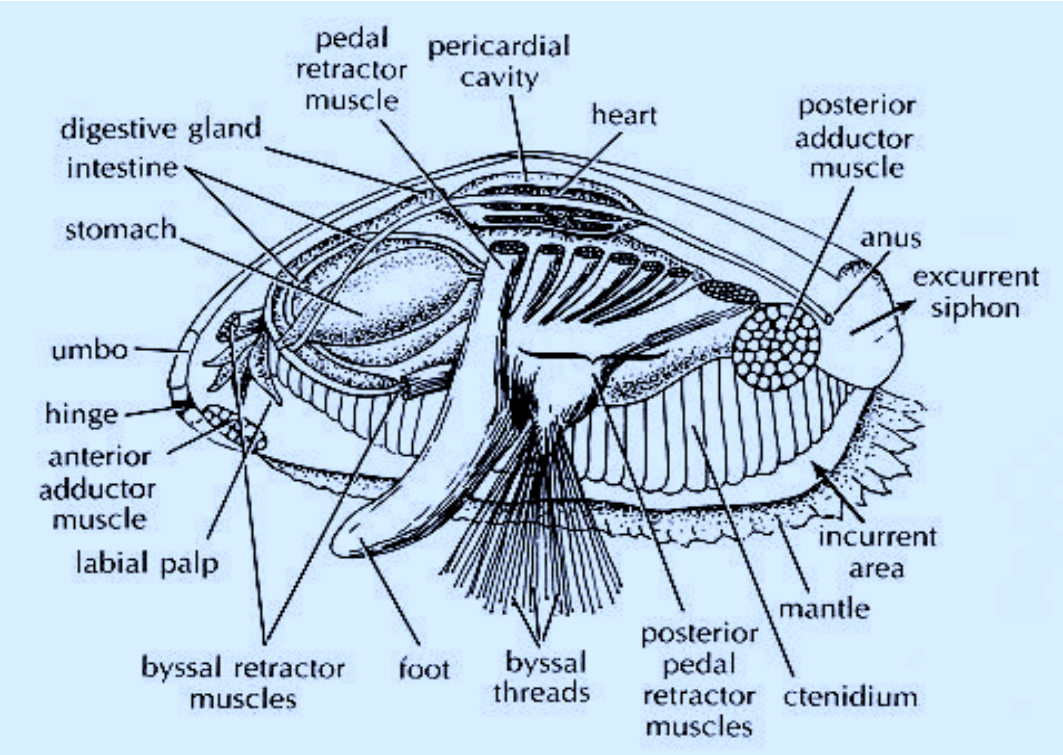


Figure 5. Anatomy of Mediterranean mussel (Mytilus galloprovincialis)

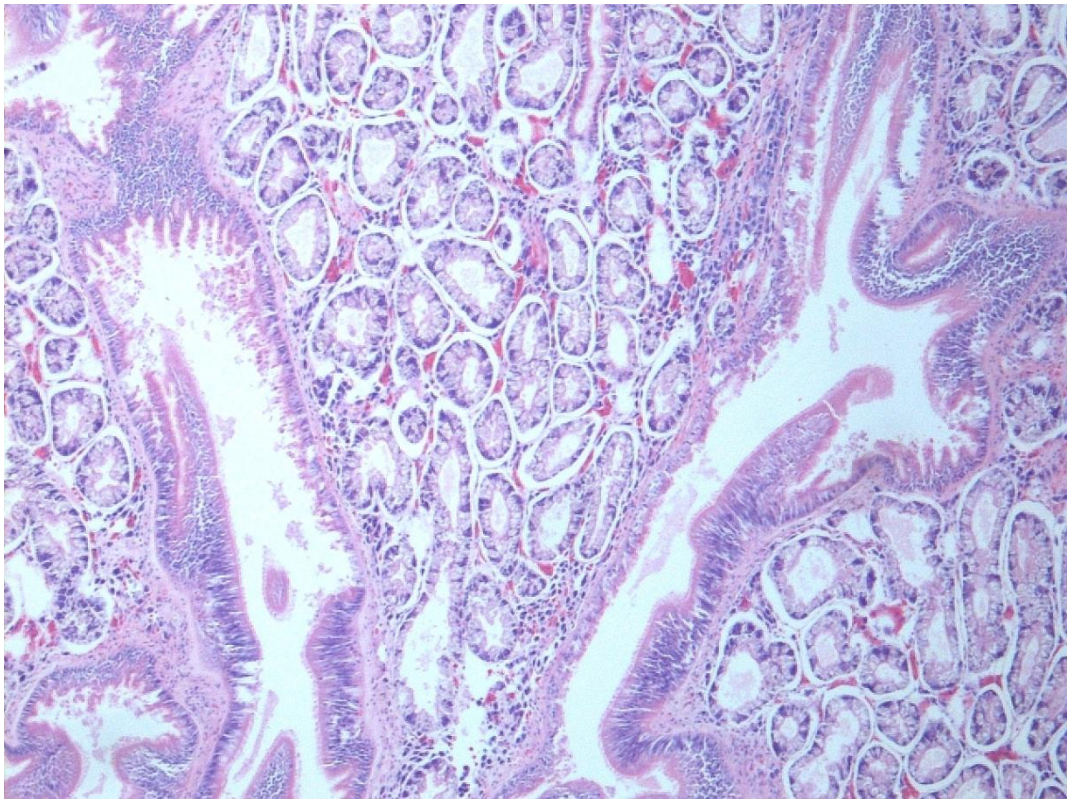


Figure 6. Digestive gland (digestive tubules and ducts) in Mediterranean mussel (Mytilus galloprovincialis), HE staining, x100

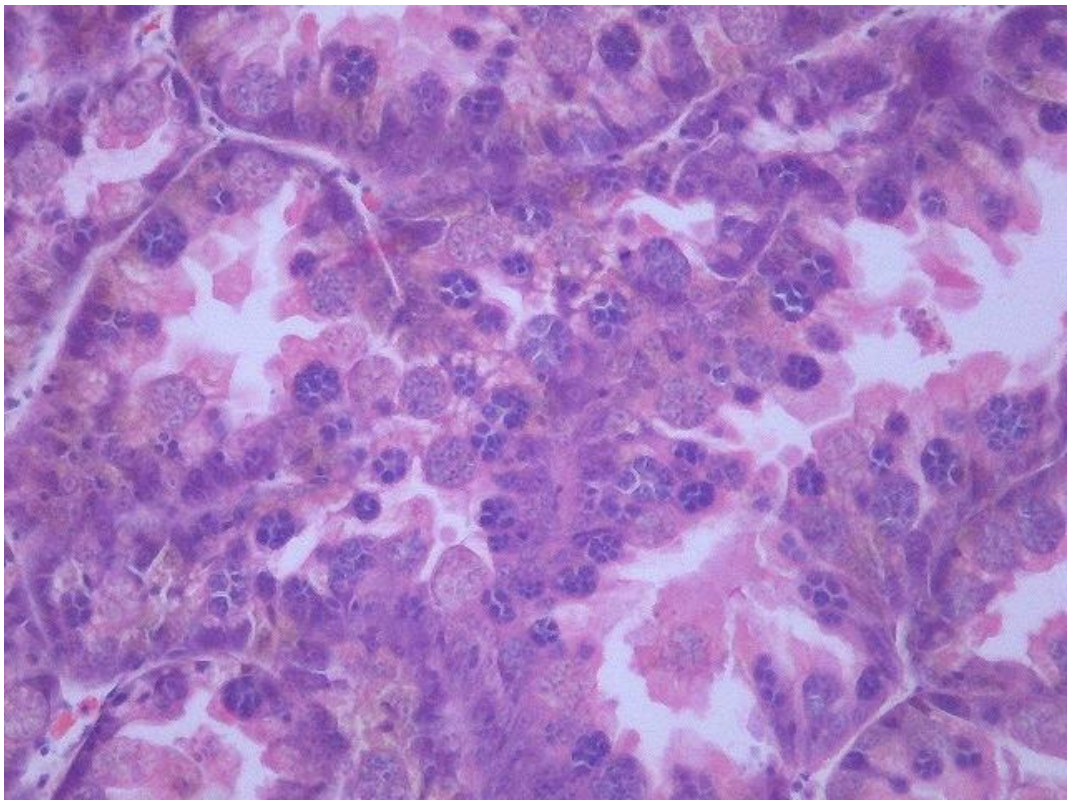


Figure 7. A severe marteiliosis due to Marteilia refringens type M, HE staining, x200

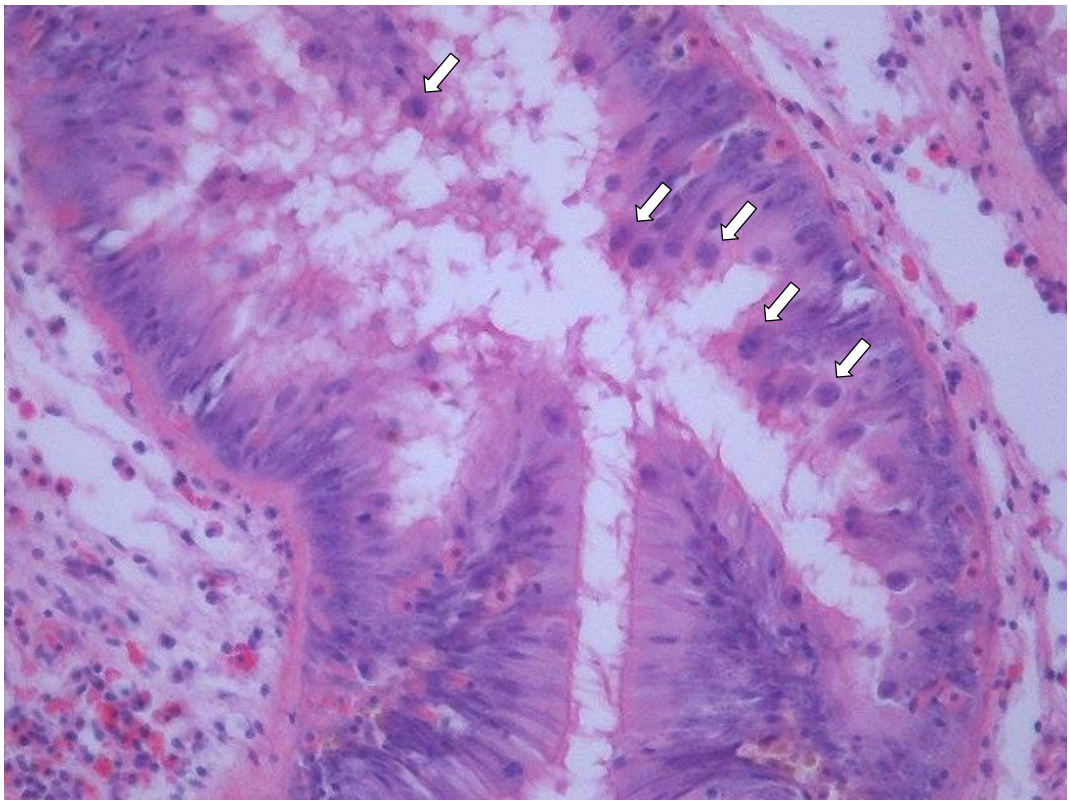


Figure 8. Early stages of Marteilia refringens type M in digestive duct (arrows), HE staining, x200

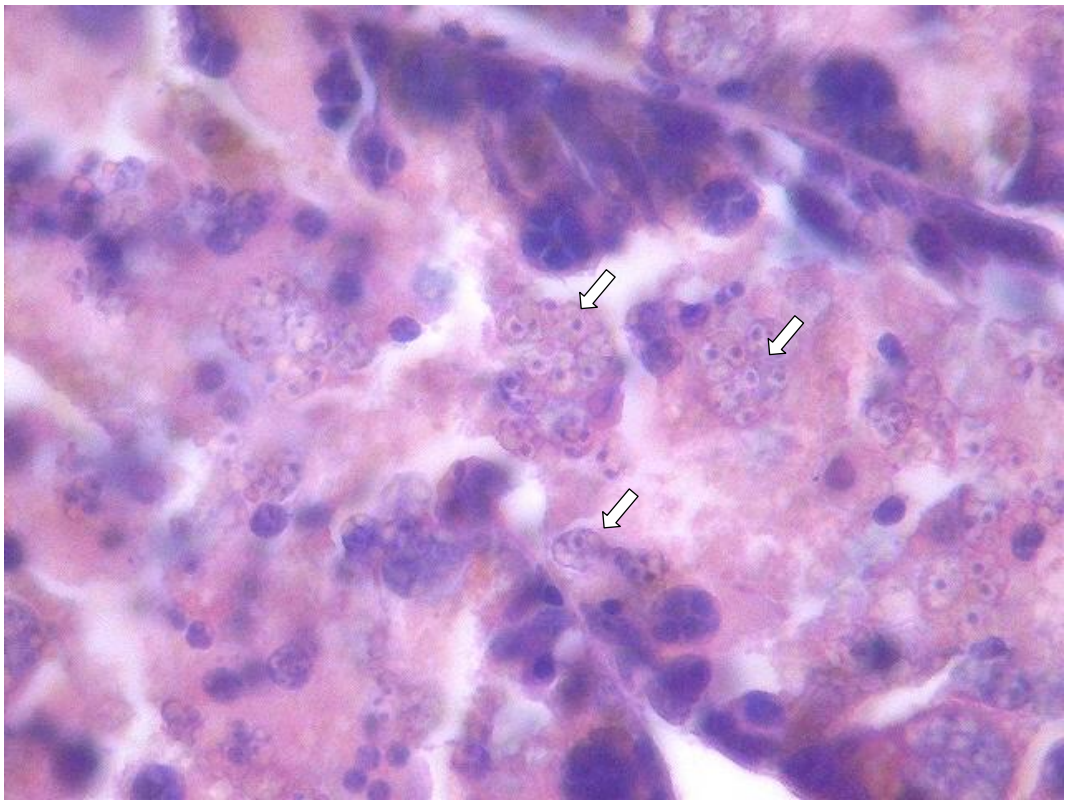


Figure 9. Spores (arrows) of Marteilia refringens type M in digestive gland lumen, HE staining, x400

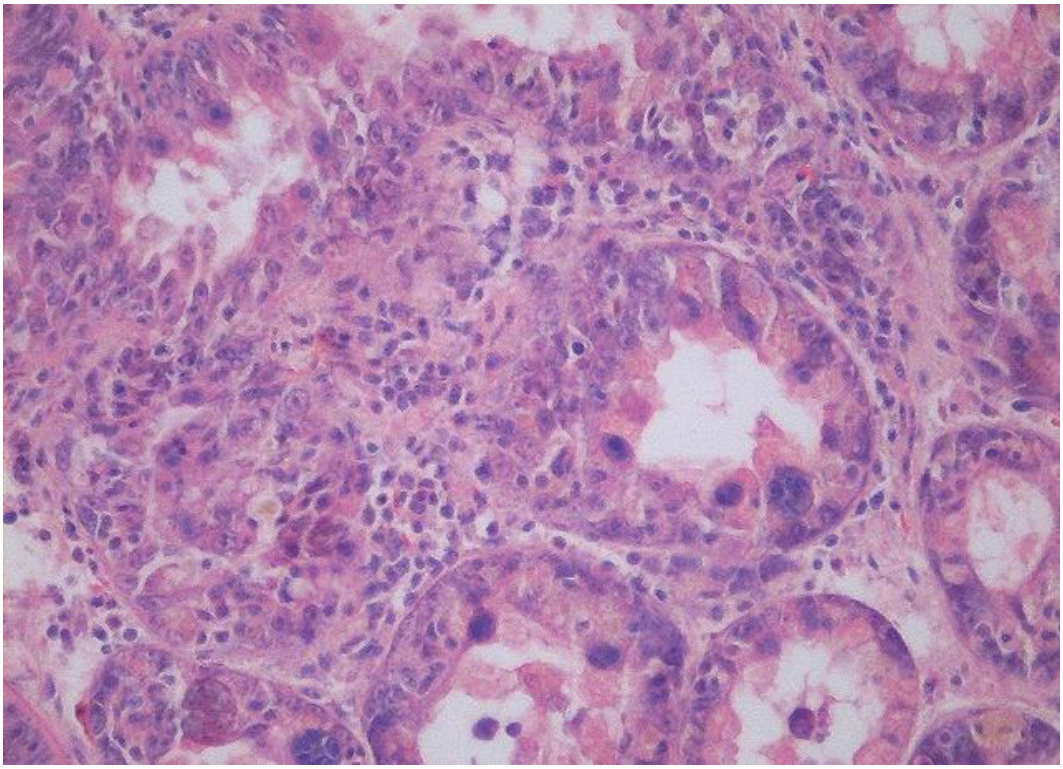


Figure 10. Marteiliosis due to Marteilia refringens type M and mild diffuse haemocytic infiltration of digestive gland, HE staining, x100

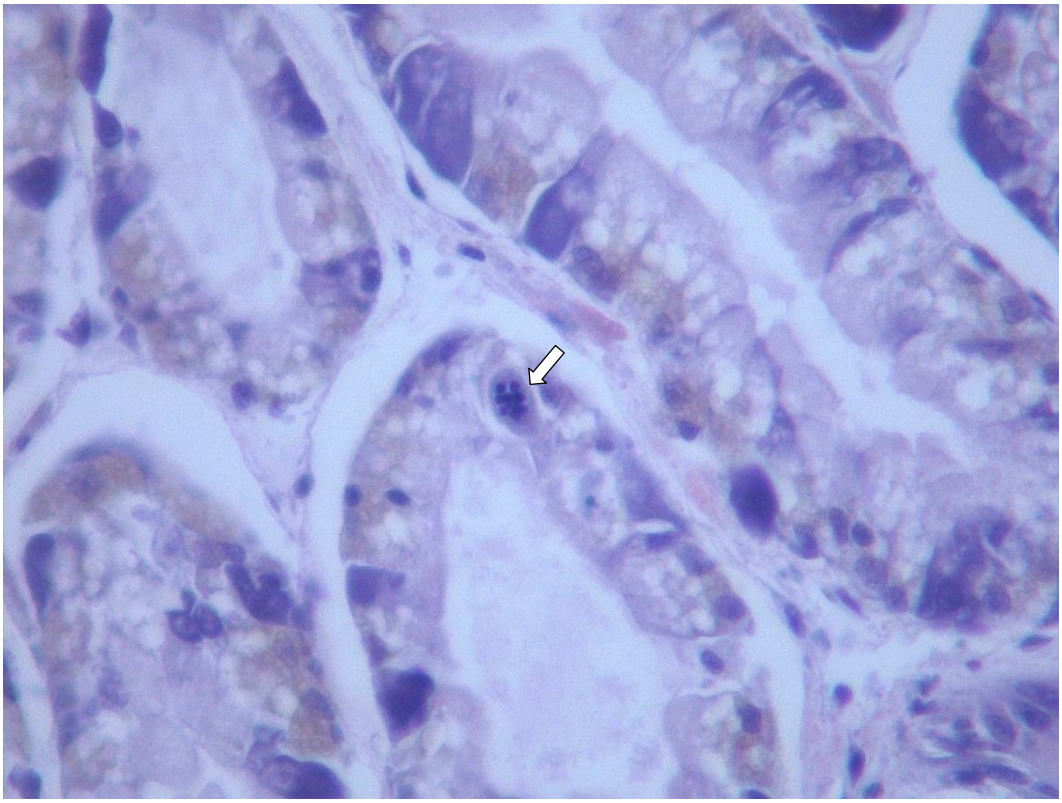


Figure 11. Intracellular ciliate (arrow) in digestive tubule, HE staining, x400

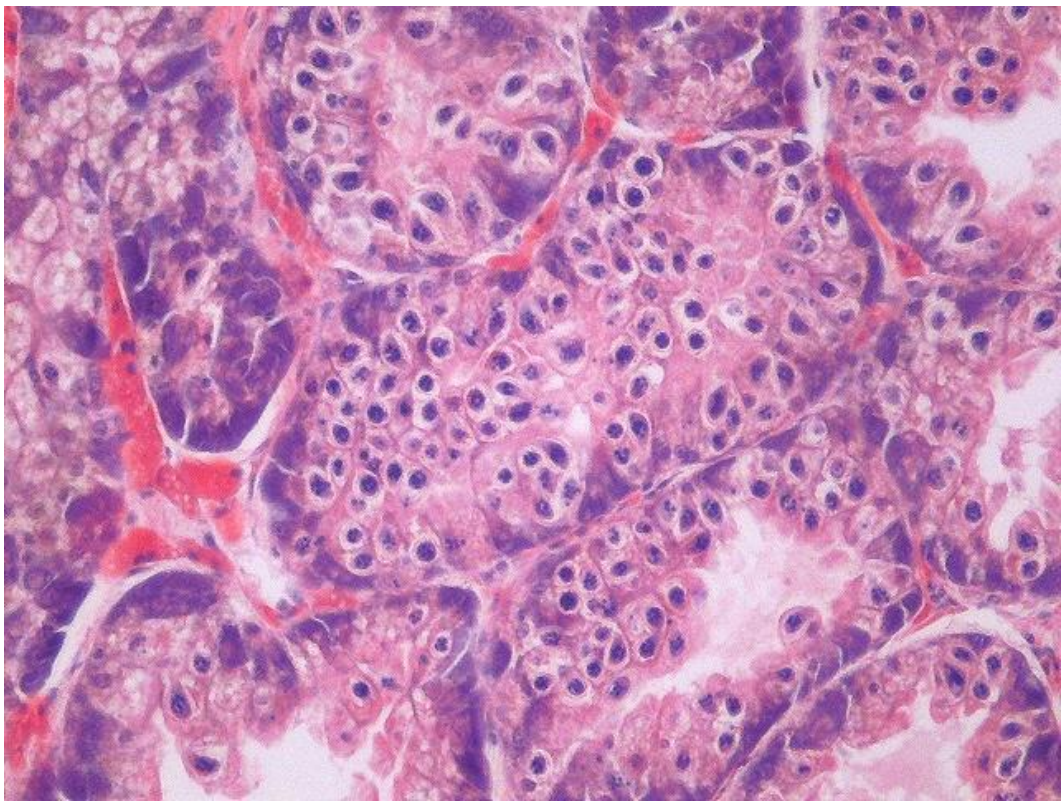


Figure 12. A severe infection with intracellular ciliates in digestive gland, HE staining, x200

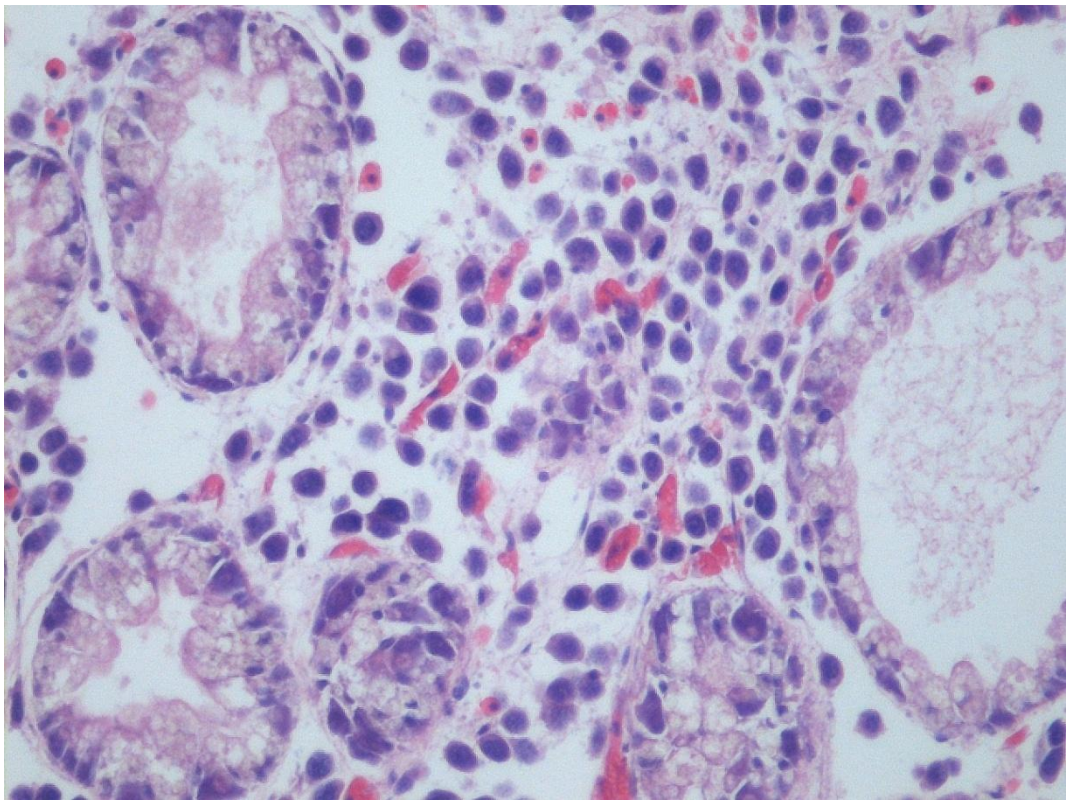


Figure 13. A diffuse form of haemocytic neoplasia in digestive gland connective tissue, HE staining, x200

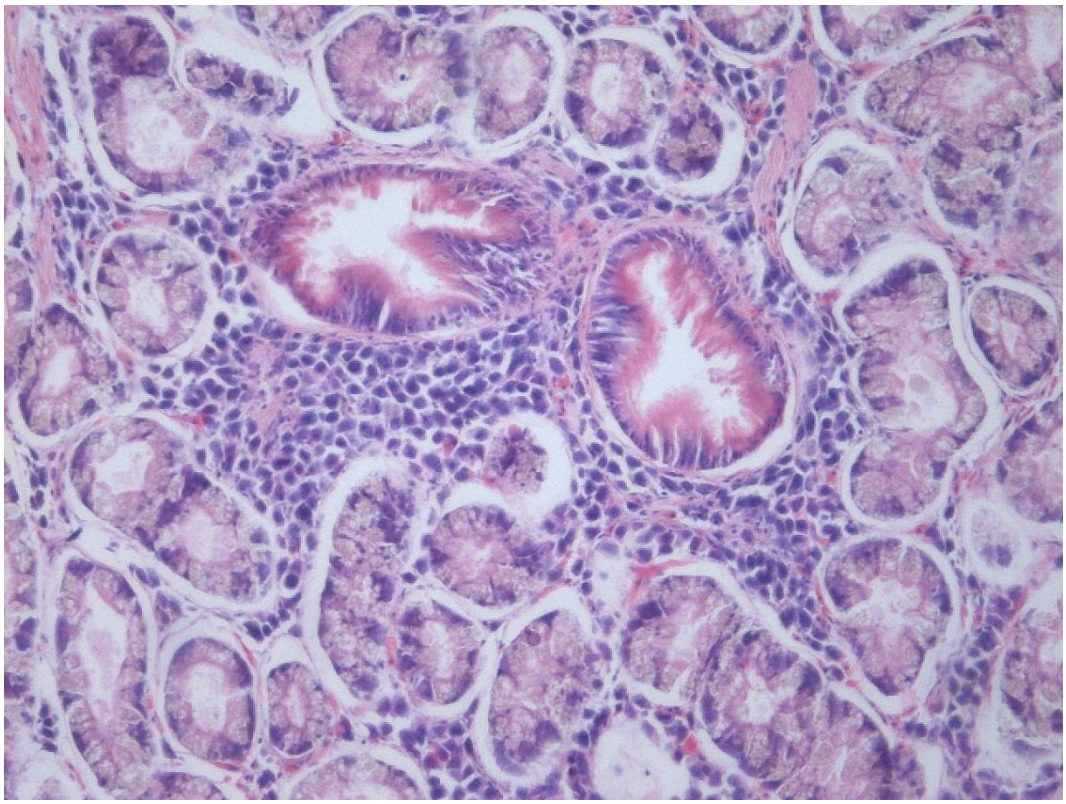


Figure 14. A multifocal form of haemocytic neoplasia in digestive gland connective tissue, HE staining, x100

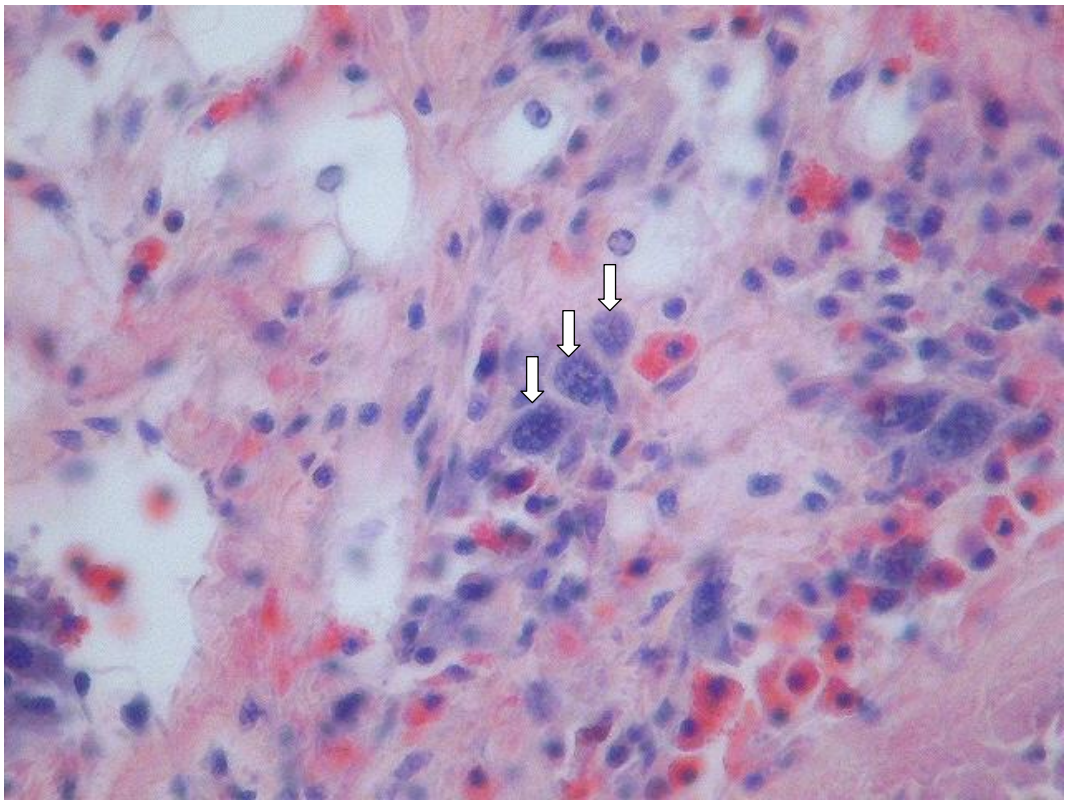


Figure 15. Single neoplastic cells (arrows) and a mild diffuse haemocytic infiltrate in digestive gland connective tissue, HE staining, x400

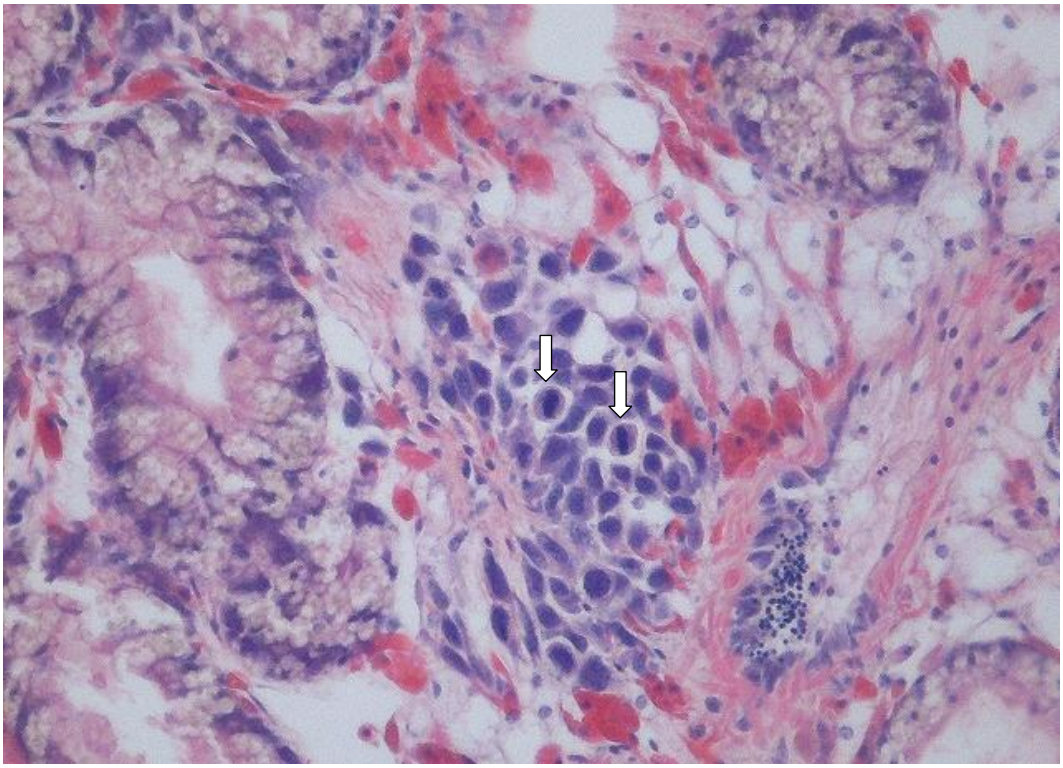


Figure 16. A multifocal form of haemocytic neoplasia with two mitoses (arrows) in digestive gland connective tissue, HE staining, x200

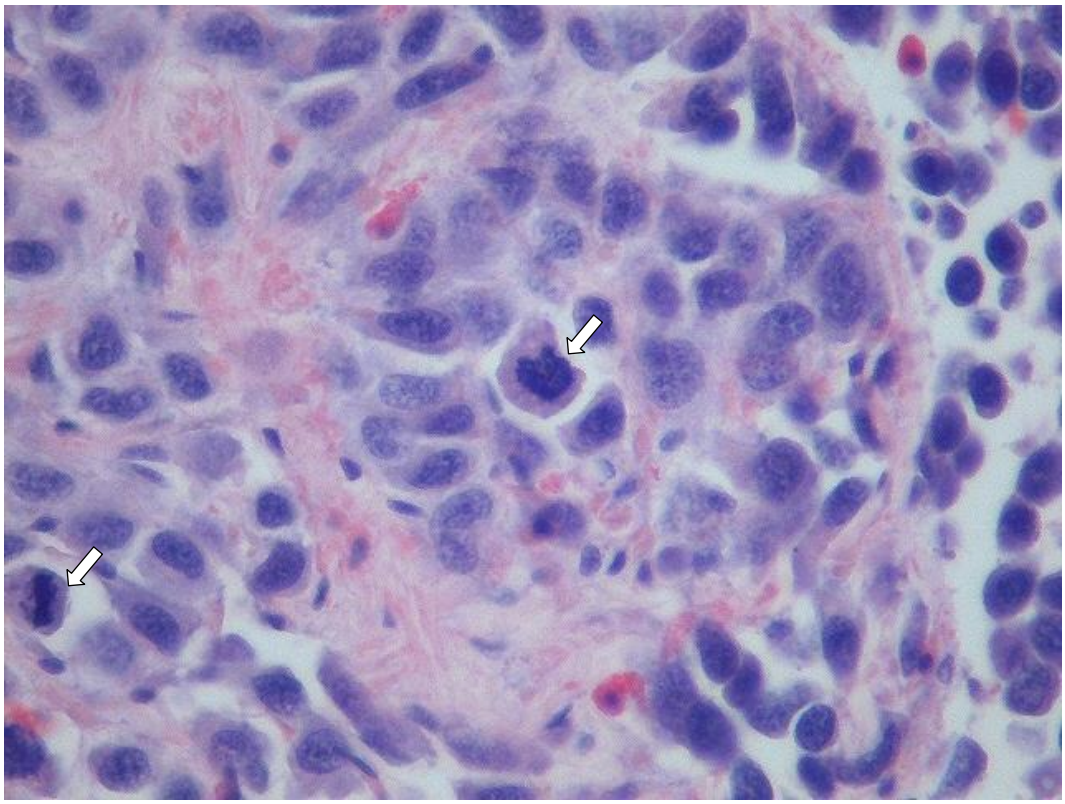


Figure 17. A diffuse form of haemocytic neoplasia in digestive gland connective tissue with two mitoses (arrows), HE staining, x400

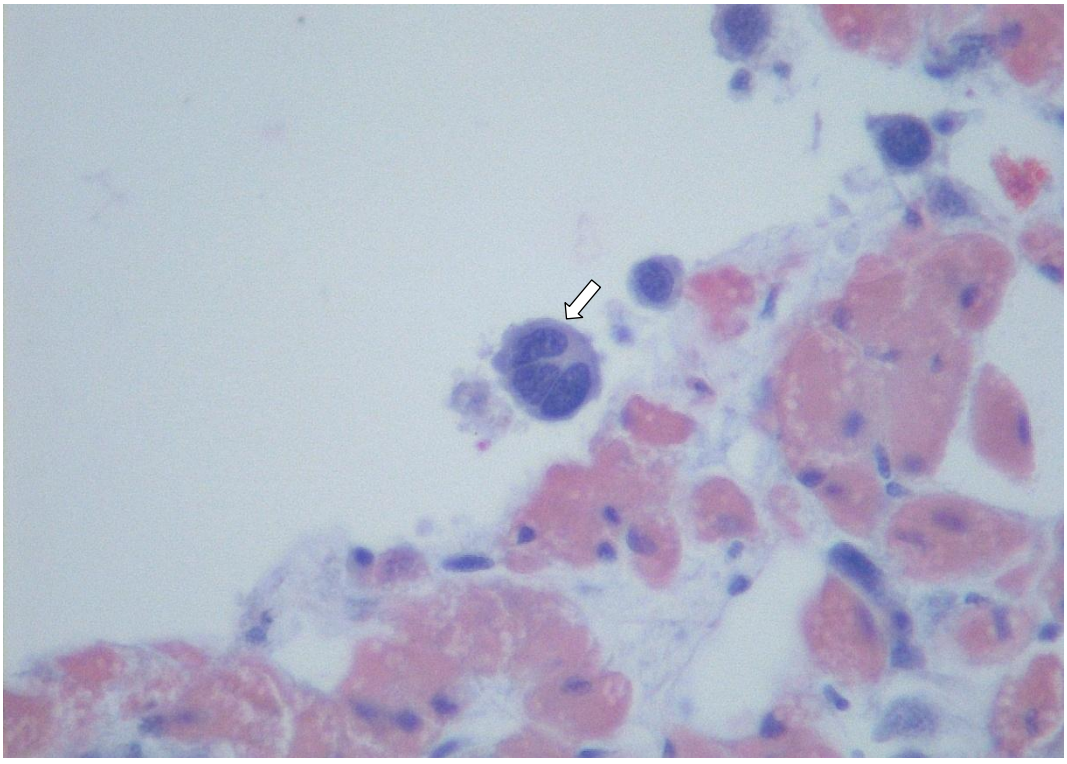


Figure 18. Tri-nucleated cell (arrow) in a diffuse form of haemocytic neoplasia, HE staining, x400

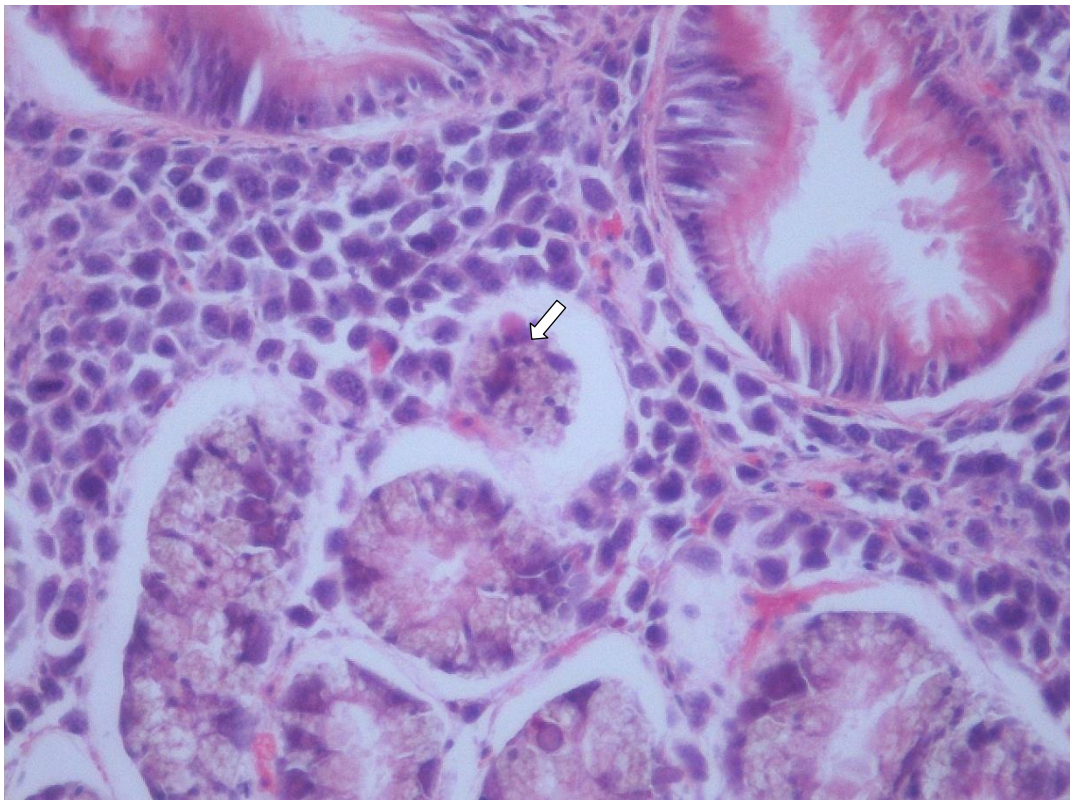


Figure 19. Tubular necrosis (arrow) in multifocal form of haemocytic neoplasia, HE staining, x200

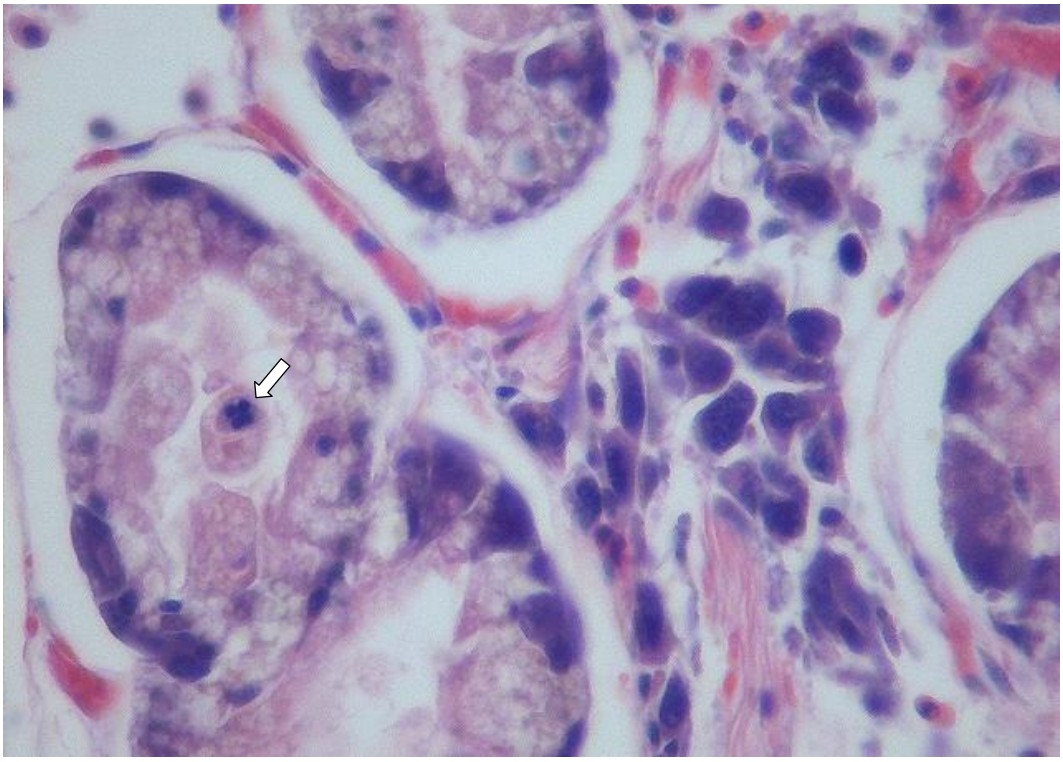


Figure 20. A multifocal form of haemocytic neoplasia in connective tissue and intracellular ciliate (arrow) in digestive tubule epithelia, HE staining, x400

6 DISCUSSION

The health status of cultured and wild Mediterranean mussels in Slovene sea was almost unknown prior to this study. In 2004 a system of surveillance of shellfish farms of Mediterranean mussels, the only shelfish species cultured in the Slovene sea, was established (EC, 1991; EC, 2002). Two sampling sites, i.e. Seča and Strunjan were established and 150 cultured Mediterranean mussels were collected annually in late spring and summer and inspected for the presence of *Marteilia* spp. To date all official samples tested negative for the presence of *Marteilia* spp., but intracellular ciliates of mussels were observed in the digestive glands of mussels (Gombač et al., 2008). Although the Slovene sea with its shallowness, constant inflow of fresh water and many other factors which highly influencing the temperature, oxygenation and salinity, represents a specific and rich habitat (Lipej et al., 2004; Richter, 2005) it was quite strange that cultured Mediterranean mussels were *Marteilia* spp. free, knowing that this protozoa are widespread from the Atlantic to the Persian Gulf (Bower and McGladdery, 2009) and were detected also in the northern Adriatic (Ceschia et al., 1992; Zrnčić et al., 2001). To clarify such a peculiarity we decided to perform a study, in which monthly collected cultured and wild Mediterranean mussels from two shellfish farms, i.e. Seča and Strunjan and from natural beds in Piran were inspected for the presence of *Marteilia* spp., intracellular ciliates of mussels and haemocytic neoplasia of mussels, and to establish their dynamics in connection with the sea temperature, oxygenation and salinity, measured at each sampling. To find out, if the protozoan infection and neoplasia have any affect on the health status of Mediterranean mussels, condition index of mussels was also calculated.

We measured the lowest sea temperature – 8.5 ^{o}C in February 2008 and the highest - 25.6 ^{o}C in July. The situation was similar in 2007: the lowest sea temperature - 10.1 ^{o}C was measured in February and the highest – 24.9 ^{o}C was measured in July (Mozetič et al., 2008), although our minimum temperature was slightly lower and the maximum higher compared to the temperatures measured in July 2007. The temperatures in 2007 were according to Mozetič et al. (2008) markedly above the 10-year average till June,

then were the same as the 10-year average, and fell slightly below it in the autumnal months.

The average sea salinities, measured in our study, were 37.25‰ in winter, 29.6‰ in spring, 38.1‰ in summer and 37.8‰ in autumn; the lowest measured salinity value – 25‰ was measured in May and the highest - 40‰ in August. Mozetič et al. (2008) reported the lowest salinity values - 34.25‰ in February and the highest – 38.2‰ in August. The very low salinity value recorded in our study in May was certainly the consequence of the long rainy period. The average salinity value in February 2008 was above 36‰ and comparable to those from 2007 (Mozetič et al. 2008).

The average sea oxygenation was 11.6mg/l in winter, 9.3mg/l in spring, 7.6mg/l in summer and 7.7mg/l in autumn. Mozetič et al. (2008) reported the highest oxygen concentration in winter and spring (9.88mg/l) and the lowest in summer (3.58 mg/l). The values were below the 10-year average (Mozetič et al., 2008) and to ours values, but they were measured at the sea bottom whereas our measurements were performed at a depth of 3 metres.

In all, 1280 adult Mediterranean mussels were collected during the one year period, 960 were cultured mussels, among them 480 from Seča and 480 from Strunjan, and 320 were wild mussels from different natural beds in Piran. The two populations were almost uniform and had more or less and equal average length (7 cm in cultured and 7.1 cm in wild mussels) and average condition index (28.14 in cultured and 29.63 in wild mussels). The condition index values were the highest in January, February, March and May, after that a significant decline was observed in June, most probably due to spawning - completely empty gonads were microscopically seen from June till September. The condition index was also very low in April, though we were not able to find a convincing reason for this.

No mortality was detected in the shellfish farms during the sampling period. 1279 mussels were macroscopically without any changes and only one mussel was severely emaciated.

The microscopic examination of digestive glands revealed *Marteilia* spp. in 4 mussels, intracellular ciliates of mussels in 293 mussels and haemocytic neoplasia of mussels in the connective tissue of 14 mussels from the Slovene sea. The severely emaciated mussel was uninfected and without any pathohistological changes so after performing only a pathohistological examination it was not possible to identify the cause of the emaciation. *Marteilia* spp. and intracellular ciliates of mussels are the only protozoa described in the digestive gland of mussels in Europe (Ceschia, 2008; Bower and McGladdery, 2009). Both have also been diagnosed in the northern Adriatic, *Marteilia spp*. in the Italian sea (Ceschia et al., 1992) and Croatian sea (Zrnčić et al., 2001), as have intracellular ciliates in the Slovene sea (Gombač et al., 2008). The haemocytic neoplasia of mussels is the only neoplasia described in mussels till date (Bower, 2006), but it was never detected in the northern Adriatic.

Marteilia spp. was detected only in four of the 1280 Slovene mussels examined. The species determination with PCR and RFLP revealed that all infected mussels carried *Marteilia refringens* type M. The taxonomic relationship and species determination of *M. refringens* and *M. maurini*, two different species or two types of a single species of genus *Marteilia* in Mediterranean mussels (*Mytilus galloprovincialis*) is still under debate (Berthe et al., 2004). *M. refringens* was first recorded in flat oysters in 1970s and linked to some massive mortality (Grizel et al., 1974). In 1982, Comps at al. discovered on the basis of ultrastructural characteristics in the Mediterranean mussels from the Venice lagoon a new species of the genus *Marteilia* - *M. maurini*. This finding was later on overruled due to the fact, that this two marteilia species could not be differentiated either on the basis of ultrastructural characteristics (Longshaw et al., 2001) or on the basis of sequences of the rDNA genes small subunits of *Marteilia* spp. isolated from oysters and mussels (Berthe et al., 2000). As such, it was proposed to consider *M. maurini* only a junior synonym of *M. refringens* (Longshaw et al., 2001). In further studies, targeting the specific ITS region of the rRNA gene, two genetic types of *Marteilia*, linked to the host species, were confirmed in Europe: *M. refringens* in oysters and *M. maurini* in mussels (Le Roux et al., 2001). Finally Lopez-Flores et al. (2004) established that there is no strict correlation of *Marteilia* types specifically to mussels or oysters and that *M. maurini* (M type) can be also found in oysters and *M. refringens* (O type) in mussels. To confirm the latter, flat oysters from the Slovene sea should also be

checked for the presence of *Marteilia* spp. and in the event of a positive result, the presence of *Marteilia* spp. should be confirmed using by PCR and RFLP. This was not done, because of the fact that flat oysters are not bred in the Slovene sea and the number of freely living oysters is quite small.

The infection in all four Slovenian *Marteilia refringens* positive mussels was severe, although some differences in the percentage were detected: above 10% of infected digestive tubules were infected in one mussel, up to 90% of digestive tubules were infected in another one and all digestive tubules resulted infected in two mussels. Sporadic disrupted epithelial cells of digestive tubules and some totally destructed digestive tubules were observed in all infected Slovene mussels. Robledo and Figueras (1995) observed that the presence of the plasmodia of *M. refringens* in the epithelial cells of the stomach and primary digestive tubules did not cause any pathohistological changes, however heavy infection resulted in almost total destruction of the digestive tubules. Figueras et al. (1991) and Villalba et al. (1997) also agreed that heavy infection results in the massive destruction of the digestive gland, which is most extensive during the release of spores from sporangia.

In the less infected Slovene mussel, a moderate diffuse haemocytic infiltration with focally distributed granulocytomas in digestive gland connective tissue and in mussel with up to 90% of infected digestive tubules a mild haemocytic infiltration was present. In the two mussels with all infected tubules, there were no alterations in connective tissue. Villalba et al. (1993a and 1997) and Carballal et al. (1998) discovered that the infection with *M. refringens* in Mediterranean mussels may cause a significant increase of haemocytes in the connective tissue. Occasionally, granulocytomas were also formed and caused the destruction of the parasites and host cells (Villalba et al., 1993a). Carballal et al. (1998) even affirmed that circulating haemocytes can decelerate or even stop the infection. Mild and moderate increases in haemocytes and focal granulocytomas were observed only in the less infected Slovene mussels, totally infected mussels were without any changes in the connective tissue. The less infected Slovene mussels probably suffered from an early infection and the haemocytes accumulation was perhaps a first body response to the invasion. Possobčy at a later, with there being no major changes to the body tissue, the mussels simply became accustomed to *M. refringens* or the host response was spent.

There was no cross infection with intracellular ciliates of mussels in any of the Slovene mussels, infected with *M. refringens* nor was there any occurrence of haemocytic neoplasia of mussels in the infected mussels. We could not find any data in the literature reporting the co-infection with intracellular ciliates of mussels or the occurrence of haemocytic neoplasia of mussels in mussels with marteiliosis.

Robledo and Figueras (1995) reported 8.3% to 29.6% mortality in Mediterranean mussels and Villalba et al. (1993a) correlated the increase of mortality with the intensity of the infection. No mortality was detected in Slovene Mediterranean mussels during our sampling and no heavy alterations in digestive gland tissue of infected mussels were microscopically seen although all *Marteilia* spp. positive mussels were severely infected.

All stages of the life cycle of *M. refringens*, i.e. spherical to elongated multinucleated cells, up to 12 µm in length, and sporangiosorus from 13.3 µm to 21.8 µm in diameter, enclosing 8 spherical sporonts from 4.7 µm to 7.2 µm in diameter, containing 2 to 4 round spores from 1 µm to 2.1 µm in diameter and light refractile inclusion bodies were seen in all four infected Slovene mussels, but early stages were predominantly seen in the less infected mussels. Perkins (1976) reported that in *M. refringens* the sporangiosorus enlarged up to 30 µm in diameter, contained 8 to 16 sporangial primordial, each about 12 µm in diameter at maturity, which matured in a sporont enclosing 2 to 4 spore primordial that matures into spores, which measured 3.5 µm to 4.5 µm. Villalba et al. (1993a and 1997) described a sporangiosorus up to 25 µm long, enclosing 8 sporonts with 12 µm in diameter containing 4 spores and 3 to 7 refringent bodies. In the research of Robledo and Figueras (1995), the sporangiosorus of *M. refringens* enlarged up to 19.6 µm at sporulation and contained 8 mature sporangia 7.3 µm in length with 3 to 4 spores 2.6 µm in length and refringent granules.

Multinucleated cells were nested in epithelial cells of digestive ducts and tubules, sporangiosori containing mature spores and refringent bodies were detected in epithelial cells of digestive gland tubules and ducts and inside their lumen and free spores were noticed in the digestive tubules lumina in all infected Slovene mussels. Other authors reported early stage *M. refringens* within the digestive tract epithelia and sporulating stages within the digestive gland epithelia (Robledo and Figuers, 1995; Villalba et al., 1997; Rayyan et al., 2006; Ifremer, 2009; Bower and McGladdery, 2009). Mature

spores have been seen in the lumen of digestive glands tubules (Bower and McGladdery, 2009).

The prevalence of infection with *M. refringens* in the Slovene sea was 0.3%. This is the lowest prevalence of marteiliosis in Mediterranean mussels described to date. The prevalence of marteiliosis in Mediterranean mussels in Delta de l'Ebre in Spain was from 1.67% to 26.67% (Carrasco et al., 2007b; Carrasco et al., 2008), in Galicia from 5.5% to 38.5% (Robledo and Figueras, 1995), 5% in Croatia (Zrnčić et al., 2001), 14.6% (Rayyan, 2006) and 21.25% (Virvilis et al., 2003) in Greece, from 16.6% to 22.2% in the Adriatic sea in Italy (Ceschia et al., 1992), 19.3% in Albania (Pëllumb et al., 2006) and from 37% to 70% in northern Brittany (Auffret and Poder, 1985).

M. refringens was detected only in cultured Slovene mussels in both shellfish farms at the same level of prevalence, all wild mussels sampled were uninfected. The fact is quite curious, because mussel seeds, designed for cultivation, are collected from wild beds in the Slovene sea and they grow in very similar conditions as wild mussels, as far the density of growth and sea parameters are concerned. This result is perhaps just the consequence of a very low prevalence and the fact that fewer mussels were collected from natural beds due to the unclement weather conditions in winter, when the infection in Slovene mussels mostly occurred. It is possible that cultured mussels are more liable to the infection due to reduced host defence caused by certain stressful events, i.e. removing the mussels from the small socks, cleaning them and redistributing them in bigger socks in the middle of the cultivation. But it is possible such a result is the consequence of the location of the shellfish farms, which lie in semi-closed small bays, whereas natural beds are located in more open areas. This view could be in accordance with Robledo and Figueras (1995) and Fuentes et al. (1995), who claimed that the infection with *Marteilia* spp. is most frequent in the shallow sea near the coast. We did not find data in the literature about the prevalence of infection with *Marteilia* spp. in wild Mediterranean mussels and to our knowledge it has never been reported that cultured mussels are more susceptible to the infection.

M. refringens was more frequently detected in Slovene mussels during winter, i.e. in December, January and February when the sea temperature was 10 ^{o}C or even lower, the oxygenation above 10 mg/l and the salinity equal to or above 37‰. Only once was the infection identified in August in high summer, when the sea temperature was

22.4 ^{o}C, oxygenation 6.9 mg/l and salinity 40‰. In all our positive cases the sporulation was in full course and free sporangia were seen in the lumen of digestive tubules. Other authors (Audemard et al., 2001; Carrasco et al., 2008; Ifremer 2009), reported that the infection of healthy mussels and oysters correlated with the sporulation of *Marteilia* spp., occuring in spring and summer when the sea temperature is higher than 17 ^{o}C and decreasing in winter, when sporangia primordia of *M. refringens* do not develop (Berthe et al., 1998). Carrasco et al. (2007b) on the other hand reported the presence of sporangiosori with mature sporangia in 50% to 75% of infected mussels also in winter with a sea temperature of 8 ^{o}C although mature spores were not released in the lumen of digestive tubules. The reported infection was very low and did not exceeded 3.34% (Carrasco et al., 2007b). *Marteilia* spp. was detected in Spain from February to October (Carrasco et al., 2008), in Greece from May to November (Photis et al., 1992), in Croatia in August (Zrnčić et al., 2001) and in Italy in April and from June till September (Ceschia et al., 1992). Ceschia et al. (1992) reported that high salinity also inhibits the infestation. In our study the infection with *Marteilia* spp. was detected also in August, when the salinity was high (39‰).

The average condition index in infected mussels from Slovene sea was just slightly lower than in uninfected mussels, but the number of infected mussels was too small to enable statistical testing. Figueras et al. (1991) and Rayyan et al. (2006) reported that heavy infection results in the lost of body condition. Rayyan et al. (2006) found out that the infection with *M. refringens*/*maurini* resulted in significant reduction of the condition index.

Fuentes et al. (1998) expressed the opinion that with mussel's growth the possibility of infection rises. The infected Slovene mussels were from 6 cm to 8 cm long, which was the average length of mussels, included in our study. The longest exemplars, with a length of up to 11.5 cm, were *Marteilia* spp. free.

The orute of transmission of the infection with *M. refringens* remains uncertain. The direct transmission of *M. refringens* (Comps and Joly, 1980; Perkins, 1988; Berthe et al., 1998) and feeding of *M. refringens* spores to small crustaceans *Carcinus maenas*, *Carcinus crangon* and *Marinogammarus marinus* (Van Banning, 1979) proved to be a failure. The PCR techniques used by Audemard et al. (2001and 2002) revealed the copepod *Paracartia* (*Acartia*) *grani* as a potential host for *M. refringens*. The

involvement of *P. grani* in the life cycle of *M. refringens* apparently is consistent with the biology of the copepod, which is mainly observed in spring and summer and absent during winter (Audemard et al., 2002) and with the fact that sporangia primordia of *M. refringens* do not develop during winter (Berthe et al., 1998). Further, Carrasco et al. (2007a), also using the PCR method established that *Acartia latisetosa, Oithona* sp. and indeterminate harpaticoida (Carrasco et al., 2008) and *Acartia discaudata, A. clausi, A. italica* and larval stages of decapods crustaceans, probably *Portumnus* sp. (Carrasco et al., 2007b) are the most probable intermediary hosts for *M. refringens*. The dominant copepod species in the Slovene sea is *Acartia clausi* (Malej, 1979; Nacionalni biološki inštitut, 2003), which is with *Oithona* spp., *Oncaea* spp. and *Paracalanus parvus* the predominant zooplankton throughout the year except in summer (Malej, 1979). *Paracartia* (*Acartia*) *grani* has to date not been reported in the northern Adriatic, whereas *Acartia latisetosa, Oithona* sp. and indeterminate harpaticoida are present in the shallow areas throughout the year, the first two most abundantly in spring and summer and indeterminate harpaticoida mostly during winter (Hure and Kršinić, 1998). Copepods found in the Slovene sea have were never been checked for the presence of *M. refringens*, but the fact that they are present also in winter, when the sporulation of *M. refringens* in the Slovene sea mostly occurred, and that they are found only in shallow sea water in which Slovene mussels are bred, confirms in part the view expressed in Carrasco's et al. (2007b, 2008) that *Acartia latisetosa, A. clausi, Oithona* sp. and indeterminate harpaticoida could be intermediary hosts for *M. refringens*. Perhaps *Oncaea* spp. and *Paracalanus parvus* should also be considered as possible intermediary hosts and should, along with *Oithona* spp., be checked for the presence of *M. refringens*.

Although three Slovene mussels were positive in winter and only one in summer and all positive mussels were from shellfish farms no statistically significant differences in infection with *Marteilia* spp. were detected between the three different locations of sampling, between cultured and wild mussels, between different months and between the sea condition parameters of temperature, oxygenation and salinity due to the fact that the number of *M. refringens* positive mussels was too small to enable reliable statistical evaluation.

Pear or spindle-shaped, 3.9 µm to 11.5 µm long and 2.9 µm to 8.4 µm width intracellular ciliates with a polymorphic oval to globular basophilic, fragmented macronucleus, were found inside the digestive tubule epithelia or were lying freely in the lumens of digestive tubules in 293 Slovene mussels. We could not find many literature data describing the intracellular ciliates of mussel. Villalba et al. (1997) described the intracellular ciliates of mussels as pear or spindle-shaped, 3.9 µm to 15 µm long parasites with a polymorphic oval to globular basophilic, fragmented macronucleus, which colonised the digestive tubule epithelia but are seen also in the lumens of digestive tubules in Mediterranean mussels from Spain.

A slight enlargement of epithelial cells that carried ciliates of a large size was the only alteration of digestive glands tubules observed in our study. A mild diffuse haemocytic infiltration in digestive gland connective tissue was noticed in 2% and haemocytic neoplasia of mussels was diagnosed in 1% of mussels infected with intracellular ciliates of mussels. No *Marteilia* spp. was detected in any of mussels infected with intracellular ciliates. No mortality was observed in our study. A slight enlargement of epithelial cells that contained ciliates of a large size, without an inflammatory response was observed also by Villalba et al. (1997) and Gombač et al. (2008). Figueras et al. (1991) on the other hand noticed a specific host response in infected mussels and McGladdery and Bower (2002) described the destruction of the digestive gland in some of infected mussels. No mortality linked to the infection with intracellular ciliates of mussels has been reported to date (McGladdery and Bower 2002).

The prevalence of infection with intracellular ciliates of Slovene mussels was 22.9%. A slightly lower rate of infection at 21.4% was detected in our previous study, performed in May and July 2004 (Gombač et al., 2008). The only data, concerning the prevalence of infection with intracellular ciliates of mussels in Mediterranean mussels we were able to find in the literature was Carrasco et al. (2007), in which a prevalence lower than 1% was observed and Villalba et al. (1997), in which a 40% prevalence of infection was reported in Spain.

The prevalence of infection was 24.4% in Slovene cultured mussels (27.3% at Seča and 21.5% at Strunjan) and 18.4% in Slovene wild mussels. The differences between cultured and wild mussels and infection with intracellular ciliates and the differences between three different locations and infection with intracellular ciliates were not

relevant. In the literature there is no data showing possibles differences in infection between cultured and wild Mediterranean mussels. In blue mussels the prevalence of infection with intracellular ciliates of mussels was lower in wild mussels (Weldon, 1999).

The highest average prevalence of infection (42%) with intracellular ciliates in Slovene mussels was detected in spring (March, April and May) with an average sea temperature of 15.2 ^{o}C, the average oxygenation of 9.3mg/l and the average salinity of 29.6‰. The lowest prevalence (12.7%) was in summer (June, July and August) with an average sea temperature of 24.1 ^{o}C, an average oxygenation of 7.6mg/l and the average salinity 38.1‰. In autumn (September, October and November) the prevalence of infection was 15.2% and the average sea temperature was of 17 ^{o}C, the average oxygenation of 7.7mg/l and the average salinity of 37.8‰. In winter (December, January and February) with an average sea temperature of 9.1 ^{o}C, average oxygenation of 11.6mg/l and average salinity of 37.25‰ the average prevalence of infection was 25.1% The highest absolute prevalence was detected in May (47%) with the average sea temperature of 20 ^{o}C, average oxygenation of 9.1mg/l and average salinity of 26.6‰, and the lowest in September (1.6) with an average sea temperature of 20.3 ^{o}C, average oxygenation of 7mg/l and average salinity of 39‰. We found the differences between prevalence of infection in different months statistically significant because of the statistically significant relationship between the salinity and the infection. The differences in temperature and the oxygenation and the infection were statistically not significant. We established that only salinity had an influence on infection with intracellular ciliates - the higher the salinity the lower the rate of infection was. Villalba et al. (1997) also found intracellular ciliates throughout the year, but the lowest prevalence was detected from October to January. Weldon (1999) on the contrary detected the highest infection (8.3%) in wild blue mussels in Canada in September with a water temperature of 15.2 ^{o}C and salinity of 34.7‰. The samplings were performed only in February, May, July, September and November and unfortunately during the February and May sampling the salinity meter broke down, so no conclusions about the influence of temperature and salinity on the infection were made.

For the evaluation of the intensity of infection with intracellular ciliates of mussels we developed our own scale. Villalba's et al. (1993a) scale, used for the evaluation of the

intensity of the infection with *Marteilia* spp., based mainly on the number of infected digestive tubules, seamed inadequate for the evaluation of the intensity of the infection with intracellular ciliates of mussels. The main reason was the uneven distribution of ciliates in Slovene mussels, since in some mussels only few tubules were infected and were carrying more than 100 ciliates, but in the others up to 100 infected tubules were inhabited by one ciliate each only.

A mild infection with less than 50 ciliates per section was detected in 74.4% of infected mussels, a moderate infection with up to 100 parasites per section in 15% of infected mussels and a severe infection with more than 100 parasites per section in 10.6% of infected mussels. In mildly infected mussels, only one ciliate inhabited the epithelia of a single infected digestive tubule and a single epithelial cell. In moderate infection, mostly more than one ciliate was seen in the epithelia of single infected digestive tubule but still only one ciliate was noticed inside a single epithelial cell. Severely infected mussels inhabited more than one ciliate in the epithelia of single digestive tubule and in some of them more than one ciliate was noticed in a single epithelial cell. In the most severe infection, more than 1000 ciliates were seen in a digestive gland and all digestive tubules were infected: up to 91 ciliates were found in a single digestive tubule and up to 3 ciliates inhabited a single infected epithelial cell. In the most severely infected blue mussel in Canada, Weldon (1999) counted 67 ciliates. No reports about the intensity of the infection were made to our knowledge to date.

The average intensity of infection with intracellular ciliates in three sampling locations was as follows: mild infection was mostly detected at Strunjan, followed by Seča and Piran, moderate infection was mostly detected at Seča, followed by Piran and Strunjan and severe infection at Piran, followed by Seča and Strunjan. The differences between three different locations and the intensity of the infection with intracellular ciliates were statistically not essential.

Mild infection was mostly seen in cultured mussels, whereas moderate and severe infection was observed in wild ones. The differences in the intensity of infection with intracellular ciliates between cultured and wild mussels were not statistically significant.

A mild infection was in all three sampling locations predominant in the summer with the highest average sea temperatures and salinity and the lowest oxygenation and the lowest infection with intracellular ciliates, and the lowest in the spring, when the

infection with intracellular ciliates was the highest. Moderate and a severe infection exhibited opposing characteristics: highest in spring, with medium average sea temperature and oxygenation but with the lowest salinity values, and the lowest in summer. Because of the constraints imposed by the sample size, we were not able to determine whether there was a statistically significant correlation between the intensity of the infection with intracellular ciliates and the month of sampling, but we did confirm that salinity had statistically significant impact on the intensity of the infection, whereas temperature and oxygenation did not.

The average condition index of all infected Slovene mussels was slightly higher than that of healthy ones. The same result was observed also in cultured and wild mussels. The condition index of infected mussels from Seča and Piran was slightly higher than that of healthy mussels, whereas the condition index of infected and healthy mussels from Strunjan was the same. In all months except in January, May, June and August, the condition index was higher for infected mussels. The differences in condition index between infected and healthy mussels, between cultured and wild mussels, between the three sampling location and between different months were not statistically significant. However, a slight connection was noticed between the infection and the length of the mussels, total mussels weight and the weight of the flesh - ciliates were most often observed in longer and heavier mussels, so perhaps intracellular ciliates more frequently inhabit older mussels. The average condition index of mussels with mild and moderate infection was higher than that of uninfected, whereas in severely infected mussels it was lower. We confirmed very small but statistically not significant differences in the intensity of the infection with intracellular ciliates of mussels and the condition index. The fact is quite interesting and could be explained as follows: intracellular ciliates of mussels perhaps prefer mussels in better condition and are harmless commensales, which don't influence the hosts' growth, when they are present in small number, but with the extensive increase of their number the condition index decreases.

Haemocytic neoplasia of mussels occurred in 14 Slovene mussels. Neoplastic cells were highly pleomorphic and anysocitotic, from 12.3 µm to 30.1 µm in diameter, with large, hyperchromatic rounded or pleomorphic nucleus from 4.3 µm to 22.7 µm in diameter with finely dispersed or dense chromatin without nucleolus. Some bi- or even tri-

nucleated cells were also observed. The nucleus to cytoplasm ratio was high and the number of mitosis was 2 mitoses per HPF. Many other authors also described haemocytic neoplasia of mussels as a proliferation of hypertrophied neoplastic cells, which are 2 to 4 times larger than normal haemocytes, with a large, hyperchromatic and often pleomorfic nucleus (Zizzo et al., 1991; Villalba et al., 1997; Usheva and Frolova, 2000; Barber, 2004; Ciocan and Sunila, 2005), up to 15 µm in diameter (Villalba et al., 1997), with finely dispersed chromatin (Villalba et al., 1997), containing one or more prominent nucleoli (Villalba et al., 1997; Usheva and Frolova, 2000; Barber, 2004) or are without it (Ciocan and Sunila, 2005). Ciocan and Sunila (2005) also noticed some bi-nucleated cells. Many other authors (Elston et al., 1992; Villalba et al., 1997; Usheva and Frolova, 2000; Barber, 2004; Ciocan and Sunila, 2005) observed that the nucleus to cytoplasm ratio and mitotic activity are high. Usheva and Frolova (2000) reported a high mitotic index from 0.9 to 1.9%.

Barber (2004) communicated that in the early stage of disease only single abnormal cells or small foci of neoplastic cells, morphologically resembling the haemocytes, are seen in the circulatory system, which supports the hemopoietic origin of neoplasia. Later, neoplastic cells progressively replace normal haemocytes and are found throughout the various tissues (Elston et al., 1992). Using Mix's (1983) stages of disease severity designed for mussels, Stage 4 (a diffuse infiltration of neoplastic cells throughout the body) was observed in 4 Slovene mussels and Stage 1 (small number of single neoplastic cells or small foci of cells in vessels and connective tissue of digestive glands) was observed in 10 Slovene mussels. Zizzo et al. (1991) and Ciocan and Sunila (2005) found a diffuse distribution of neoplastic cells in the connective tissue of various organs and in blood vessels in all affected mussels, whereas Villalba et al. (1997) found a diffuse form of neoplasia also only in blood vessels and sinuses around the stomach.

The necrosis and multifocal atrophy of digestive tubules were observed in Slovene mussels with diffuse neoplasia whereas severe haemocytic infiltration of connective tissue was seen in mussels with single neoplastic cells. In 2 mussels with severe haemocytic infiltration and single neoplastic cells and in a mussel with focuses of neoplastic cells, a mild infection with intracellular ciliates was observed. In other cases of haemocytic neoplasia there were no intracellular ciliates. Fibrisos, displacement, compression of gills, gonad and connective tissue, atrophy of digestive diverticula and

general degeneration and necrosis of tissues has been described in the diffuse form of haemocytic neoplasia (Villalba et al., 1997; Barber, 2004). No tissue damage was observed in mussels with only single neoplastic cells (Villalba et al., 1997). Haemocytic neoplasia of mussels is normally lethal, but remission has also been reported (Elston et al., 1988a; Elston et al., 1988b; Elston et al., 1990b).

The prevalence of haemocytic neoplasia of mussels in the Slovene sea was 1.1%. Other authors also reported only sporadic cases of the disease in Mediterranean mussels and subsequently very low prevalence: 0.27% in Rias of Galicia in Spain (Villalba et al., 1997), 0.45% in the Mediterranean sea in Italy (Zizzo et al., 1991), 0.5% in the Black sea in Romania (Ciocan and Sunila, 2005) and 3.4% in Delta de l'Ebre in Spain (Carrasco et al., 2008).

The prevalence of haemocytic neoplasia in cultured mussels was 1.25% and 0.6% in wild mussels. Mussels from Seča and Strunjan exhibited the same prevalence of 1.25%. Haemocytic neoplasia was more frequently (5 cases) observed in spring (in March and May), when the sea temperature was between 11 ^{o}C and 20.2 ^{o}C, the oxygenation below 9 mg/l and the salinity between 28‰ and 32‰ and in autumn (5 cases, in September and October) when the sea temperature was between 17.8 ^{o}C and 20.3 ^{o}C, the oxygenation below 7.1 mg/l and the salinity between 26‰ and 39‰. Three mussels were affected in summer (in June and July), when the sea temperature was above 24.2 ^{o}C, the oxygenation below 8.3 mg/l and the salinity above 36‰. Only one affected mussel was detected in winter in December with the sea temperature 9.5 ^{o}C, the oxygenation 10.95 mg/l and the salinity 35‰. In January, February, April, August and November no haemocytic neoplasia of mussels was detected. Elston (1990a) also discovered out that the haemocytic neoplasia of mussels mostly occurs in the late autumn but also in winter, from October to March, Carrasco et al. (2007b) found affected mussels in June and October, whereas Barber (2004) reported the major occurrence from January to March. No statistically significant differences in the occurrence of haemocytic neoplasia in Slovene mussels between the three different locations, between cultured and wild mussels, between different months and sea temperatures, oxygenation and salinities were detected, but the number of mussels with haemocytic neoplasia was too small to enable reliable statistical testing.

In Elston's (1990a) opinion older mussels are more frequently affected. The average length of Slovene mussels with haemocytic neoplasia of mussels was 6.7 cm, which is slightly below the average length of all collected mussel (7 cm). The longest and unquestionablely older mussels, with the length up to 11.5 cm, were unaffected.

The average condition index of Slovene mussel with haemocytic neoplasia was higher than that of healthy ones. The same result was obtained comparing the average condition index ofaffected and healthy cultured mussels from both shellfish farms. The average condition index of affected wild mussels was on contrary lower than that of healthy ones. The average condition index of mussels with single neoplastic cells and with multifocal form of haemocytic neoplasia of mussels was higher than that of healthy mussels, whereas the condition index of mussels with the diffuse form of haemocytic neoplasia was equal to the condition index of unaffected mussels. Although the average condition index of mussels with haemocytic neoplasia of mussels was slightly higher than that of healthy ones, the number of affected mussels was too small to enable the reliable confirmation of the differences between affected and healthy mussels. We can conclude that haemocytic neoplasia of mussels did not influence the condition index of Slovene mussels, but the fact that the average condition index of mussels with a diffuse form of haemocytic neoplasia was lower than in other forms of distribution should not be overlooked. Barber (2004) reported that neoplastic haemocytes lose the capability of digestion, absorption and food transportation, which leads to the starvation of affected mussel. The only data regarding the condition index of affected bivalves found in the literature, was Leavitt's et al. (1990) claim that the condition index of diseased clams was significantly lower than thet of healthy ones.

The potential etiological factors of haemocytic neoplasia of mussels are viruses (retrovirus) (Elston et al., 1988b), environmental contamination and biotoxins (Barber, 2004). Hillman (1993) observed significantly higher morbidity in mussels along the both United States coasts in areas contaminated with polycyclic aromatic hydrocarbons (PHA). In areas polluted by pesticides, chrome, mercury and cadmium the morbidity was significantly lower. Usheva and Frolova (2000) found the connection between haemocytic neoplasia and pollution also in Japan. Wolowicz et al. (2006) on the other hand belive that the cause of haemocytic neoplasia in shellfish is heavily polluted sea sediments. Landsberg (1996) noticed that the occurrence of haemocytic neoplasia

coincided with the outbreaks of several species of toxic dinoflagellates, which may increase the susceptibility to neoplasia, particularly viral agents. It is also presumed that all kinds of stress may have a negative impact on the host defence mechanisms and cause also haemocytic neoplasia (Barber, 2004).

No virus has been isolated from the mussels affected with haemocytic neoplasia to date and affected Slovene mussels were not checked for the presence of presumable viruses. Environmental contamination and bio toxins were also not evaluated in the Slovene sea during our sampling, but measurements of physical-chemical parameters, halogenated organic compounds and metals in the Slovene sea, cadmium and mercury content in sea sediments and mussels flesh and the concentration of toxic phytoplankton, performed from 2003 to 2005 (Turk et al., 2005; Ambrožič et al., 2007) in 2006 (Turk et. al., 2007) and in 2007 (Mozetič et al., 2008) at Seča and Strunjan revealed that all data were under limiting values.

The highest concentration of all phytoplankton was reported in February (NIB, 2002; Mozetič et al., 2008) - the month, in which in our study, performed in 2008, no haemocytic neoplasia of mussels was detected, and elevated values in May (NIB, 2002) (3 cases of haemocytic neoplasia in our study), in June (Mozetič et al., 2008) (2 cases of haemocytic neoplasia in our study), in July (NIB, 2002) (1 case of haemocytic neoplasia in our study) and August (NIB, 2002) (0 case of haemocytic neoplasia). The genus *Alexandrium*, the potential causative agent of paralytic shellfish poisoning (PSP), was in 2001 most abundant in September, February and April (NIB, 2002) in 2006 in June (Mozetič et al., 2007) and in 2007 in September (Mozetič et. al., 2008). In our study two cases of haemocytic neoplasia were detected in June and 3 cases in September, but mussels were unaffected in February and April. The concentration of *Prorocentrum lima*, the potential agent of diarrhoea shellfish poisoning (DSP), was in 2001 on the contrary highest in July and August (NIB, 2002), in 2006 in June and September (Mozetič et al., 2007) and in 2007 in July and September (Mozetič et. al., 2008). In June two cases, in July only one case, in August none and in September three cases of haemocytic neoplasia were observed in our study. The elevated concentrations of diatoms from the genus *Pseudo-nitzschia*, the potential agents of amnesia shellfish poisoning (ASP), were in 2006 (Mozetič et. al., 2007) measured in June and September and in 2007 in June and October (Mozetič et. al., 2008). In June and October two cases

of haemocytic neoplasia of mussels and in September 3 cases of haemocytic neoplasia were detected in our study. If the phytoplankton concentrations at the time of our sampling were comparable to those, obtained in 2001, 2006 and in 2007, we can presume that bio toxic phytoplankton from genus *Alexandrium* and *Prorocentrum lima* has no influence on the occurrence of haemocytic neoplasia in Slovene mussels, but a high concentration of phytoplankton of the genus *Pseudo-nitzschia* might induce haemocytic neoplasia of mussels. To confirm this hypothesis the exact measurements of phytoplankton should have been performed at the time of our sapling in all three locations, which was not done.

The concentrations of heavy metals in mussels were in 2001 higher at Strunjan than at Seča (NIB, 2002), although the prevalence of haemocytic neoplasia of mussels was in our study the same at both locations. In 2006 the concentrations of heavy metals in mussels were comparable to those obtained in 2001 and from 2002 to 2005 (Turk et. al., 2007). The concentrations of heavy metals were under limiting values from 2001 to 2006, so we can suppose that also in 2007 and in 2008 at the time of our sampling the situation was quite the same. On this basis we can presume that heavy metals don't cause haemocytic neoplasia of mussels in the Slovene sea.

The analysed DNA damage in Slovene mussels, a consequence of mutagen substances, were under the level of normal damage caused by normal cellular mitoses and their values were the same at Seča and Strunjan in 2001 (NIB, 2002). Unfortunately, we could not find any data about DNA damages in mussels in later years, so it was impossible to link them to the occurrence of haemocytic neoplasia in mussels.

All kinds of stress may also have a negative impact on the host defence mechanisms and cause also haemocytic neoplasia (Barber, 2004). Although the difference in the degree to which Slovene cultured and wild mussels were affected was not statistically significant, it was established that haemocytic neoplasia occurred mostly in cultured mussels. Perhaps such a stress may be caused by the collection of seeds after their anchorage, their embedding in nylon socks and their removal, cleaning and redistribution in bigger socks at halftime of cultivation.

7 CONCLUSIONS

1. Slovene Mediterranean mussels (*Mytilus galloprovincialis*) are infected with *Marteilia* spp. and intracellular ciliates of mussels. Haemocytic neoplasia of mussels also occurs in Slovene Mediterranean mussels.
2. *Marteilia refringens* type M was identified using PCR-RFLP in all *Marteilia* spp. positive mussels.
3. The prevalence of infection with *Marteilia refringens* in Slovene Mediterranean mussels is very low (0.3%), but the infection is severe.
4. *Marteilia refringens* causes sporadic disruption of epithelial cells of digestive tubules, multifocal destruction of digestive tubules, haemocytic infiltration and focal granulocytomas but no mortality in Slovene mussels.
5. *Marteilia refringens* was detected only in cultured Slovene mussels.
6. The infection with *Marteilia refringens* is not seasonal and is concentrated in winter.
7. The sporulation of *Marteilia refringens* occurs also in sea temperatures lower than 10 ^{o}C at very high salinity and low oxygenation values.
8. No cross infection with intracellular ciliates and haemocytic neoplasia of mussels occured in *M. refringens* positive mussels.
9. The condition index of *Marteilia refringens* positive mussels is slightly lower than that of uninfected mussels.
10. Sea temperature, oxygenation and salinity do not seem to influence the incidence of infection with *M. refringens.*
11. The number of Slovene *M. refringens* positive mussels was too small to enable reliable statistical evaluation of differences between different locations of sampling, between cultured and wild mussels, between different months and the condition index of infected and healthy mussels.
12. The prevalence of infection with intracellular ciliates of mussels in Slovene Mediterranean mussels is moderate (22.9%), the intensity of the infection is predominantly mild.
13. The infection with intracellular ciliates of mussels causes only a slight enlargement of epithelial cells that carried ciliates of a large size.

14. The infection with intracellular ciliates of mussels is not seasonal and is highest in spring and lowest in summer.
15. The infection with intracellular ciliates is not linked to the manner and location of mussels growing neither to the time (month) of the year.
16. Salinity has negative influence on the incidence of infection with intracellular ciliates, whereas sea temperatures and oxygenation have no influence on the incidence of infection.
17. Intracellular ciliates more often inhabit heavier and longer mussels, but have no influence on the condition index of infected mussels.
18. Haemocytic neoplasia of mussels occurs only sporadically in Slovene Mediterranean mussels with a1.1% prevalence.
19. Diffuse haemocytic neoplasia causes necrosis and multifocal atrophy of digestive tubules, single and multifocal neoplastic cells have no effect on the digestive gland.
20. The affection is not seasonal and is highest in spring and in autumn and lowest in winter.
21. The number of Slovene mussels with haemocytic neoplasia of mussels was too small to enable reliable statistical evaluation of differences between different locations of sampling, between cultured and wild mussels, between different months, between sea temperatures, oxygenation and salinities and the condition index of diseased and healthy mussels.

ZAKLJUČKI

1. Slovenske mediteranske klapavice (*Mytilus galloprovincialis*) so invadirane s praživalmi iz rodu *Marteilia* in z znotrajceličnimi migetalkarji klapavic, ugotovili pa smo tudi novotvorbo hemocitov klapavic.
2. *Marteilio refringens* M-tip smo z molekularno metodo PCR-RFLP determinirali pri vseh *Marteilia* spp. pozitivnih klapavicah.
3. Prevalenca invazije z *M. refringens* je v slovenskih klapavicah zelo nizka (0,3-odstotna), invazija pa zelo močna.
4. *M. refringens* povzroča propad posameznih epitelnih celic prebavnih kanalčkov, žariščno destrukcijo prebavnih kanalčkov, infiltracijo hemocitov in posamezne granulocitome, vendar ne povzroča smrtnosti klapavic.
5. *M. refringens* smo ugotovili le pri gojenih klapavicah.
6. Invazija z *M. refringens* je najpogostejša pozimi, vendar ni sezonska.
7. Sporulacija *M. refringens* poteka tudi pri temperaturah morja, nižjih od 10 ^{o}C, visoki slanosti in nizki oksigenaciji.
8. Pri klapavicah z marteiliozo nismo ugotovili niti znotrajceličnih migetalkarjev klapavic niti novotvorbe hemocitov klapavic.
9. Kondicijski indeks klapavic z marteiliozo je nekoliko nižji kot pri zdravih klapavicah.
10. Temperatura, oksigenacija in slanost morja ne vplivajo na invazijo z *M. refringens*.
11. Število z *M. refringens* invadiranih klapavic v slovenskem morju je bilo prenizko, da bi lahko statistično ustrezno ovrednotili razlike med različnimi lokacijami vzorčenja klapavic, med gojenimi in divjimi klapavicami, med različnimi meseci in razlike v kondicijskem indeksu med invadiranimi in zdravimi klapavicami.
12. Prevalenca invazije z znotrajceličnimi migetalkarji klapavic je v slovenskih klapavicah zmerna – 22,9-odstotna, invazija pa pretežno blaga.
13. Invazija znotrajceličnih migetalkarjev povzroča blago razširitev le tistih epitelnih celic prebavnih kanalčkov slovenskih klapavic, v katerih so zelo veliki migetalkarji.

14. Invazija z znotrajceličnimi migetalkarji ni sezonska; odstotek invadiranih klapavic je najvišji spomladi in najnižji poleti.
15. Invazija z znotrajceličnimi migetalkarji ni v povezavi z načinom in mestom rasti klapavic, prav tako ni povezana z različnimi meseci.
16. Visoka slanost morja ima negativen vpliv na invazijo z znotrajceličnimi migetalkarji, temperatura in oksigenacija pa nanjo nimata vpliva.
17. Znotrajcelični migetalkarji pogosteje naseljujejo težje in večje klapavice, vendar ne vplivajo na kondicijski indeks invadiranih klapavic.
18. Prevalenca novotvorbe hemocitov klapavic je v slovenskih klapavicah 1,1-odstotna.
19. Difuzna oblika novotvorbe hemocitov povzroča nekrozo in žariščno atrofijo prebavnih kanalčkov, posamezne neoplastične celice in neoplastične celice v majhnih skupinah pa na prebavne žleze nimajo vpliva.
20. Pojav novotvorbe hemocitov ni sezonski; bolezen je najpogostejša spomladi in jeseni in najredkejša pozimi.
21. Število slovenskih klapavic z novotvorbo hemocitov je bilo prenizko, da bi lahko statistično ustrezno ovrednotili razlike med različnimi lokacijami vzorčenja klapavic, med gojenimi in divjimi klapavicami, med različnimi meseci, vpliv temperature, slanosti in oksigenacije morja in razlike v kondicijskem indeksu med bolnimi in zdravimi klapavicami.

8 SUMMARY

The health status of cultured and wild Mediterranean mussels (*Mytilus galloprovincialis*) in the Slovene sea was to date unknown. To find out which protozoa and neoplasia affect the digestive gland of Slovene Mediterranean mussels a year-long study was performed. In all 1280 adult Mediterranean mussels from three sampling sites, 480 from a shellfish farm at Seča, 480 from a shellfish farm at Strunjan and 320 from natural beds at Piran (960 cultured and 320 wild mussels) were collected in twelve monthly samplings and included in our study. Water temperature, oxygenation and salinity were measured and shellfish farms were also checked for eventual mortality at each sampling. All mussels were macroscopically inspected and measured. The flesh condition index was calculated for each mussel by weighting the fresh mussel. One slide per mussel containing digestive gland was stained with haematoxylin and eosin and microscopically examined for the presence of protozoa and neoplasia.

No mortality occurred in shellfish farms during the sampling period.

Cultured and wild mussels were almost uniform in length and condition index: the average length was 7 cm in cultured and 7.1 cm in wild mussels and the average condition index was 28.14 in cultured and 29.63 in wild mussels.

Only one mussel showed a severe emaciation all the others were without macroscopically observable changes.

Microscopic examination of digestive glands revealed *Marteilia* spp. in 4 mussels, intracellular ciliates of mussels in 293 mussels and haemocytic neoplasia of mussels in the connective tissue of 14 mussels. The severely emaciated mussel was uninfected and without any tissue alterations.

The prevalence of *Marteilia* spp. was very low, 0.3%. The species determination with PCR-RFLP revealed that all infected mussels carried *Marteilia refringens* type M.

All stages of the life cycle of *M. refringens*, i. e. spherical to elongated multinucleated cells, up to 12 µm in length, and sporangiosorus from 13.3 µm to 21.8 µm in diameter, enclosing 8 spherical sporonts from 4.7 µm to 7.2 µm in diameter, containing 2 to 4 round spores from 1 µm to 2.1 µm in diameter and light refractile inclusion bodies were seen in all four infected mussels. Early stages were predominantly seen in the less infected mussels. Multinucleated cells were nested in epithelial cells of digestive ducts

and tubules, sporangiosori were detected in epithelial cells of digestive gland tubules and ducts and inside their lumen and free spores were sporadically seen in the digestive tubules lumina in all infected mussels.

The infection with *M. refringens* was severe in all four mussels, from above 10% to 100% of infected digestive tubules. Sporadic disrupted epithelial cells of digestive tubules and some totally destructed digestive tubules were noticed in all infected mussels and diffuse haemocytic infiltration and focal granulocytoma in connective tissue of two less infected mussels. No cross infection with intracellular ciliates of mussels and no occurrence of haemocytic neoplasia of mussels was noticed in any of the infected mussels.

M. refringens was detected only in cultured mussels in both shellfish farms at the same prevalence, all wild mussels sampled were uninfected.

M. refringens was more frequently detected in winter (one infected mussel in December, one in January and one in February), when the sea temperature was lower than or equal to 10 ^{o}C, the oxygenation above 10 mg/l and the salinity equal to or above 37‰. Once the infection was noticed in August, when the sea temperature was 22.4 ^{o}C, oxygenation 6.9 mg/l and salinity 40‰. The sampled mussels were uninfected in the other months.

Reliable statistical evaluation of differences in infection with *M. refringens* between cultured and wild mussels, the three different locations of sampling, among different months and sea temperatures, oxygenation and salinities was impossible due to the low number of *M. refringens* positive mussels.

The average condition index of mussels, infected with *M. refringens* was lower than that of uninfected mussels. Statistical confirmation of differences was not possible duo to the small number of positive mussels.

The prevalence of infection with intracellular ciliates of mussels was 22.9%. Intracellular ciliates were pear or spindle-shaped, 3.9 µm to 11.5 µm long and 2.9 µm to 8.4 µm wide with a polymorphic oval to globular basophilic, fragmented macronucleus. They were found inside the digestive tubule epithelia or were in the lumina of digestive tubules.

A slight enlargement of epithelial cells that carried ciliates of a large size was the only alteration of digestive glands tubules observed. A mild diffuse haemocytic infiltration in digestive gland connective tissue was noticed in 2% and haemocytic neoplasia of mussels in 1% of mussels infected with intracellular ciliates of mussels. No *Marteilia* spp. was detected in any of mussels infected with intracellular ciliates.

The prevalence of infection with intracellular ciliates of mussels was 24.4% in cultured mussels (27.3% at Seča and 21.5% at Strunjan) and 18.4% in wild mussels. The differences between cultured and wild mussels and the difference between three different locations and the infection with intracellular ciliates were statistically not relevant.

The highest average prevalence of infection (42%) with intracellular ciliates was detected in spring with an average sea temperature of 15.2 ^{o}C, average oxygenation of 9.3 mg/l and average salinity of 29.6‰. The lowest prevalence (12.7%) was in summer with an average sea temperature of 24.1 ^{o}C, average oxygenation of 7.6mg/l and average salinity of 38.1‰. The highest absolute prevalence was detected in May (47%) with a sea temperature of 20 ^{o}C, oxygenation of 9.1 mg/l and salinity of 26.6‰, and the lowest in September (1.6%) with the sea temperature of 20.3 ^{o}C, oxygenation of 7 mg/l and salinity of 39‰. The differences between the month of sampling and the infection were statistically significant because of the statistically significant relationship between the salinity and the infection. The differences in temperature and the oxygenation and the infection were not statistically significant. We concluded that only salinity had an influence on the incidence of infection with intracellular ciliates - the higher was the salinity the lower was the infection.

A mild infection with intracellular ciliates was detected in 74.4% of infected mussels, a moderate infection in 15% and a severe infection in 10.6% of infected mussels. A mild infection was mostly detected at Strunjan, followed by Seča and Piran, a moderate infection was mostly detected at Seča, followed by Piran and Strunjan and a severe infection at Piran, followed by Seča and Strunjan. The differences between three different locations and the intensity of the infection with intracellular ciliates were statistically not essential. A mild infection was mostly seen in cultured mussels, whereas moderate and severe infection was mostly seen in wild mussels. The differences between cultured and wild mussels and the intensity of the infection with

intracellular ciliates were not statistically significant. A mild infection was in all three sampling locations predominant in the summer with the highest average sea temperatures and salinity and the lowest oxygenation and lowest in the spring. Moderate and a severe infection were highest in spring with medium average sea temperature and oxygenation but with the lowest salinity values and the lowest in summer. Due to the constraints imposed by the sample size, we were not able to determine the significance between the intensity of the infection with intracellular ciliates and month of sampling, but we confirmed that salinity had statistically significant influence on the intensity of the infection, whereas temperature and oxygenation did not.

The average condition index of all infected mussel and of cultured and wild mussels was slightly higher than that of healthy ones. The average condition index of infected mussels from Seča and Piran was slightly higher than that of healthy mussels whereas the condition index in infected and healthy mussels from Strunjan was the same. In all months except in January, May, June and August, the condition index was higher in infected mussels. The differences in condition index between infected and healthy mussels, between cultured and wild mussels, between the three sampling location and between different months were not statistically significant, but ciliates were statistically significantly most often observed in longer and heavier mussels. The average condition index of mussels with a mild and a moderate infection was higher than that of uninfected, whereas in severe infected mussels the condition index was lower than that of uninfected mussels. The differences in the intensity of the infection with intracellular ciliates of mussels and the condition index were not significant.

Haemocytic neoplasia of mussels occurred in 1.1% of mussels. Neoplastic cells were highly pleomorphic and anysocitotic, from 12.3 μm to 30.1 μm in diameter and had large, hyperchromatic rounded or pleomorphic nucleus from 4.3 μm to 22.7 μm in diameter with finely dispersed or dense chromatin without nucleolus. Bi- or even tri-nucleated cells were noticed. The nucleus to cytoplasm ratio was high and the number of mitoses was 2 mitoses per HPF. A diffuse infiltration of neoplastic cells was noticed in four mussels and a small number of single neoplastic cells or small foci of neoplastic cells in connective tissue of digestive glands were observed in 10 mussels.

Necrosis and multifocal atrophy of digestive tubules were observed in Slovene mussels with diffuse neoplasia whereas severe haemocytic infiltration of connective tissue was seen in mussels with single neoplastic cells. In mussels with multifocal form of haemocytic neoplasia no alterations were seen. In 2 mussels with severe haemocytic infiltration and single neoplastic cells and in a mussel with focuses of neoplastic cells a mild infection with intracellular ciliates was observed.

The prevalence of haemocytic neoplasia in cultured mussels was 1.25% and 0.6% in wild mussels. Mussels from Seča and Strunjan exhibited the same prevalence of 1.25%.

Haemocytic neoplasia was more frequently observed in spring, when the sea temperature was between 11 ^{o}C and 20.2 ^{o}C, the oxygenation below 9 mg/l and the salinity between 28‰ and 32‰ and in autumn, when the sea temperature was between 17.8 ^{o}C and 20.3 ^{o}C, the oxygenation below 7.1 mg/l and the salinity between 26‰ and 39‰. Only one affected mussel was detected in winter (in December) with a sea temperature of 9.5 ^{o}C, oxygenation of 10.95 mg/l and salinity of 35‰. In January, February, April, August and November no haemocytic neoplasia of mussels was detected.

The average condition index of all mussels with haemocytic neoplasia and in affected cultured mussels from both shellfish farms was higher than that of healthy ones, but lower in wild mussels. The average condition index of mussels with single neoplastic cells and with multifocal form of haemocytic neoplasia of mussels was higher than that of healthy mussels, whereas the average condition index of mussels with diffuse form of haemocytic neoplasia was equal to the average condition index of unaffected mussels.

Reliable statistical testing of differences in the occurrence of haemocytic neoplasia between the three different locations, between cultured and wild mussels, between different months and between sea temperatures, oxygenation and salinities and in condition index of healthy and that of diseased mussels was not possible due to the low number of mussels with haemocytic neoplasia.

POVZETEK

Zdravstveno stanje gojenih in divjih mediteranskih klapavic (*Mytilus galloprovincialis*) v slovenskem morju je bilo do danes neznano. Da bi ugotovili, katere praživali invadirajo prebavne žleze slovenskih mediteranskih klapavic in katere novotvorbe se pojavljajo v njih, smo opravili enoletno raziskavo, v katero smo vključili 1280 odraslih mediteranskih klapavic – 960 gojenih in 320 divjih. Klapavice smo enkrat mesečno odvzeli iz treh rastišč: školjčišč v Seči in Strunjanu in naravnega rastišča v okolici Pirana, pri čemer smo iz vsakega rastišča ob vsakem vzorčenju odvzeli 40 klapavic. V enoletnem obdobju smo tako odvzeli 480 klapavic iz školjčišča v Strunjanu, 480 iz školjčišča v Seči in 320 iz naravnega rastišča v okolici Pirana. Ob vsakem odvzemu smo na vsakem rastišču izmerili temperaturo, oksigenacijo in slanost morja ter v školjčiščih preverili odstotek smrtnosti klapavic. Pri vseh klapavicah smo z natančnim makroskopskim pregledom ugotavljali morebitne vidne spremembe. Vsaki klapavici smo izmerili dolžino in jo stehtali. Iz lupine smo odstranili mehki del, ga osušili na vpojnem papirju in stehtali ter na podlagi formule teža mesa školjke x 100/težo cele školjke Poševnico za pomen ulomljeno, skozi, na pišemo stično. izračunali školjkin kondicijski indeks. Vsaki školjki smo odvzeli del prebavne žleze, iz nje izdelali tkivno rezino, jo obarvali s hematoksilinom in z eozinom ter s svetlobnim mikroskopom ugotavljali praživali in novotvorbe.

V školjčiščih med vzorčenjem nismo ugotovili smrtnosti klapavic.

Gojene in divje klapavice so imele primerljivo dolžino in kondicijski indeks: povprečna dolžina gojenih klapavic je bila 7 cm, divjih 7,1 cm, povprečen kondicijski indeks gojenih je bil 28,14, divjih pa 29,63.

Z makroskopskim pregledom smo le pri eni klapavici ugotovili močno shiranost, preostale klapavice so bile brez vidnih sprememb.

S svetlobnim mikroskopom smo v prebavnih žlezah štirih klapavic ugotovili praživali iz rodu *Marteilia* (0,3-odstotna prevalenca), znotrajcelične migetalkarje klapavic pri 293 klapavicah (22,9-odstotna prevalenca) in novotvorbo hemocitov pri 14 klapavicah (1,1-odstotna prevalenca). V močno shujšani klapavici nismo opazili praživali niti novotvorb, prebavna žleza je bila nespremenjena.

Vrstna determinacija praživali iz rodu *Marteilia*, opravljena z molekularno metodo PCR-RFLP, je pokazala, da so vse slovenske klapavice invadirane z marteilijami vrste *Marteilia refringens* M-tip.

V vseh štirih invadiranih klapavicah smo opazili vse oblike razvojnega kroga *M. refringens*: okrogle do ovalne večjedrne celice, premera do 12 µm, in sporangiosore s premerom 13,3 do 21,8 µm. V sporangiosorih so bili okrogli sporonti, premera od 4,7 do 7,2 µm, ki so vsebovali dve do štiri okrogle spore s premerom 1 do 2,1 µm in svetlobo lomeče inkluzije. Zgodnje razvojne oblike so prevladovale v dveh manj invadiranih klapavicah. Večjedrne celice so bile ugnezdene v epitelnih celicah prebavnih vodov in kanalčkov, sporangiosore smo opazili v epitelnih celicah prebavnih vodov in kanalčkov in v njihovih svetlinah, proste spore pa smo ugotovili v svetlinah prebavnih kanalčkov vseh invadiranih klapavic.

Invazija z *M. refringens* je bila v vseh klapavicah zelo močna, odstotek invadiranih prebavnih kanalčkov se je gibal od 10 do 100 %. V vseh invadiranih klapavicah smo opazili posamezne propadle celice prebavnih kanalčkov in posamezne nekrotične prebavne kanalčke, difuzno hemocitno infiltracijo in posamezne granulocitome v intersticiju prebavne žleze pa smo ugotovili pri dveh klapavicah s šibkejšo invazijo. V klapavicah, invadiranih z *M. refringens*, nismo našli znotrajceličnih migetalkarjev in novotvorbe hemocitov.

M. refringens smo ugotovili le pri gojenih klapavicah z enako prevalenco v obeh školjčiščih.

Marteilije smo najpogosteje ugotovili pozimi (ena klapavica je bila odvzeta v decembru, ena v januarju in ena v februarju), ko je bila temperatura morja enaka ali nižja 10 ^{o}C, oksigenacija višja od 10 mg/l in slanost enaka ali višja od 37 ‰. Le enkrat smo marteilije diagnosticirali poleti (avgusta), ko je bila temperatura morja 22,4 ^{o}C, oksigenacija 6,9 mg/l in slanost 40 ‰. Klapavice, odvzete v drugih mesecih, so bile negativne na praživali iz rodu *Marteilia*.

Kondicijski indeks z marteilijami invadiranih klapavic je bil nižji kot pri zdravih.

Število klapavic, invadiranih z *M. refringens*, je bilo prenizko, da bi lahko statistično ustrezno ovrednotili razlike med različnimi lokacijami vzorčenja klapavic, med gojenimi in divjimi klapavicami, med različnimi meseci in razlike v kondicijskem indeksu med invadiranimi in zdravimi klapavicami.

Znotrajcelični migetalkarji, ugotovljeni v slovenskih klapavicah, so bili hruškaste ali trnaste oblike, dolgi 3,9 do 11,5 µm in široki 2,9 do 8,4 µm, s polimorfnim, ovalnim do okroglim, bazofilnim, fragmentiranim jedrom. Naseljevali so epitelne celice prebavnih kanalčkov, nekateri so bili tudi prosto v lumnu prebavnih kanalčkov.

Epitelne celice, v katerih so bili zelo veliki migetalkarji, so bile blago razširjene, drugih sprememb v prebavnih kanalčkih nismo ugotovili. Blago difuzno hemocitono infiltracijo veziva prebavnih žlez smo opazili pri 2 %, novotvorbo hemocitov pa pri 1 % invadiranih klapavic. V klapavicah, invadiranih z znotrajceličnimi migetalkarji, nismo ugotovili marteilij.

Prevalenca invazije z migetalkarji je bila pri gojenih školjkah 24,4-odstotna (27,3-odstotna v Seči in 21,5-odstotna v Strunjanu), pri divjih pa 18,4-odstotna. Razlika med gojenimi in divjimi klapavicami in razlike med tremi mesti vzorčenja statistično niso bile bistvene. Prevalenca invazije z migetalkarji je bila najvišja spomladi (42-odstotna), ko je bila povprečna temperatura morja 15,2 ^{o}C, povprečna oksigenacija 9,3 mg/l in povprečna slanost 29,6 ‰, najnižja pa poleti (12,7-odstotna), ko je bila povprečna temperatura morja 24,1 ^{o}C, povprečna oksigenacija 7,6 mg/l in povprečna slanost 38,1 ‰. Najvišjo prevalenco (47-odstotno) smo ugotovili maja, ko je bila temperatura morja 20 ^{o}C, oksigenacija 9,1 mg/l in slanost 26,6 ‰, najnižjo(1,6-odstotno) pa septembra, ko je bila temperatura morja 20,3 ^{o}C, oksigenacija 7 mg/l in slanost 39 ‰. Razlike med posameznimi meseci so bile statistično značilne zaradi statistično signifikantnega vpliva slanosti na invazijo z znotrajceličnimi migetalkarji klapavic. Razlike v temperaturi in oksigenaciji v povezavi z invazijo niso bile statistično značilne. Ugotovili smo, da slanost vpliva na invazijo z znotrajceličnimi migetalkarji klapavic – višja kot je slanost, nižja je invazija.

Blago invazijo smo ugotovili pri 74,4 %, zmerno pri 15 % in močno pri 10,6 % invadiranih klapavic. Odstotek blage invazije je bil najvišji v Strunjanu, sledila sta Seča in Piran, zmerne v Seči, sledila sta Piran in Strunjan, odstotek močne invazije pa v Piranu, sledila sta Seča in Strunjan. Razlike med posameznimi kraji in stopnjo invazije statistično niso bile značilne. Blaga invazija je bila najpogostejša v gojenih, zmerna in močna pa v divjih klapavicah. Razlika med gojenimi in divjimi klapavicami in stopnjo invazije migetalkarjev statistično ni bila značilna. Blaga invazija je na vseh lokacijah vzorčenja prevladovala poleti, ko sta bili temperatura in slanost najvišji, oksigenacija pa

najnižja, najnižja pa je bila spomladi. Zmerna in močna invazija sta bili najvišji spomladi, ko sta bili povprečni vrednosti temperature in oksigenacije morja srednji, slanost pa najnižja, in najnižji poleti. Zaradi premajhnega števila invadiranih klapavic v posameznih skupinah nismo mogli ugotoviti signifikatnosti razlik med stopnjo invazije z znotrajceličnimi migetalkarji klapavic in različnimi meseci, potrdili pa smo signifikantnost vpliva slanosti morja na stopnjo invazije z znotrajceličnimi migetalkarji klapavic.

Povprečni kondicijski indeks vseh klapavic z migetalkarji ter gojenih in divjih klapavic je bil malce višji kot pri zdravih. Povprečni kondicijski indeks invadiranih klapavic iz Seče in Pirana je bil malo višji kot pri zdravih, pri klapavicah iz Strunjana pa je bil v obeh skupinah enak. Z izjemo januarja, maja, junija in avgusta je bil kondicijski indeks invadiranih klapavic višji kot pri zdravih. Razlike v kondicijskem indeksu med invadiranimi in zdravimi gojenimi in divjimi klapavicami, med klapavicami na različnih lokacijah in v različnih mesecih leta statistično niso bile značilne, migetalkarji pa so bili statistično signifikantno večkrat ugotovljeni v daljših in težjih klapavicah. Povprečni kondicijski indeks klapavic z blago in zmerno stopnjo invazije migetalkarjev je bil višji kot pri zdravih, pri tistih z visoko stopnjo invazije pa nižji. Razlike v stopnji invazije z znotrajceličnimi migetalkarji in kondicijskim indeksom školjk statistično niso bile značilne.

Neoplastične celice novotvorbe hemocitov klapavic so bile zelo pleomorfne in anizocitotične, s premerom od 12,3 µm do 30,1 µm. Imele so veliko, hiperkromatično okroglo ali pleomorfno jedro, premera 4,3 µm do 22,7 µm, z dobro razpršenim kromatinom brez jedrca. Med njimi so bile posamezne dve- ali trijedrne celice. Razmerje med jedrom in citoplazmo je bilo visoko, našteli smo dve mitozi pri veliki povečavi (HPF). Difuzno infiltracijo neoplastičnih celic smo ugotovili pri štirih klapavicah, posamezne neoplastične celice in majhna žarišča neoplastičnih celic pa pri 10 klapavicah.

Pri klapavicah z difuzno obliko novotvorbe smo opazili nekrozo in večžariščno atrofijo prebavnih kanalčkov, pri klapavicah s posameznimi neoplastičnimi celicami pa smo ugotovili močno difuzno hemocitno infiltracijo v intersticiju prebavne žleze. Pri dveh klapavicah z difuzno obliko novotvorbe, dveh klapavicah s posameznimi neoplastičnimi

celicami in klapavici z žariščno obliko novotvorbe smo ugotovili blago invazijo z znotrajceličnimi migetalkarji. Marteilij pri klapavicah z novotvorbo hemocitov nismo diagnosticirali.

Prevalenca novotvorbe hemocitov je bila pri gojenih klapavicah 1,25-odstotna, pri divjih pa 0,6-odstotna. Pri gojenih klapavicah iz Seče in Strunjana je bila prevalenca enaka – 1,25-odstotna.

Novotvorbo hemocitov smo najpogosteje zaznali spomladi, ko je bila temperatura vode med 11 ^{o}C in 20,2 ^{o}C, oksigenacija pod 9 mg/l in slanost med 28 ‰ in 32 ‰, in jeseni, ko je bila temperatura morja med 17,8 ^{o}C in 20,3 ^{o}C, oksigenacija pod 7,1 mg/l in slanost med 26 ‰ in 39 ‰. Pozimi (v decembru), ko je bila temperatura morja 9,5 ^{o}C, oksigenacija 10,95 mg/l, slanost 35 ‰, smo le v eni klapavici ugotovili novotvorbo hemocitov. Bolezni nismo zaznali januarja, februarja, aprila, avgusta in novembra.

Povprečni kondicijski indeks vseh klapavic z novotvorbo hemocitov in gojenih klapavic je bil višji kot pri zdravih, pri bolnih divjih klapavicah pa nižji. Povprečni kondicijski indeks klapavic s posameznimi neoplastičnimi celicami in z majhnimi skupinami neoplastičnih celic je bil višji kot pri zdravih, pri klapavicah z difuzno obliko novotvorbe pa enak povprečnemu kondicijskemu indeksu zdravih klapavic.

Število klapavic z novotvorbo hemocitov je bilo prenizko, da bi lahko statistično ustrezno ovrednotili razlike med različnimi lokacijami vzorčenja klapavic, med gojenimi in divjimi klapavicami, med različnimi meseci in razlike v kondicijskem indeksu med bolnimi in zdravimi klapavicami ter vpliv temperature, slanosti in oksigenacije morja na pojav novotvorbe hemocitov klapavic.

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