MOLECULAR IDENTIFICATION OF FRESHWATER PATHOGENIC OOMYCETES AND DETERMINATION OF NOVEL FACTORS THAT PROMOTE THEIR SPREADING

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DOCTORAL DISSERTATION

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Supervisor:

PhD, Ana Bielen, Associate Professor

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The doctoral dissertation was carried out as a part of the Doctoral Programme in Biology at the University of Zagreb, Faculty of Science, Department of Biology, under the supervision of Assoc. Prof. Ana Bielen, PhD. The research presented in this dissertation was supported by the Croatian Science Foundation project: "Interactions of freshwater pathogenic oomycetes and the environment" (InteractOomyc, UIP-2017-05-6267, PI: Assoc. Prof. Ana Bielen, PhD). This doctoral dissertation was made at the University of Zagreb, Faculty of Food Technology and Biotechnology, Laboratory for Biology and Microbial Genetics.

Mentor’s Biography

Ana Bielen (July 31st 1979), PhD, Associate Professor at the Faculty of Food Technology and Biotechnology, University of Zagreb

Assoc. Prof. Ana Bielen has been working in the Laboratory for Biology and Microbial Genetics at the Faculty of Food Technology and Biotechnology, University of Zagreb, since 2003. She received her PhD in molecular microbiology from the Faculty of Science, University of Zagreb in 2011.

Her scientific interest is in the microbial ecology of pathogenic freshwater oomycetes with a focus on the development of molecular methods for their detection and monitoring as well as environmentally friendly methods for their control in aquaculture. To date, she has led three national and one international scientific project and collaborated on ten national and three international projects. Currently, she is the PI of the installation research project of the Croatian Science Foundation "Interactions of freshwater pathogenic oomycetes and the environment" (InteractOomyc). She has co-authored 30 scientific articles in internationally recognised journals in the field of environmental science and molecular microbiology, most of them in the first and second quartile. She presented the results of her research at several scientific conferences. She has conducted parts of her research at prestigious foreign institutions (Graz University of Technology, Austria; University of Pau, France; Helmholtz Centre Munich, Germany) and participated in numerous national and international courses.

She has supervised 2 PhD theses, 5 master and 8 bachelor theses, as well as 3 student research projects awarded with the Rector's Award at the University of Zagreb. She is a co-author of two university textbooks "Methods in Molecular Biology" and "Handbook of Biology 1". She is a member of several professional societies and is particularly active in the Croatian Microbiological Society, where she held the position of Secretary from 2018 to 2019. She has participated in the organisation of several international scientific conferences and was the editor of two international books of abstracts. She received the Award of the Society of University Teachers and Other Scientists in Zagreb for Young Scientists and Artists in 2009, the first prize in the competition "ddPCR challenge" in 2019 and the Award of the Faculty of Food Technology and Biotechnology for an outstanding contribution to research in 2020. She is continuously involved in science communication and popularisation.

Zahvale

Prvo želim zahvaliti svojoj mentorici, izv. prof. dr. sc. Ani Bielen, što me je uvela u svijet molekularne mikrobiologije i bila velika podršku tijekom svih ovih godina. Hvala joj na savjetima, ukazanom povjerenju kao i svim pohvalama i kritikama. Ana, hvala što si mi bila više od mentorice, veselim se svim budućim mozganjima, projektima i druženjima. Uz tebe svaki problem ima rješenje.

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University of Zagreb
Faculty of Science
Department of Biology

Doctoral dissertation

MOLECULAR IDENTIFICATION OF FRESHWATER PATHOGENIC OOMYCETES AND DETERMINATION OF NOVEL FACTORS THAT PROMOTE THEIR SPREADING

DORA PAVIĆ

Department of Biology, Faculty of Science, University of Zagreb, Rooseveltov trg 6, 10 000 Zagreb

Aphanomyces astaci and *Saprolegnia* spp. are considered as the most destructive Oomycota pathogens of freshwater animals, both in natural habitats and in aquaculture, and this dissertation addressed several understudied aspects of their ecology. First, a highly sensitive *S. parasitica*-specific ddPCR assay was developed, which allowed absolute quantification of the pathogen in environmental DNA samples. Further, the results obtained provided information on the microbiological and physico-chemical properties of natural waters that favour the sporulation and spread of these pathogens, such as environmentally relevant concentrations of humic acids. Finally, the role of aquaculture facilities as points of spreading of *Saprolegnia* spp. into downstream freshwater environments was demonstrated. Overall, the knowledge gained provides the basis for sensitive monitoring and predicting the various factors that could promote the spread of *A. astaci* and *Saprolegnia* spp. in the wild and aquaculture.

(111 pages, 11 figures, 3 tables, 287 references, original in English)

Key words: *Aphanomyces astaci*, crayfish, ddPCR, *Saprolegnia parasitica*, trout, water quality

Supervisor: PhD Ana Bielen, Associate Professor

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PhD Perla Tedesco, Researcher

Sveučilište u Zagrebu
Prirodoslovno-matematički fakultet
Biološki odsjek

Doktorska disertacija

MOLEKULARNA IDENTIFIKACIJA SLATKOVODNIH PATOGENIH OOMYCOTA I UTVRĐIVANJE NOVIH ČIMBENKA KOJI POTIČU NJIHOVO RASPROSTRANJIVANJE

DORA PAVIĆ

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Aphanomyces astaci i *Saprolegnia* spp. najznačajniji su oomicetni patogeni slatkovodnih životinja, kako u prirodnim staništima tako i u akvakulturi. U sklopu ove disertacije dobivena su nova saznanja o nekim aspektima njihove ekologije. Najprije je razvijen visoko osjetljiv i specifičan ddPCR-test za detekciju vrste *S. parasitica*, čime je po prvi puta omogućena apsolutna kvantifikacija ovog patogena u uzorcima okolišne DNA. Nadalje, utvrđena su mikrobiološka i fizikalno-kemijska svojstva prirodnih voda koja potiču sporulaciju i širenje oomicetnih patogena, kao što je prisutnost huminskih kiselina u okolišno relevantnim koncentracijama. Naposlijetku, demonstrirana je uloga uzgajališta pastrve u širenju patogena *Saprolegnia* spp. u nizvodna prirodna staništa. Zaključno, rezultati ove disertacije omogućit će praćenje oomicetnih patogena u prirodnim slatkovodnim staništima i akvakulturi, kao i predviđanje različitih čimbenika koji potiču njihovo rasprostranjivanje.

(111 stranice, 11 slike, 3 tablice, 287 literaturnih navoda, jezik izvornika: engleski)

Ključne riječi: *Aphanomyces astaci*, ddPCR, kvaliteta vode, pastrve, *Saprolegnia parasitica*, slatkovodni rakovi

Mentor: Izv. prof. dr. sc. Ana Bielen

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Doc. dr. sc. Marin Ježić
Doc. dr. sc. Perla Tedesco

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List of publications

This doctoral dissertation is based on the publications listed below. The publications are referred to in the text by their assigned Roman numerals.

I. **Pavić D**, Grbin D, Hudina S, Prosenc Zmrzljak U, Miljanović A, Košir R, Varga F, Ćurko J, Marčić Z, Bielen A (2022) Tracing the oomycete pathogen *Saprolegnia parasitica* in aquaculture and the environment. Scientific Reports 12:16646.

II. **Pavić D**, Miljanović A, Grbin D, Šver L, Vladušić T, Galuppi R, Tedesco P, Bielen A (2021) Identification and molecular characterization of oomycete isolates from trout farms in Croatia, and their upstream and downstream water environments. Aquaculture 540:736652.

III. **Pavić D**, Grbin D, Gregov M, Ćurko J, Vladušić T, Šver L, Miljanović A, Bielen A (2022) Variation in the sporulation efficiency of pathogenic freshwater oomycetes in relation to the physico-chemical properties of natural waters. Microorganisms 10:520.

1. INTRODUCTION

1.1. Oomycota as pathogens of freshwater animals

Oomycota, commonly referred to as water moulds, are fungal-like filamentous microorganisms (Beakes et al., 2012; Phillips et al., 2008). According to available phylogenomic studies, the Oomycota are positioned within the eukaryotic supergroup Stramenopiles-Alveolata-Rhizaria (SAR), which also includes other parasites (e.g. *Plasmodium* spp.) and photosynthetic microbial lineages (e.g. diatoms) (Burki, 2014; Grattepanche et al., 2018; Judelson, 2012; Tsui et al., 2009). Recent whole-genome phylogenetic analyses have confirmed the existence of four well-supported orders of Oomycota (Peronosporales, Pythiales, Albuginales and Saprolegniales), with the Saprolegniales (including the genera *Aphanomyces* and *Saprolegnia*) representing the basal order (Figure 1) (McCarthy and Fitzpatrick, 2017).

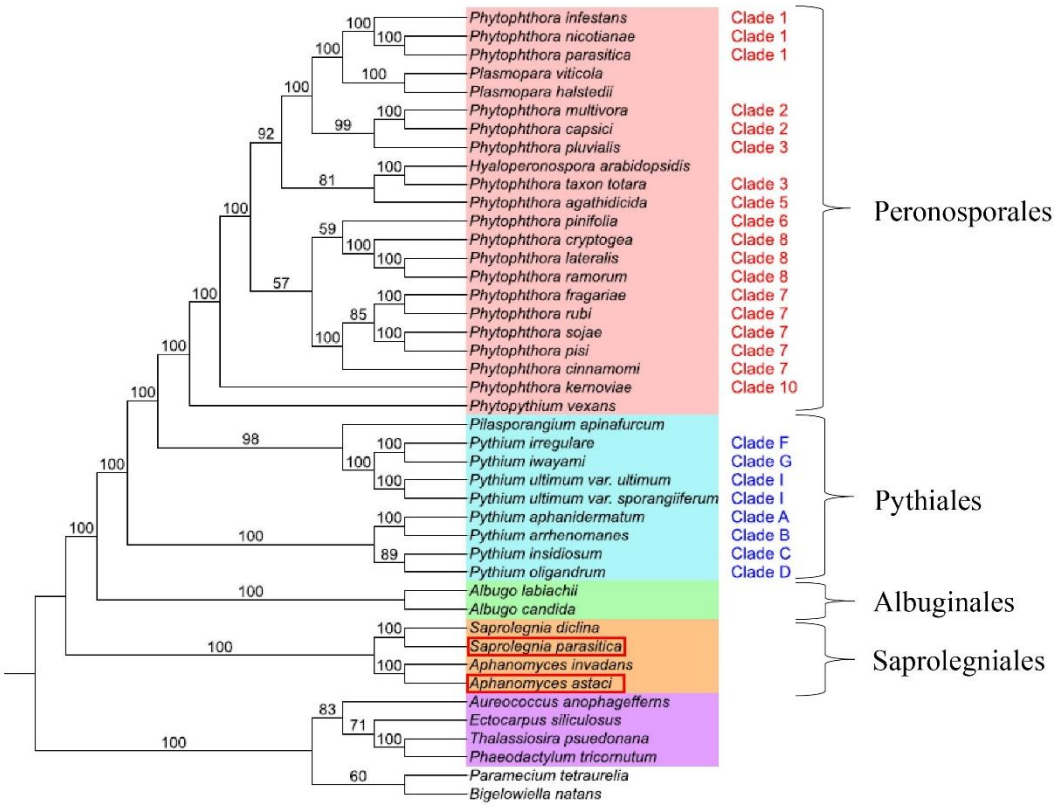


Figure 1. Whole-genome phylogenetic tree of Oomycota. Species that were used as model organisms in this dissertation, *Saprolegnia parasitica* and *Aphanomyces astaci*, are marked by red rectangles. Non-Oomycota species belonging to the SAR clade (purple and no colour) represent an outgroup (McCarthy and Fitzpatrick, 2017).

Oomycota have a diverse lifestyle, either feeding on non-living organic matter (saprotrophs) or living as parasites on various host species (biotrophs, pathogens). Pathogenic Oomycota frequently cause disease outbreaks in agriculture and aquaculture as well as in natural ecosystems, making them a threat to biodiversity and food security worldwide (Fisher et al., 2012; Phillips et al., 2008). Most of the Oomycota species described to date are plant pathogens (McGowan and Fitzpatrick, 2020). Among them, members of the genus *Phytophthora* are of particular importance, including *P. infestans*, the causal agent of potato late blight, which is responsible for significant economic losses in the potato industry (Cooke and Lees, 2004). Compared to these plant-pathogenic species important for agriculture, animal pathogens are understudied, although they are also responsible for serious diseases worldwide (Becking et al., 2022; Derevnina et al., 2016; Fisher et al., 2012; Gozlan et al., 2014). The most important animal pathogenic Oomycota cause diseases in freshwater animals and belong to the genera *Aphanomyces* and *Saprolegnia* (order Saprolegniales). Crayfish plague, caused by the pathogen *Aphanomyces astaci* Schikora, 1906, is considered a major cause of mass mortality of native crayfish species in Europe and other parts of the world (i.e. Asia and South America) (Holdich et al., 2009; Martín-Torrijos et al., 2018, 2021b; Peiró et al., 2016; Putra et al., 2018). Saprolegniosis is mainly a fish disease caused by several *Saprolegnia* species and affects all developmental stages, from eggs to juveniles and adults. It is a significant problem in salmonid farms and hatcheries with annual economic losses of more than 10% and occasionally up to 50% (Bruno et al., 2011; Diéguez-Uribeondo et al., 2007; Gozlan et al., 2014; van den Berg et al., 2013; van West, 2006). *Saprolegnia* species have a much wider host range than the crayfish pathogen *A. astaci*. Besides a wide range of fish species, especially salmonids (Gozlan et al., 2014), they can also infect other aquatic animals such as crayfish (Dieguez-Uribeondo et al., 1994), amphibians (Fernández-Benéitez et al., 2008; Kiesecker et al., 2001) and insects (Holt et al., 2021).

The complete life cycle of Oomycota consists of a sexual and an asexual part and is shown schematically in Figure 2 using *S. parasitica* as an example. The asexual stage involves the coenocytic, multinucleate hyphae that produce sporangia and release motile zoospores, crucial for the pathogen's dispersal. Sexual part of the life cycle involves the production of oospores, which play an important role in increasing genetic diversity and enable survival in harsh conditions (Diéguez-Uribeondo, 2009; van den Berg et al., 2013). However, a sexual stage has not been documented for all Oomycota species and *A. astaci* is an example of such a species where oospores

have not yet been observed (Diéguez-Uribeondo, 2009; Royo et al., 2004; Söderhäll and Cerenius, 1999). The most important asexual infection stage are zoospores, which are released from sporangia into the water (Diéguez-Uribeondo, 2009; Oidtmann et al., 2002; Phillips et al., 2008). Zoospores have two lateral flagella and can travel long distances until they find a suitable host through chemotaxis (Alderman and Polglase, 1986; Cerenius and Söderhäll, 1984a; Unestam, 1969; van den Berg et al., 2013). After arriving on the surface of the crayfish cuticle and/or the skin of fish, they encyst, the cysts germinate into hyphae and invade the host's body, affecting internal tissues and organs (Bruno and Wood, 1999; Cerenius et al., 1988; Hatai and Hoshiai, 1994; Willoughby, 1994). When the host is overcome with the disease, hyphal tips with zoosporangia grow out of the body surface and the zoospores are released into the water again, completing the life cycle (Diéguez-Uribeondo et al., 2009; Oidtmann et al., 2002). If the zoospores do not find a suitable host, they encyst and the cysts release new zoospores in a process called repeated zoospore emergence (RZE) or polyplanetism. RZE occurs in both *A. astaci* and *S. parasitica*, meaning that the zoospores have multiple opportunities to find and infect a suitable host (Cerenius and Söderhäll, 1984b, 1984a; Diéguez-Uribeondo et al., 2007, 1994)

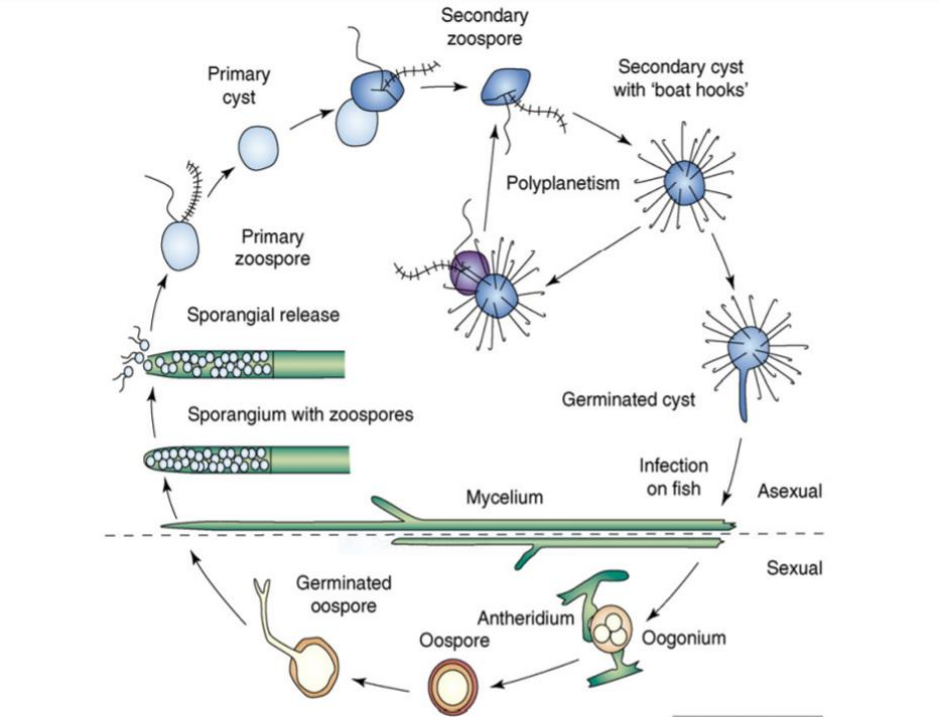


Figure 2. *Saprolegnia parasitica* life cycle (Phillips et al., 2008).

1.1.1. Oomycota pathogens from the genus *Saprolegnia*, causative agents of saprolegniosis

Several species from the genus *Saprolegnia* (*S. parasitica, S. diclina, S. ferax, S. australis* and others) are known to cause saprolegniosis, a disease that affects a wide range of animals, mostly freshwater fish (Cao et al., 2012; Eissa et al., 2013; Gozlan et al., 2014; Hatai and Hoshiai, 1994; Rezinciuc et al., 2014b; Sandoval-Sierra et al., 2014b). Species from genus *Saprolegnia* are usually considered opportunistic secondary pathogens that infect host individuals under stress, with the possible exception of *S. parasitica* that might act as a primary pathogen (Gozlan et al., 2014; Noga, 1993; van den Berg et al., 2013). Conditions such as other infections, injuries, changes in water temperature, fish reaching sexual maturity associated with increased cortisol levels, reduced water quality, poor hygiene or high densities of host species can weaken immune defences and lead to higher infection rates (Bly et al., 1993; Casas-Mulet et al., 2021; Diéguez-Uribeondo et al., 2007; Pickering, 1994; Sarowar et al., 2019a; Stewart et al., 2018; Stueland et al., 2005).

Saprolegnia species have a wide host range and usually cause major problems in farmed fish, but also infect other aquatic animals such as amphibians and crustaceans (Blaustein et al., 1994; Bruno et al., 2011; Dieguez-Uribeondo et al., 1994; Gozlan et al., 2014; Holt et al., 2021). Although infections usually occur and are more severe in aquaculture facilities than in the natural environment, disease outbreaks have also been reported in wild fish (Diéguez-Uribeondo et al., 1996; Neitzel et al., 2004; Ravasi et al., 2018; Richards and Pickering, 1978; White, 1975). Among the *Saprolegnia* spp., *S. parasitica* has the highest virulence and is therefore sometimes even considered the primary pathogen (Elameen et al., 2021; Neish and Hughes, 1980; Whisler, 1996; Willoughby and Pickering, 1977). In addition, saprolegniosis is a serious problem for hatcheries, with fish eggs predominantly infected with *S. australis* and *S. diclina* (Cao et al., 2012; Fregeneda-Grandes et al., 2007; Meyer, 1991; Sandoval-Sierra et al., 2014a; Thoen et al., 2011). The main symptom of the disease are white and grey patches of cottony mycelium growing anywhere on the fish body (Figure 3) or on the surface of the eggs or embryos (Bruno et al., 2011; Fregeneda Grandes et al., 2001; Willoughby, 1994, 1989). In adult fish, the final stage of infection leads to impaired osmoregulation, followed by the destruction of the epidermis and respiratory arrest, while the infected fish eggs die due to hyphal rupture of the chorionic membrane, leading to disruption of the osmotic balance of the embryo (Liu et al., 2014; Pickering and Willoughby, 1982).

Saprolegnia spp. are considered ubiquitous in freshwater environments (Bruno et al., 2011; Johnson et al., 2002; van West, 2006), although the lack of rapid and effective molecular

monitoring methods limits the collection of data on their distribution, especially in the wild. In aquaculture, *S. parasitica* is the most commonly reported *Saprolegnia* species on adult fish (Hussein and Hatai, 2002; Ravasi et al., 2018; Sandoval-Sierra et al., 2014a; Sarowar et al., 2019a; van den Berg et al., 2013), while *S. australis*, *S. diclina*, *S. delica* and *S. ferax* are mostly detected on fish embryos, i.e. eggs and alevins (Cao et al., 2012; Fregeneda-Grandes et al., 2007; Magray et al., 2021; Rezinciuc et al., 2014b; Sandoval-Sierra et al., 2014a; Tandel et al., 2021; Thoen et al., 2011).

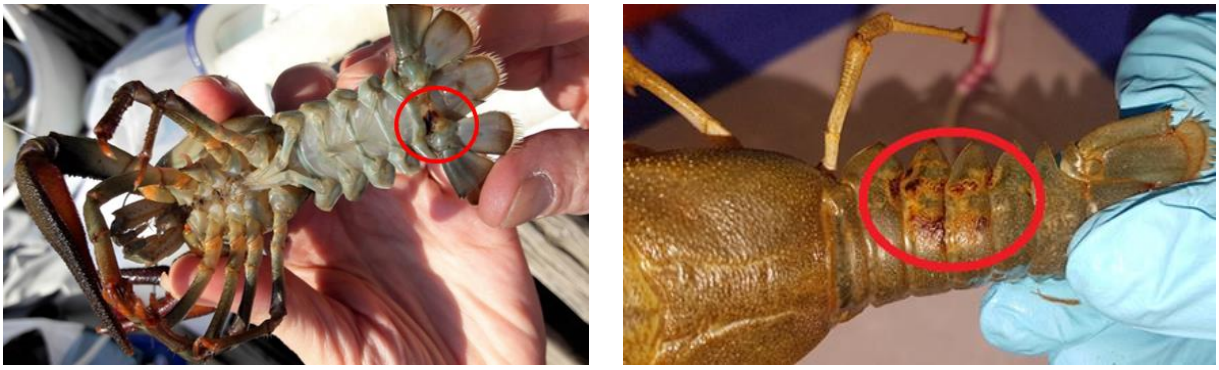
Figure 3. Rainbow trout (*Oncorhynchus mykiss*) infected with saprolegniosis (own photograph). Oomycota mycelium is visible on the fish tail (encircled in red).

1.1.2. *Aphanomyces astaci*, the causative agent of crayfish plague

Aphanomyces astaci causes crayfish plague, a disease responsible for the decimation and near-extinction of many native crayfish populations in Europe, Asia and South America (Alderman, 1996; Holdich et al., 2009; Hsieh et al., 2016; Martín-Torrijos et al., 2018; Peiró et al., 2016). Due to its rapid spread and destructive impact, it has been listed among the 100 worst invasive alien species in the world (Lowe et al., 2000). The pathogen was introduced to Europe in the 19th century along with North American invasive crayfish species, which are mostly resistant to infection and act as pathogen carriers in European waters (Aydin et al., 2014; Holdich et al., 2009; Jussila et al., 2014; Svoboda et al., 2017). In contrast, when native crayfish come into contact with the zoospores of *A. astaci*, they usually develop a lethal disease (Becking et al., 2015; Makkonen et al., 2014). However, factors such as moulting, reproduction, poor environmental conditions or infestation by various parasites can weaken even the more resistant non-native

American crayfish's immune defences and thus lead to the development of crayfish plague (Aydin et al., 2014; Cerenius et al., 2003; Thomas et al., 2020).

Crayfish plague is caused by the penetration of hyphae through the cuticle and their subsequent spread into the internal tissues of crayfish (Cerenius et al., 2003; Soderhall and Cerenius, 1992). The first signs of the disease are melanisation spots seen on the crayfish carapace or abdomen (Figure 4). Such melanisations result from the localised crayfish's innate immune response to the hyphal penetration and can also be found during latent infection in carrier individuals (Jussila et al., 2014; Svoboda et al., 2017). As the disease progresses, more serious symptoms occur, such as daytime activity, loss of limbs or abdominal paralysis, leading to death (Alderman et al., 1987; Martín-Torrijos et al., 2017).



Astacus astacus *Pontastacus leptodactylus*

Figure 4. Symptoms characteristic of *Aphanomyces astaci* infection: melanisation spots on the abdomen/exoskeleton of the noble crayfish *Astacus astacus* (own photo) and narrow-clawed crayfish *Pontastacus leptodacytalus* (photo by Anđela Miljanović).

Five genotype groups of *A. astaci* have been identified so far, which differ in virulence and the host they were originally isolated from: A (As), B (PsI), C (PsII), D (Pc) and E (Or) (Diéguez-Uribeondo et al., 1995; Huang et al., 1994; Rezinciuc et al., 2014a). Genotype group A (As) was isolated during an outbreak of crayfish plague in the native European noble crayfish *Astacus astacus* and is associated with the first invasion of *A. astaci* in Europe in the 19th century, but the original host is still unknown (Huang et al., 1994). The remaining genotype groups are associated with non-native crayfish species. Genotype groups B (PsI) and C (PsII) have been isolated from the North American signal crayfish *Pacifastacus leniusculus*. Genotype C (PsII) was isolated

during a disease outbreak in Sweden in 1978 from signal crayfish which originated from Canada (Lake Pitt) and has not been detected since (Huang et al., 1994; Makkonen et al., 2018; Söderhäll and Cerenius, 1999), while genotype B (PsI) is now the most common genotype group of *A. astaci* in Europe (Kouba et al., 2014). Group D (Pc) was isolated from the red swamp crayfish *Procambarus clarkii*, which was introduced to Spain (Diéguez-Uribeondo et al., 1995; Diéguez-Uribeondo and Söderhäll, 1993; Rezinciuc et al., 2014a), while group E (Or) was isolated from the spiny-cheek crayfish *Faxonius limosus*, which was first introduced from North America to a fish farm in Poland in 1890 (Diéguez-Uribeondo et al., 2006; Kozubíková-Balcarová et al., 2013; Kozubíková et al., 2011). Strains of different genotype groups may differ in virulence, with genotypes B and D being more virulent than genotype A towards native species such as noble crayfish (Becking et al., 2015; Jussila et al., 2011; Kokko et al., 2012; Makkonen et al., 2014, 2013).

Different *A. astaci* genotype groups have spread unevenly across European continent (Figure 5), with genotype group B being the most widespread, together with its non-native carrier, signal crayfish (Kouba et al., 2014). Outbreaks of crayfish plague caused by the group B genotype have been recorded in southern Finland, Norway, United Kingdom, France and northern Spain, as well as in central Europe and Italy. Group B has also been isolated from *Pontastacus leptodactylus*, which occurs in Turkey and eastern European rivers, but without disease outbreaks, suggesting elevated resistance of this crayfish species towards the pathogen (Jussila et al., 2020; Kokko et al., 2018). Outbreaks caused by group A have been recorded in Fennoscandia, the Czech Republic, Croatia, Italy and Bosnia. Similar to group B, chronic infections were found in native European crayfish in Finland, the Julian and Dinaric Alps and Turkey. Group D was found mainly in Spain, but some outbreaks of this genotype were also recorded in France, Italy and Central Europe, while group E is prevalent in the Czech Republic and has been reported in France and Estonia (Ungureanu et al., 2020).

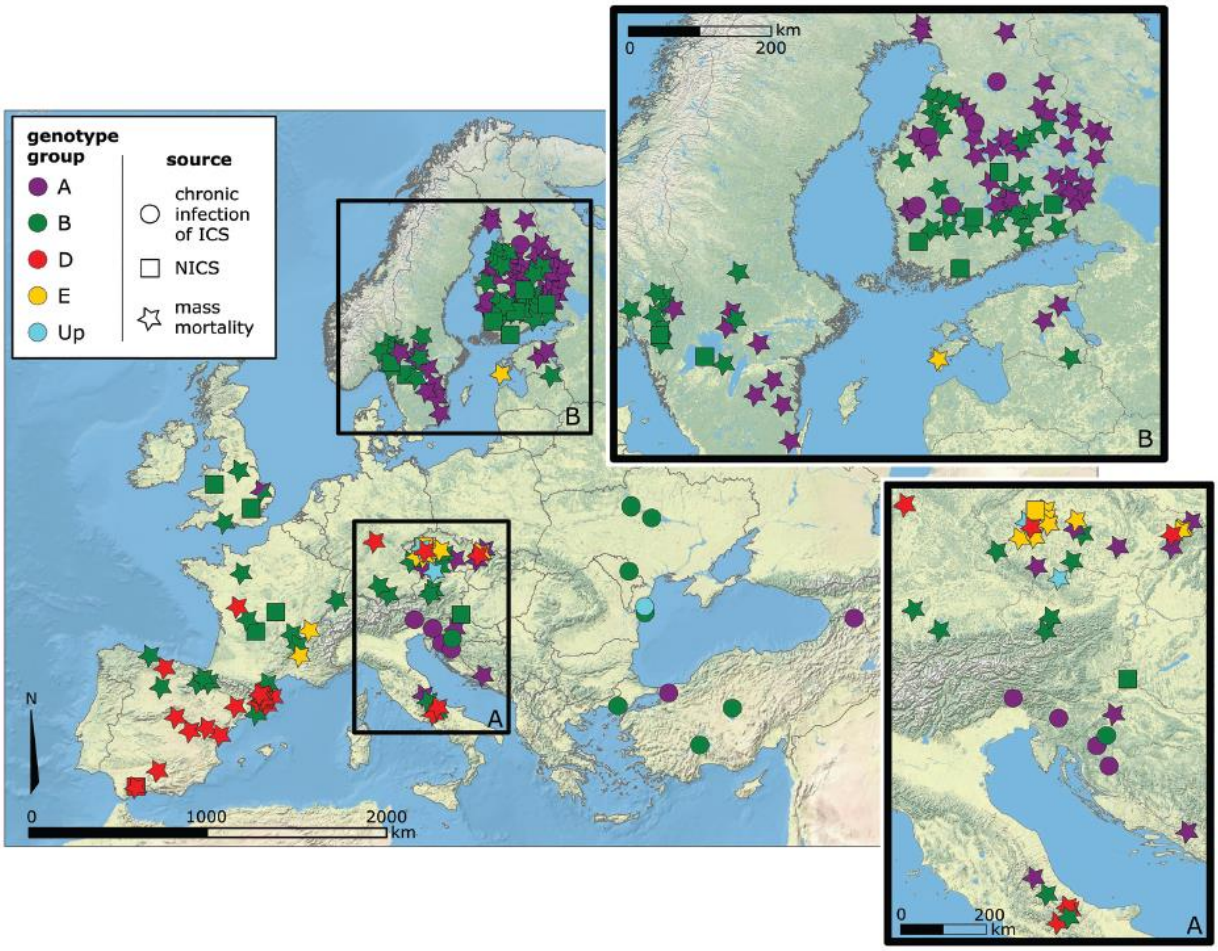


Figure 5. Distribution of *A. astaci* genotype groups in Europe in the last three decades. ICS – indigenous crayfish species, NICS – non-indigenous crayfish species, Up – unusual microsatellite multilocus genotype reported in Czechia and Croatia (Maguire et al., 2016; Ungureanu et al., 2020).

Croatian freshwater ecosystems are inhabited by four native crayfish species (noble crayfish *As. astacus*, white-clawed crayfish *Austropotamobius pallipes*, stone crayfish *Au. torrentium* and narrow-clawed crayfish *Po. leptodactylus*) and by three invasive crayfish species (spiny-cheek crayfish *F. limosus*, signal crayfish *Pa. leniusculus* and marbled crayfish *Procambarus virginalis*) (Figure 6a) (Maguire et al., 2018). *Aphanomyces astaci* distribution was monitored and genotyped in Croatia since 2003 (Figure 6b). The pathogen has been detected in all native crayfish species, with genotype A identified in all four species, while genotype group B has only been detected in *Po. leptodactylus* (Maguire et al., 2016; Pavić et al., 2021). As far as non-native crayfish species are concerned, group B was detected in *Pa. leniusculus*, as expected

(Maguire et al., 2016). *Aphanomyces astaci* presence was also detected in *F. limosus* but the level of infection was too low for genotype group determination, therefore the assumption that it carries genotype E still needs to be confirmed experimentally (Maguire et al, 2016). In the case of *Pr. virginalis*, it is still not known whether this species carries a new, undescribed *A. astaci* genotype group or one of the groups already described (Keller et al., 2014). Therefore, further studies on the *A. astaci* status of these two invasive crayfish species in Croatia are needed.

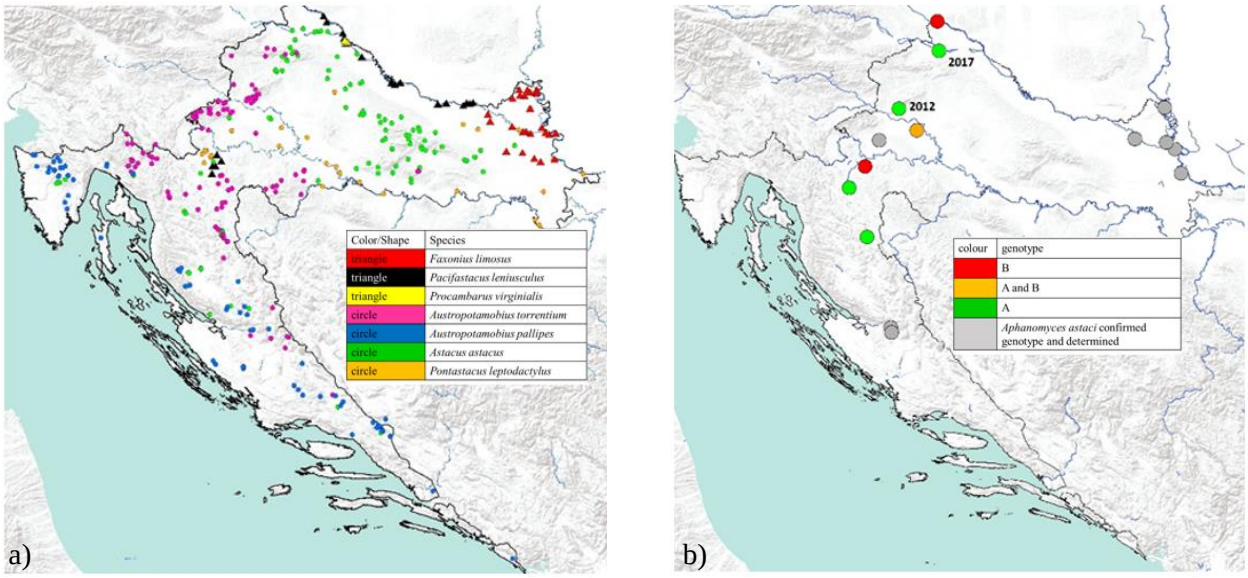


Figure 6. (a) Distribution of crayfish species in Croatia (Maguire et al., 2018, I. Maguire unpublished data). (b) Distribution in of *Aphanomyces astaci* genotypes in Croatia (Maguire et al., 2016, Pavić et al. 2021, 2020). Years of the recently reported crayfish plague outbreaks are noted on the map.

1.2. Development of molecular methods for detection and identification of pathogenic Oomycota in freshwater ecosystems

Pathogenic Oomycota have recently been classified as emerging pathogens due to their ever-increasing geographical distribution, increase in virulence and in number of known hosts (Fisher et al., 2012; Walker and Winton, 2010). For example, the rapid spread of the crayfish pathogen *A. astaci* that has been demonstrated in recent years is mainly linked to the spread of invasive North American crayfish species, which serve as vectors and contribute to the transmission of the pathogen in Europe and worldwide (Holdich et al., 2009; Kouba et al., 2014;

Svoboda et al., 2017). Continuous monitoring of pathogenic freshwater Oomycota is needed to gain an accurate insight into changes in their distribution both in the natural environment and in aquaculture. However, for most pathogenic Oomycota in freshwater systems there are virtually no tests for detection and genotyping, regardless of their rapid spread and detrimental effects. The only exception is *A. astaci*, for which molecular monitoring methods are fairly well developed (Di Domenico et al., 2021; Francesconi et al., 2021; Grandjean et al., 2014; Makkonen et al., 2018; Minardi et al., 2019, 2018; Oidtmann et al., 2006; Tuffs and Oidtmann, 2011; Vrålstad et al., 2009).

The ideal monitoring method for detecting pathogens of interest in freshwater ecosystems should be (i) rapid, (ii) specific (detects only the target species, not closely related species), (iii) sensitive (can detect low levels of the target in the sample), (iv) quantitative (allows the quantification of the pathogen load in the sample), and (v) non-destructive (can detect the target in environmental sample without killing the often endangered host species individuals). In addition, one might aim to detect the pathogen at the species level (as described in section 1.2.1.) or at the genotype level (as described in section 1.2.2.), as genotypes may differ in virulence.

1.2.1. *Aphanomyces astaci* and *Saprolegnia parasitica* identification/detection methods

Traditionally, Oomycota species are distinguished based on the structures produced during asexual or sexual reproduction, i.e. asexual sporangia, and sexual oogonia, antheridia and oospores. The prerequisite for such identification is the isolation of the target species in pure culture and the microscopic determination of the morphological characteristics (Cerenius et al., 1988; Diéguez-Uribeondo et al., 2007; Johnson et al., 2002; Viljamaa-Dirks and Heinikainen, 2006). However, this process is laborious, time-consuming and often unsuccessful. For example, isolation of *A. astaci* from infected crayfish is known to be difficult because it grows slowly and when grown *in vitro* is often overgrown by other water moulds or fungi from the cuticle of the crayfish. In addition, its growth can also be inhibited by the present bacteria (Cerenius et al., 1988; Oidtmann et al., 1999). Even after successful isolation of the Oomycota pathogen, the structures needed for identification are often difficult to induce in the laboratory conditions and/or cannot be used to distinguish between closely related species. For example, although both sexual and asexual stages have been described in the genus *Aphanomyces*, for animal pathogenic *Aphanomyces* species, including *A. astaci*, the sexual stage has not been observed (Diéguez-Uribeondo et al., 2009; Royo et al., 2004; Söderhäll and Cerenius, 1999). Therefore, the identification of *A. astaci* has

traditionally relied on characteristics that arise during asexual reproduction. As these were not sufficient for the identification of *A. astaci*, it was necessary to perform an infection experiment with susceptible crayfish (Alderman and Polglase, 1986; Cerenius et al., 1988; Oidtmann et al., 1999). Moreover, some closely related *Aphanomyces* spp. such as *A. astaci* and *A. fennicus* are morphologically indistinguishable (Figure 7; Viljamaa-Dirks and Heinikainen, 2018). In the case of *Saprolegnia* species, until recently pure cultures for identification had to be obtained from clinical samples (e.g. tissue samples from embryonic and adult fish) or from hemp seed baits that were used to capture the pathogen from water (Sandoval-Sierra et al., 2014a; Seymour, 1970; Tedesco et al., 2021). Then, the examination of sexual structures such as oogonia, antheridia and oospores was done (Coker, 1923; Johnson et al., 2002; Seymour, 1970). However, isolates sometimes fail to develop sexual structures *in vitro*, and species identification based only on asexual structures is not possible (Seymour, 1970). Also, some species, such as the closely related *S. parasitica* and *S. diclina*, have very similar sexual structures, making it impossible to distinguish one species from the other (Diéguez-Uribeondo et al., 1996; Neish and Hughes, 1980; Willoughby, 1978).

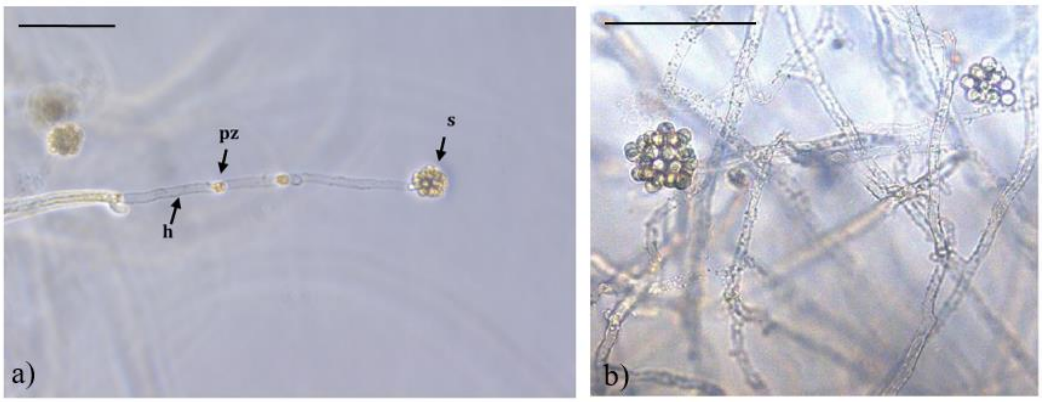


Figure 7. Similarity of asexual morphological features of (a) *Aphanomyces astaci* and (b) *Aphanomyces fennicus* sp. nov. Scale bar: 100 μm. h - hypha, pz - primary zoospore, s - spore ball sporangium. Modified from Martín-Torrijos et al. (2021a) and Viljamaa-Dirks and Heinikainen (2018).

In recent decades, the development of molecular methods has made it possible to overcome these problems, as the identification of isolates could be performed by PCR amplification and Sanger sequencing of the selected marker genes and their comparison with the sequences in publicly available databases (Martín-Torrijos et al., 2021b; Masigol et al., 2020; Mojžišová et al.,

2020; Sandoval-Sierra et al., 2014a; Sarowar et al., 2019b; Tandel et al., 2021). For Oomycota, the internal transcribed spacer region (ITS) between the *18S* and *28S* rRNA genes is most commonly used (Lévesque and De Cock, 2004; Masigol et al., 2020; Oidtmann et al., 2006; Robideau et al., 2011; Sandoval-Sierra et al., 2014b; Sarowar et al., 2019b; Tandel et al., 2021), although other markers such as cytochrome c oxidase subunits I and II (COX1 and COX2) are occasionally used (Robideau et al., 2011; Sarowar et al., 2019a; Schroeder et al., 2013; Yang and Hong, 2018). However, sequencing of marker genes for species identification has its drawbacks, including but not limited to the difficulties of the isolation process itself. For example, a large number of sequences in databases (e.g. GenBank) was shown to be misassigned (i.e. many sequences were erroneously classified), leading to further misclassifications (Sandoval-Sierra et al., 2014b). In the case of the genus *Saprolegnia*, this problem was recently solved by a robust ITS-based phylogeny, which now allows correct identification of *Saprolegnia* isolates down to the species level (Sandoval-Sierra et al., 2014b).

To circumvent the difficult and time-consuming isolation procedure, faster and more reliable molecular methods are being developed that allow pathogen detection in DNA isolated directly from environmental samples. In recent decades, several such assays for the detection of *A. astaci* in infected tissues (Oidtmann et al., 2006, 2004), cuticle swabs (Pavić et al., 2020) and water samples (Strand et al., 2014, 2012) were developed using standard polymerase chain reaction (PCR) (Oidtmann et al., 2006) and/or quantitative polymerase chain reaction (qPCR) (Vrålstad et al., 2009), in which the ITS marker region is amplified with *A. astaci*-specific primers. First, Oidtmann et al. (2004, 2006) developed an *A. astaci*-specific PCR assay for direct detection of the pathogen in DNA isolated from clinical specimens, such as soft abdominal cuticle, walking legs, dorsal abdomen and telson. Later, Vrålstad et al. (2009) developed a more sensitive TaqMan qPCR assay that also targets the *A. astaci* ITS region. This assay can detect down to one zoospore or 50 fg of genomic DNA (gDNA) of the pathogen, which is about 10 times more sensitive than conventional PCR (Tuffs and Oidtmann, 2011). The concentration of *A. astaci* DNA detected by qPCR in the crayfish tissue samples is expressed as semi-quantitative agent levels ranging from A0 to A7, where A0 means that no *A. astaci* DNA can be detected in the sample, while A7 means an exceptionally high level of pathogen DNA (observed PCR-forming units = $PFU_{obs} \geq 10^6$ PFU) (Vrålstad et al., 2009). This test is widely used today for monitoring *A. astaci* (see e.g.: Filipová et al., 2013; Maguire et al., 2016; Mojžišová et al., 2020; Peiró et al., 2016), but has some drawbacks.

For example, qPCR is known to be susceptible to inhibitors commonly found in environmental DNA (eDNA) samples (Agersnap et al., 2017; Hunter et al., 2019; Jane et al., 2015). Also, although this assay can detect extremely low levels of pathogen DNA, false positives are difficult to detect because no-target controls sometimes get Ct values, as reported by several laboratory groups (personal communication). This could be because the assay and probe can sometimes produce a signal in the absence of the template, or because of non-specific amplification of an unidentified microorganism (contamination can come from laboratory products or the infrastructure environment; Borst et al., 2004; Loeffler et al., 1999). Furthermore, Viljamaa-Dirks and Heinikainen (2018) recently reported that both conventional PCR and qPCR *A. astaci* primers amplify the ITS region of a recently described new species *Aphanomyces fennicus* sp. nov. that is closely related to *A. astaci*. Therefore, additional sequencing is required to confirm the positive results obtained with these assays (Tuffs and Oidtmann, 2011; Viljamaa-Dirks and Heinikainen, 2018), but in some cases this is not possible due to the low amount of target/amplicon DNA. For this reason, a modified, more specific qPCR primer pair has recently been proposed that also targets the *A. astaci* ITS region (Francesconi et al., 2021).

Until recently, DNA for the detection of *A. astaci* was extracted from tissue samples, i.e. the procedure involved killing the animals to remove a piece of crayfish tissue/cuticle or the collection of one walking leg. As such approaches involve the time-consuming capture of crayfish and are not suitable for the detection of crayfish plague in endangered native populations that are latently infected or suspected to be infected with crayfish plague (Maguire et al., 2016; Strand et al., 2011; Viljamaa-Dirks et al., 2013), new non-destructive detection methods have been developed. For example, Pavić et al. (2020) and demonstrated that *A. astaci* DNA (from zoospores, cysts or hyphae) can be detected in the mixed epibiotic microbial communities from the surface of living crayfish. In addition, *A. astaci* can be detected in eDNA isolated from water samples (Robinson et al., 2018; Rusch et al., 2020; Strand et al., 2019, 2014, 2011). Briefly, water samples are collected and filtered, and then eDNA is extracted from the filters and used the for detection and quantification of *A. astaci* by qPCR (Strand et al., 2014; Vrålstad et al., 2009; Wittwer et al., 2018). Overall, the eDNA-based method for monitoring *A. astaci* has proven to be an effective alternative to traditional destructive sampling, especially when combined with eDNA-based monitoring of native and non-native crayfish species (Rusch et al., 2020; Strand et al., 2019). It can even be used to confirm the *A. astaci*-negative status of unpopulated habitats for restocking the

native crayfish or Ark site selection (Nightingale et al., 2017). It is widely expected that non-destructive eDNA-based methodology will gradually replace traditional capture-based monitoring (Bohmann et al., 2014; Sieber et al., 2020). For example, eDNA-based *A. astaci* monitoring protocols have already been introduced in the official Norwegian crayfish and crayfish plague monitoring programmes, and other countries should adopt similar approaches in the coming years (Strand et al., 2019).

In contrast to the well-developed and widely used molecular assays for the detection and quantification of the causative agent of crayfish plague described above, the detection of *S. parasitica* is still based on cultivation and isolation in pure culture, followed by microscopy and molecular identification of the pathogen by sequencing of the ITS region (Sandoval-Sierra et al., 2014b). Therefore, it is necessary to develop highly sensitive and specific molecular methods that allow the detection of *S. parasitica* directly from environmental samples. This could be achieved by PCR or its more advanced variants, such as loop-mediated isothermal amplification (LAMP), qPCR and droplet digital PCR (ddPCR). For example, a LAMP assay has recently been developed that enables highly sensitive and rapid on-site detection of *Saprolegnia* spp. (Ghosh et al., 2021). The drawback is that this assay amplifies the ITS fragment of all species within the genus *Saprolegnia*, although some species, such as *S. parasitica*, are much more deleterious than others (Phillips et al., 2008; Sandoval-Sierra et al., 2014a; Sarowar et al., 2019a; van den Berg et al., 2013). Furthermore, a multiplex PCR assay for the detection of *S. parasitica* was recently developed that amplifies in parallel the ITS region and a hypothetical protein gene containing the highly conserved Puf RNA-binding domain (Chanu et al., 2022). The newly developed method showed high specificity and sensitivity (with a detection limit of 16 pg of *S. parasitica* genomic DNA), but the applicability of this protocol to detect the pathogen in environmental samples (e.g. water or skin swabs) remains to be tested.

Sensitive quantitative techniques such as qPCR and ddPCR are often better suited for pathogen detection as they allow monitoring of the pathogen load in the environment (Blaya et al., 2016; Davison et al., 2017; Pavić et al., 2020; Rusch et al., 2020; Tuffs and Oidtmann, 2011). The qPCR assay for the detection of *S. parasitica* was recently developed by Rocchi et al. (2017) and the high specificity and sensitivity of the assay were confirmed by Korkea et al. (2022), making it suitable for monitoring *S. parasitica* in both fish and the environment. ddPCR is a relatively new technology considered advantageous over the more traditional qPCR as it allows absolute

quantification of target DNA without the need for standards and is less susceptible to inhibitors that are often present in environmental samples (Hindson et al., 2013; Hoshino and Inagaki, 2012; Pinheiro et al., 2012). ddPCR has been successfully used for the detection and quantification of several oomycete plant pathogens such as *Aphanomyces euteiches* (Gibert et al., 2021), *P. infestans* (Ristaino et al., 2020) and *P. nicotianae* (Blaya et al., 2016), while no ddPCR protocols have yet been established for oomycete pathogens of animals, including *S. parasitica*.

1.2.2. *Aphanomyces astaci* and *Saprolegnia parasitica* genotyping methods

In many cases, considerable variability in virulence has been observed between different isolates/genotypes of the same pathogen species, as exemplified by variability in virulence of different *A. astaci* genotypes (Diéguez-Uribeondo et al., 1995; Huang et al., 1994; Rezinciuc et al., 2014a) and many other host-pathogen systems (Dutta et al., 2021; Rokas, 2022; Singh et al., 2021). Therefore, the ability to distinguish specific genotypes of the pathogen is an extremely important task in monitoring the spread of the pathogen and identifying host populations at greatest risk for disease outbreaks.

Initially, different genotype groups of *A. astaci* were identified by DNA fingerprinting methods. First, genotypes A, B, C, D and E were identified from pure cultures using random amplification polymorphic DNA polymerase chain reaction (RAPD-PCR) (Diéguez-Uribeondo et al., 1995; Huang et al., 1994; Kozubíková et al., 2011) and amplified fragment length polymorphism (AFLP) (Rezinciuc et al., 2014), which showed better reproducibility, sensitivity and resolution compared to RAPD-PCR. However, a major drawback of fingerprinting methods is that they require axenic cultures of the pathogen, which are usually difficult to obtain and maintain over a long period of time (Oidtmann et al., 1999). Therefore, culture-independent molecular assays have been developed to detect different *A. astaci* genotypes directly from tissue samples (e.g. soft abdominal cuticle, telson or uropods) (Di Domenico et al., 2021; Grandjean et al., 2014; Makkonen et al., 2018; Minardi et al., 2019, 2018). The first assay of this type was developed by Grandjean et al. (2014). It is based on nine *A. astaci* microsatellite markers with a variable number of repeats and can be used to distinguish all five genotype groups defined previously by RAPD. The limitation of this method is its low sensitivity, i.e. it only allows genotype determination if the pathogen load in the samples (agent levels according to Vrålstad et al., 2009) is ≥ A3 ($PFU_{obs} \geq 10^3$). Furthermore, the specificity of the amplification must be confirmed by sequencing to exclude

cross-reactions with other Oomycota species that are frequently found in crayfish. In a later study, Makkonen et al. (2018) focused on the available mitochondrial genome of *A. astaci* (Makkonen et al., 2016) and strain-specific transcriptomic data to develop two sets of PCR primers that amplify the ribosomal subunits of mitochondrial DNA (mtDNA) rnnS and rnnL. The rationale was that the mtDNA genes have a higher copy number than the nuclear DNA (which is analysed with the microsatellite markers) and therefore the mtDNA-based assay will increase the sensitivity of genotyping. Phylogenetic analysis of the sequences obtained has confirmed the ability of the method to discriminate between four different haplogroups (A, B, D and E) and five haplotypes (a, b, d1, d2 and e). Although the sensitivity of this mtDNA-based amplification was slightly better than microsatellite markers, the pathogen load in the samples still needs to be ≥ A3 to allow successful genotyping. Relatively recently, Minardi et al. (2018) used whole genome sequencing of different *A. astaci* genotypes, identified genotype-specific genomic regions and developed genotype-specific PCR primers. However, similar to other genotyping methods, the genotype can only be determined in highly infected crayfish tissue (agent level ≥ A5 or ≥ 10^4 PFU_{obs}). In addition, the primer pair for genotype C cross-amplified with *Phoma*-like and *Pythium flevoense* DNA isolates, so that the genotyping results must be confirmed by sequencing. To increase the sensitivity of *A. astaci* genotyping, Minardi et al. (2019) identified single nucleotide variants (SNVs) in the mtDNA of five known *A. astaci* genotype groups (A, B, C, D and E) and developed genotype-specific PCR primer pairs. The sensitivity of the assay was significantly higher than that of other genotyping methods going down to agent level A1 (PFU_{obs} ≥ 5 PFU). Finally, based on the genomic regions previously described by Minardi et al. (2018), Di Domenico et al. (2021) developed a TaqMan qPCR assay to distinguish five genotype groups of *A. astaci* (A-E) in crayfish plague outbreaks and also in latent infections. Although this method is specific and rapid, it still does not surpass the sensitivity of most previous assays (i.e. the pathogen load in the samples must be ≥ A3), presumably because it targets single-copy genes. Furthermore, genotyping results are sometimes inconclusive. It was therefore recommended to use this method in combination with other genotyping methods to obtain more accurate genotyping results.

Compared to the relatively abundant literature data on the distribution and virulence of different *A. astaci* genotypes, the molecular epidemiology of *S. parasitica* is largely unexplored, with only a few available studies examining differences between *S. parasitica* genotypes (Bangyeekhun et al., 2003; de la Bastide et al., 2018; Naumann, 2014; Ravasi et al., 2018).

Bangyeekhun et al. (2003) used RAPD-PCR to characterise the genetic variation of *Saprolegnia* spp. isolates from infected salmon and trout from Finland and Sweden and identified four genetic groups. One of them was suspected to be highly virulent because it was widely distributed in different salmonid species and water samples. Recently, Ravasi et al. (2018) applied multilocus sequence typing (MLST) to genotype 77 *S. parasitica* isolates from different fish host species and different sites in Switzerland. They developed an MLST scheme that compares nucleotide polymorphisms within seven housekeeping genes and identified ten different genotypes (diploid sequence types DST1 - DST10). They found that one of them, DST3, was involved in more than half of the saprolegniosis outbreaks in Switzerland between 2015 and 2017. Further studies are needed to assess the spread of these genotypes beyond Switzerland and the differences in their impact on host populations in aquaculture and in the wild.

1.3. Factors that promote the spreading of pathogenic Oomycota in freshwater ecosystems

A variety of abiotic and biotic factors can influence the incidence and spread of animal Oomycota pathogens in different types of freshwater ecosystems (subsections 1.3.1. to 1.3.4.) and most of them are not sufficiently explored.

1.3.1. Effect of human-mediated transfer

One of the factors influencing the geographic distribution of Oomycota pathogens in freshwater ecosystems is the dispersal of their hosts, by intentional or unintentional movement of fish/crayfish individuals by humans (Fisher et al., 2012; Gozlan et al., 2014; Svoboda et al., 2017; Tedesco et al., 2021; Ungureanu et al., 2020). For example, invasive North American crayfish species such as *Pa. leniusculus* were intentionally introduced into Europe for breeding and commercial purposes, bringing with them the crayfish plague pathogen *A. astaci* (Holdich et al., 2009; Kouba et al., 2014; Martín-Torrijos et al., 2021b; Svoboda et al., 2017). In addition, some carrier non-native crayfish species, such as the marbled crayfish *Pr. virginalis* or the red swamp crayfish *Pr. clarkii*, have been introduced into European freshwaters through the ornamental trade, followed by garden pond escapes and aquarium releases (Chucholl, 2013; Mrugała et al., 2015; Souty-Grosset et al., 2016). Native latently infected crayfish species, such as *Po. leptodactylus*, have also been transferred across Europe (mostly for human consumption), contributing to the

spread of *A. astaci* (Caprioli et al., 2018). *Aphanomyces astaci* can also spread from an *A. astaci*-positive to an *A. astaci*-negative location via the equipment contaminated with zoospores or mycelium, e.g. equipment used to transport host species for aquaculture purposes (Alderman, 1996; Alderman et al., 1987), fishing gear and other equipment (e.g. traps, boots, nets), and water (i.e. in which the host species were kept) (OIE, 2019). As for *Saprolegnia* species, although they are considered ubiquitous in freshwater habitats worldwide (Tedesco et al., 2021; van den Berg et al., 2013), knowledge of the distribution and virulence of specific genotypes is still virtually non-existent (Ravasi et al., 2018). However, as the disease develops most rapidly in highly populated fish farming systems, it can be expected that specific, highly virulent genotypes could be introduced from aquaculture facilities into wild freshwater ecosystems (Tedesco et al., 2021).

Considering all these risks, it is important to develop appropriate management plans to prevent the unintentional spread of Oomycota pathogens into freshwater ecosystems. This includes disinfecting all equipment that may have come into contact with the pathogen before it is moved into new waterways, avoiding restocking of natural habitats with animals from infected breeding areas, educating the public and stakeholders about the risks of introducing animals into waterways, etc. (OIE, 2019; Pavić et al., 2021; Tedesco et al., 2021).

1.3.2. Effect of host health status

Most studies on the factors affecting the virulence of Oomycota pathogens focus on the health status of the host (Aydin et al., 2014; Bly et al., 1993; Svoboda, 2015). In aquaculture facilities (e.g. fish farms) or the natural environment, stressful conditions such as temperature shock, crowding (i.e. dense populations), injury, infection by bacteria or fungi, moulting, reproduction or altered environmental conditions can promote the development of diseases caused by Oomycota (Aydin et al., 2014; Bly et al., 1993; Cerenius et al., 2003; Edsman et al., 2015; Kozubíková et al., 2009; Noga, 1993; Thomas et al., 2020). *Saprolegnia* spp. are opportunistic pathogens with a broad host range that can infect many different species, including crayfish, but in these hosts infection usually occurs when pre-existing wounds are present on the epicuticular layer of the host (Dieguez-Uribeondo et al., 1994). In addition, experimental infection of fish species with *Saprolegnia* spp. is favoured when the animals are net-shaken to cause multiple skin erosions and increase stress (Casas-Mulet et al., 2021; Ellison et al., 2018; Hussein and Hatai, 2002). Similar findings were recently reported by Tedesco et al. (2022), where it was shown that stress associated

with fish handling in aquaculture facilities could be one of the main factors positively influencing the prevalence of *Saprolegnia* spp. Crayfish plague is also more likely to develop when crayfish individuals are immunocompromised. For example, invasive signal crayfish, which are normally resistant to infection, may develop crayfish plague if they are simultaneously infected with another pathogen such as *Psorospermium haeckeli* or *Fusarium* spp. (Edsman et al., 2015; Thörnqvist and Söderhäll, 1993) or are in a certain physiological state, e.g. moulting (Aydin et al.; 2014). On the other hand, latent crayfish plague infection with genotype group A without mass mortality has been reported in native crayfish species such as noble crayfish (Maguire et al., 2016; Pavić et al., 2021), narrow-clawed crayfish (Jussila et al., 2020; Kokko et al., 2018), stone crayfish (Kušar et al., 2013) and white-clawed crayfish (Manfrin and Pretto, 2014), suggesting that animals can resist the pathogen if they are in a good condition. However, if the physiological condition of the crayfish is compromised, for example, due to increased population density, non-optimal water temperature, increased pathogen load, etc., disease outbreaks may still occur (Cerenius et al., 2003; Jussila et al., 2011; Makkonen et al., 2014, 2012; Svoboda et al., 2017).

1.3.3. Effect of abiotic properties of the environment

Numerous studies, mostly conducted *in vitro*, have shown that various abiotic properties of the environment, such as water temperature, pH and ion content, can influence the growth and reproduction of Oomycota pathogens (Bly et al., 1992; Cerenius and Söderhäll, 1984b; Diéguez-Uribeondo et al., 1994; Kitancharoen et al., 1996; Rantamäki et al., 1992; Tedesco et al., 2022; Unestam, 1969, 1966).

The optimal *in vitro* temperature for mycelial growth of both *A. astaci* and *S. parasitica* is around 20°C, although growth occurs over a much wider temperature range. On the other hand, the motility of zoospores, which represent the crucial infective stage of the life cycle, is maximal at about 10°C (Diéguez-Uribeondo et al., 1995; Kitancharoen et al., 1996; Matthews, 2019; Rezinciuc et al., 2014b; Strand et al., 2012). Consistent with this, mortality of the native narrow-clawed crayfish *Po. leptodactylus* of *A. astaci* genotype B was significantly higher at 18°C than at 22°C (Bielen et al., unpublished data). *Aphanomyces astaci* genotype D has been shown to adapt to warmer temperatures of up to 26°C, in contrast to genotypes A, B and C (Rezinciuc et al., 2014a). Furthermore, the temperature is the abiotic environmental factor most commonly associated with outbreaks of *Saprolegnia*, which is consistent with the fact that immune defences in their

stenothermic hosts are highly temperature dependent. However, various *in vivo* studies show different effects of thermal disturbances. Disease outbreaks are more frequent in winter, probably because sudden drop in water temperature can simultaneously cause host immunosuppression and trigger sporulation of the pathogen (Bly et al., 1993). On the other hand, a modest 2°C increase in water temperature was reported to have a significant positive impact on the progression of *Saprolegnia* infection, but the host component, namely the immunocompetence of the three-spined stickleback *Gasterosteus aculeatus*, had at least as great an influence as the thermal effect (Stewart et al., 2018). Finally, it was shown that *S. parasitica*-exposed brown trout alevins and fry experienced increased mortality when a constant temperature increase was combined with intermittent thermal increases (Casas-Mulet et al., 2021).

The growth and survival of pathogens are also influenced by the pH of the medium. In most cases, *S. parasitica* prefers acidic conditions, with a pH between 4.8 and 5.5 being most suitable for mycelial growth, while a higher pH of 6.2 is most optimal for zoospore production (Kitancharoen et al., 1996). In *A. astaci*, zoospore motility is maximal between pH 4.5 and 9, the optimal range for zoospore production is between pH 5 and 7, and mycelial growth is most rapid between pH 6 and 7 (Unestam, 1966, 1965).

In addition, lack of nutrients (e.g. contact of mycelium with natural autoclaved water) is known to trigger the formation of Oomycota zoospores (Bly et al., 1992; Unestam, 1969). Most protocols for sporulation of Oomycota are therefore based on washing the mycelium with a stream or lake water (Diéguez-Uribeondo et al., 1994; Heungens and Parke, 2000; Makkonen et al., 2012). Also, the mycelial growth of *S. parasitica*, but not the development of sporangia and zoospores, is inhibited by environmentally relevant concentrations of humic substances, which make up the majority of dissolved organic matter in oligotrophic freshwater ecosystems (Meinelt et al., 2007).

Finally, ions present in water and host tissues can influence the life stages of Oomycota pathogens, as shown in several *in vitro* studies (Appiah et al., 2005; Barszcz et al., 2014; Cerenius and Söderhäll, 1984b; Rantamäki et al., 1992; Rezinciuc et al., 2018). For example, the addition of Ca^{2+} ions to a zoospore suspension of *S. parasitica* has been shown to promote cyst formation and adhesion of cysts to the host (Burr and Beakes, 1994; Rezinciuc et al., 2018). Similarly, it has been shown that 1 mM Ca^{2+} ions can stimulate *A. astaci* zoospore production and cyst formation, while 1 mM Mg^{2+} has no such effect and even inhibits sporulation at higher concentrations (20 mM) (Cerenius and Söderhäll, 1984b; Rantamäki et al., 1992). However, the concentrations of Ca^{2+} and

Mg^{2+} ions used in these laboratory studies were much higher than those usually present in natural freshwaters (Weyhenmeyer et al., 2019), so the results cannot be easily extrapolated to the real environmental setting. In a recent study by Preuett et al. (2016), a sea salt mixture was dissolved in distilled water to mimic naturally occurring saline water ranges (from non-saline to moderately saline to very saline, 0 to 45 g/L) and it was found that survival, growth and infectivity of the plant Oomycota pathogen *Phytophthora ramorum* were negatively correlated with salt concentration. At concentrations > 20 g/L (high salinity) no zoospores were released, but infection could still occur via mycelial growth.

1.3.4. Effect of microbial communities present in the environment

Diverse microbial communities are present in the environment of freshwater Oomycota pathogens, in the water, in the sediment, on the surface and in the internal organs of their hosts (Austin, 2006; Carbajal-González et al., 2011; Dragičević et al., 2021; Karner et al., 2001; Liu et al., 2014; Mahmoud and Magdy, 2021; Orlić et al., 2021; Sagova-Mareckova et al., 2021; Skelton et al., 2017). Oomycota pathogens thus constantly interact with other microbes in their habitat, and these interactions can range from mutualistic to antagonistic. Accordingly, microbial taxa living in and on host species have been shown to both promote and inhibit the Oomycota infection process in plant hosts (Larousse and Galiana, 2017). For example, a motile unicellular *Vorticella* has been shown to disseminate *Phytophthora parasitica* by acting as a sower of Oomycota propagules (Galiana et al., 2011). Also, co-infection by two plant-pathogenic Oomycota, *Albugo laibachii* and *P. infestans*, enables *P. infestans* to colonise a non-host plant *Arabidopsis thaliana* (Belhaj et al., 2017). On the other hand, host-associated microbial communities have been shown to help protect the host from various pathogens. For example, certain bacterial species found on fish skin and crayfish cuticle can inhibit Oomycota pathogens, and it has therefore been suggested that they could be used to protect the host (i.e. biocontrol) (Carbajal-González et al., 2011; Orlić et al., 2021). The low incidence of saprolegniosis on salmon eggs correlated with the species diversity of certain commensal Actinobacteria, with the presence of genus *Frondihabitans* associated with strong inhibition of *Saprolegnia* attachment to eggs (Liu et al., 2014). In addition, bacterial strains isolated from water, skin lesions, fish eggs and crayfish cuticle, mainly belonging to the genera *Aeromonas* and *Pseudomonas*, have been shown to reduce vegetative growth and germination of *Saprolegnia* spp. and *A. astaci* cysts *in vitro* (Bly et al., 1997; Carbajal-González et al., 2011; Hussein and Hatai,

2001; Liu et al., 2015; Orlić et al., 2021). Such results are important for understanding the interactions between epibiotic bacteria of host species (fish and crayfish) and pathogenic Oomycota (*S. parasitica* and *A. astaci*), but also open the perspective of testing the application of selected bacterial inoculums for the prevention and/or treatment of diseases caused by Oomycota in fish and crayfish aquaculture.

Furthermore, the physico-chemical parameters of water quality can simultaneously influence the composition and diversity of bacterial communities present in the water (Niu et al., 2019; Tiquia, 2010) and the survival and reproduction of Oomycota pathogens (as explained in subsection 1.3.3.). As it is evident that the diversity and composition of microbial communities are highly reflective of changes in the environment, there is increasing evidence that they should be used as bioindicators of water quality (Sagova-Mareckova et al., 2021). Accordingly, certain microbial taxa present at some freshwater sites could not only predict water composition and quality but also serve as indicators of the presence of Oomycota pathogens and thus help predict disease outbreaks or their spread.

Overall, a complex variety of factors (described in detail in subsections 1.3.1. to 1.3.4.) can influence the load of Oomycota pathogens in a given environment and make disease outbreaks more or less likely. However, further studies on each of these factors are needed for accurate prediction of disease 'hot spots'.

1.4. Aims and hypotheses

This dissertation addresses several previously understudied aspects of the ecology of the pathogenic freshwater Oomycota *Aphanomyces astaci* and *Saprolegnia parasitica*. The results of the dissertation were published in three scientific articles (**I-III**).

1. To test the hypothesis that *S. parasitica* can be detected directly in environmental DNA (e.g. water, host biofilm), **Aim 1** was to develop a sensitive, specific and quantitative *S. parasitica* ddPCR assay. The developed assay is presented in **Publication I**.
2. To test the hypothesis that trout farms can act as points of spreading of *Saprolegnia* spp. to natural waters, **Aim 2** was to isolate and identify Oomycota species present in trout farms in Croatia and compare them with Oomycota isolates obtained from upstream and downstream locations. These results are presented in **Publication II**.

3. To test the hypothesis that certain microbiological and physico-chemical characteristics of natural waters influence the life cycle stages of freshwater Oomycota pathogens, **Aim 3** was to identify the characteristics of natural waters that promote sporulation and spread of *A. astaci* and *S. parasitica*. The analyses that correlated specific characteristics of natural waters with the load and sporulation intensity of Oomycota pathogens are presented in **Publications I and III**, and in Appendices 1 - 3.

2. ORIGINAL SCIENTIFIC PAPERS

2.1. Publication I: Tracing the oomycete pathogen *Saprolegnia parasitica* in aquaculture and the environment

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Tracing the oomycete pathogen *Saprolegnia parasitica* in aquaculture and the environment

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Saprolegnia parasitica **causes saprolegniosis, a disease responsible for significant economic losses in aquaculture and declines of fish populations in the wild, but the knowledge of its distribution and prevalence in the environment is limited. We developed a fast, sensitive and specific *S. parasitica* droplet digital PCR (ddPCR) assay and demonstrated its applicability for the detection and quantification of the pathogen in environmental samples: swab DNA collected from the host (trout skin, surface of eggs) and environmental DNA extracted from water. The developed assay was used to assess how abiotic (i.e. physico-chemical parameters of the water) and biotic (health status of the host) factors influence the *S. parasitica* load in the environment. The pathogen load in water samples was positively correlated with some site-specific abiotic parameters such as electrical conductivity (EC) and calcium, while fluorides were negatively correlated, suggesting that physico-chemical parameters are important for determining *S. parasitica* load in natural waters. Furthermore, skin swabs of injured trout had significantly higher pathogen load than swabs collected from healthy fish, confirming that *S. parasitica* is a widespread opportunistic pathogen. Our results provide new insights into various environmental factors that influence the distribution and abundance of *S. parasitica*.**

Oomycete pathogens cause diseases in a wide range of plant and animal species, both in the wild and cultured environments, and threaten biodiversity and food security worldwide[1,2]. One of the most destructive oomycete pathogens in freshwater ecosystems is *Saprolegnia parasitica* (Coker, 1923). It causes saprolegniosis, a disease that mainly affects salmonids, from eggs to adult fish, but also other fish species, as well as amphibians, crayfish and other hosts[3,4]. Existing studies on *S. parasitica* mostly focus on its negative impacts in aquaculture: it is responsible for significant economic losses in salmonid farms and hatcheries worldwide[3,5], and aquaculture facilities can act as pathogen pools from which *S. parasitica* spreads to natural environments[6,7]. In comparison, the potential negative impacts of *S. parasitica* on wild populations of salmonids and other animals remain largely unexplored, although declines in wild salmon populations caused by saprolegniosis have been reported[8,9].

Existing knowledge on the abiotic and biotic factors influencing the incidence and spread of *S. parasitica* comes either from aquaculture research focusing on host health[10] or from in vitro microbiological studies[11,12]. Stressful conditions commonly encountered in aquaculture facilities (e.g. temperature shock, infection by bacteria and fungi, stress due to overcrowding or injury) can weaken the host's immune system and increase its susceptibility to *S. parasitica*[10,13]. On the other hand, laboratory studies have shown that zoospore production decreases when water temperature rises above 20 °C[14,15], while their germination is inhibited under acidic conditions (pH < 4[15]). Humic substances, which constitute the majority of dissolved organic matter in oligotrophic freshwater ecosystems, can inhibit mycelial growth of the pathogen[16], while Ca^{2+} ions regulate processes such as adhesion, encystment and germination[11,17]. Despite numerous laboratory studies, knowledge about the prevalence of *S. parasitica* in the natural environment is very limited[9,18–20], making it impossible to define the pathogen's distribution range and analyse ecological parameters that could influence its occurrence. Determining the relationship between the ecological and physico-chemical status of natural waters and the occurrence of *S. parasitica* is central to any attempt to predict the possibility of disease outbreaks in a realistic natural setting.

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Currently, the biggest obstacle to such studies is the lack of a fast, effective, sensitive and non-invasive monitoring tool for *S. parasitica*. Traditional *S. parasitica* diagnostic procedures are usually performed after the disease outbreak and are laborious and invasive, requiring the capture and culling of host animals[12,21,22]. Pathogen identification further relies on growing isolates in pure culture and then sequencing their ITS region[6,23,24]. In recent decades, environmental DNA (eDNA) has been increasingly used to detect pathogens in aquatic environments to circumvent invasive and labour-intensive standard methods[25–28]. The target species can be detected in eDNA samples by specific primers designed to amplify only the marker DNA region of the taxon of interest by standard polymerase chain reaction (PCR) or loop-mediated isothermal amplification (LAMP). For example, a LAMP assay has recently been developed that enables highly sensitive and rapid on-site detection of the genus *Saprolegnia*[29]. However, sensitive techniques that allow quantification are often more appropriate, such as quantitative PCR (qPCR) or droplet digital PCR (ddPCR), as they allow monitoring of the dynamics of target species in the environment[26,30–33]. Droplet digital PCR is a relatively new technology and is considered advantageous over the more traditional qPCR since it allows absolute quantification of target DNA without the need for standards and is less susceptible to inhibitors[34–36]. qPCR assay for detection of *Saprolegnia parasitica* was recently reported, but it has very low specificity[37], leaving the need for development of more specific and effective detection methods. Further, ddPCR has been used for the detection and quantification of several oomycete plant pathogens such as *Aphanomyces euteiches*[38], *Phytophthora infestans*[39] and *P. nicotianae*[30], while no ddPCR protocols have yet been established for oomycete pathogens of animals, including *S. parasitica*.

To improve the knowledge on environmental factors that might influence the distribution and abundance of *S. parasitica* in the environment, in the scope of this study we have: (i) developed *S. parasitica*-specific ddPCR assay; (ii) analysed the effect of different physico-chemical parameters of water on the presence and load of *S. parasitica* in the natural environment; and (iii) analysed the effect of host health status on the *S. parasitica* load.

Results

ddPCR assay for the detection of *Saprolegnia parasitica*. The assay was developed to target the internal transcribed spacer region 2 (ITS 2), a common marker sequence for *S. parasitica* and other oomycetes[40–42] (Fig. 1a). The primer pair 333F and 580R was designed to be *S. parasitica*-specific (Fig. 1b), which was confirmed in silico using the Primer-BLAST tool[43] and in vitro using gDNA from *S. parasitica* and closely related non-target species (Table 1). Four different *S. parasitica* isolates yielded > 10,000 ITS copies per ng gDNA, while gDNA from non-*S. parasitica* oomycetes, including the closely related species *S. diclina* and *S. ferax*, and trout/crayfish gDNA did not yield positive droplets. *Saprolegnia* sp. 1 gDNA was the only exception and yielded ~ 6 ITS copies per ng gDNA. In addition, the sensitivity of the assay was tested using a dilution series of *S. parasitica* gDNA (Fig. 2). The limit of detection (LOD), i.e. the lowest concentration of pathogen DNA that could be reliably detected with the developed assay conditions, was estimated to be 14 fg *S. parasitica* gDNA per reaction (with 91.3% confidence level).

Next, the applicability of the developed ddPCR assay for quantifying *S. parasitica* load in environmental DNA (eDNA) samples was demonstrated using trout eggs infected with *S. parasitica* in the laboratory. Swab samples taken from the infected eggs had a significantly higher *S. parasitica* load (average 6,195 ITS copies per ng of total eDNA, min = 63, max 18,148) than healthy eggs from the hatchery (average 1.5 ITS copies per ng of total eDNA, min = 0, max 7; Mann–Whitney U test, $W = 0$, $p < 0.01$) and healthy eggs used as negative controls in the infection trial (average 2.2 ITS copies per ng of total eDNA, min = 0, max 9; Mann–Whitney U test, $W = 0$, $p = 0.01$). No significant difference in *S. parasitica* load was found between the healthy eggs from the hatchery and the healthy eggs used as negative controls in the infection trial (Mann–Whitney U test, $W = 12$, $p = 1$).

Variations of *Saprolegnia parasitica* load in the environment. We used the newly developed ddPCR assay to analyse the effect of water composition and health status of the host on *S. parasitica* load in water and on the skin surface of its fish hosts, respectively.

Effect of physico-chemical parameters of water quality on *Saprolegnia parasitica* load in the water. We collected water samples from stagnant and flowing water bodies in Croatia (Fig. 3, Supplementary Table S1) and *S. parasitica* was detected in 13 of 21 water samples (62%). The average *S. parasitica* load in water samples was 3.24 ITS copies per ng of total eDNA (min = 0, max 14). Physico-chemical analyses showed overall good quality of collected water samples, but some samples exceeded legal limits (Official Gazette, 96/2019): nitrates were elevated in 6 out of 21 samples (28.6%), ammonium in 2/21 (9.5%) and total phosphorus in 2/21 (9.5%) (Supplementary Table S2).

Using the PLS-R modelling, we analysed the relationship between *S. parasitica* load (response variable, Y) and various physico-chemical parameters of the water (explanatory variables, X). As for the model quality indices R^2X and R^2Y in component 1, 35% of the variance in the set of explanatory variables (X) was used to explain 37% of the variance for the response variable (Y). In component 2, 61% of the set of explanatory variables (X) was used to explain 41% of the response variable (Y). Q^2, as a measure of goodness of prediction, showed that component 1 contributed 15% and component 2 contributed 5% to model quality. The relationship between blocks of predictor and response variables is visually represented in the form of a correlation radar (Fig. 4a), with positively correlated variables close together and negatively correlated ones far apart. Multivariate PLS-R analysis showed that Ca^{2+} ($r = 0.56$) and EC ($r = 0.49$) were the most important parameters that positively influenced the *S. parasitica* load in the water samples, followed by Na^+ ($r = 0.47$), SO_4^{2-} ($r = 0.45$), Cl^- ($r = 0.44$) and NO_3^- ($r = 0.35$), while the most pronounced negative correlation was found for F^- ($r = -0.35$), followed by COD ($r = -0.27$), TOC ($r = -0.27$), K^+ ($r = -0.26$), TP ($r = -0.23$), NH_4^+ ($r = -0.21$) and pH ($r = -0.20$) (Fig. 4a, Supplementary

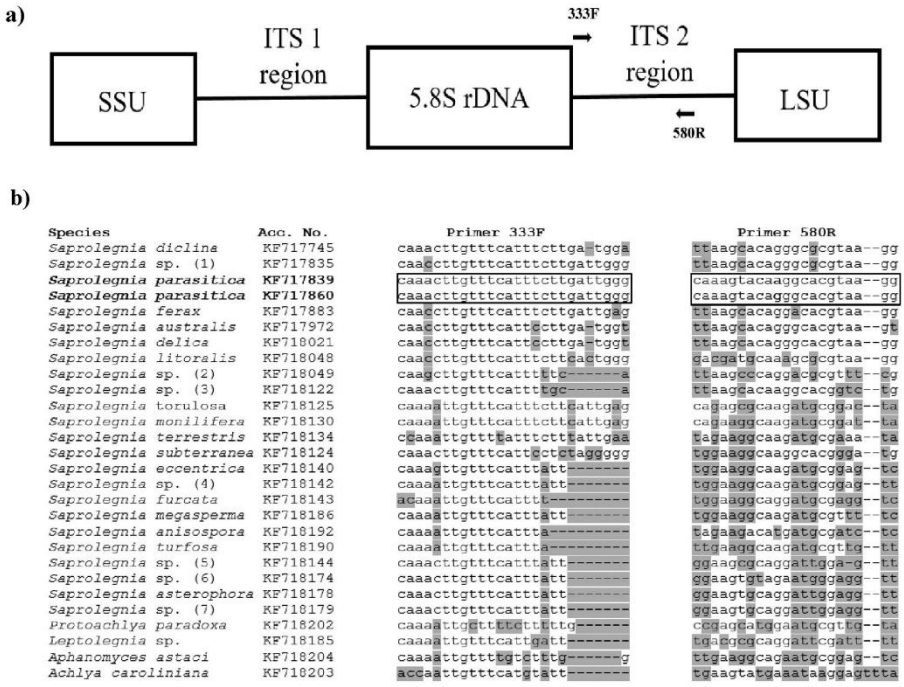

Figure 1. (**a**) Position of *Saprolegnia parasitica*-specific primers 333F and 580R in the internal transcribed spacer (ITS) region (including ITS 1, 5.8S rDNA, and ITS 2) between small subunit (SSU) and large subunit (LSU) rDNA. (**b**) Multiple sequence alignment (MSA) of primer sequences and ITS 2 segments of a range of *Saprolegnia* spp. and other oomycetes. Primer sequences are framed, with a degenerate position (A/G) in the primer 580F marked in bold. Nucleotides that differ between *S. parasitica* and other species within a particular MSA column are shaded in grey. Acc. No.—Genbank accession numbers.

Species	Isolate/code	Amplification with primers 333F/580R	Source/reference of the isolate/tissue
Saprolegnia parasitica	BF1	+	Pavić et al. [6]
Saprolegnia parasitica	BF2	+	Pavić et al.[6]
Saprolegnia parasitica	Z42	+	Pavić et al.[6]
Saprolegnia parasitica	Z46	+	Pavić et al.[6]
Saprolegnia australis	Z25	–	Pavić et al.[6]
Saprolegnia delica	BF5	–	Pavić et al.[6]
Saprolegnia diclina	SAP-1	–	Provided by J. Diéguez-Uribeondo
Saprolegnia ferax	Z106	–	Pavić et al.[6]
Saprolegnia litoralis	SAP-2	–	Provided by J. Diéguez-Uribeondo
Saprolegnia sp. 1	SAP-3	–	Provided by J. Diéguez-Uribeondo
Aphanomyces astaci	PEC8	–	Provided by F. Grandjean
Pythium sp.	VU3 3	–	Pavić et al.[6]
Oncorhynchus mykiss	T-DNA	–	Provided by E. Teskeredžić
Pacifastacus leniusculus	C-DNA	–	Provided by I. Maguire

Table 1. Specificity of the assay. The *S. parasitica* isolates yielded > 10,000 ITS copies per ng of genomic DNA (marked with +), while the other tested species yielded no ITS copies (no positive droplets, marked with –), except for *Saprolegnia* sp. 1 (SAP-3) that yielded 6 ITS copies per ng of genomic DNA.

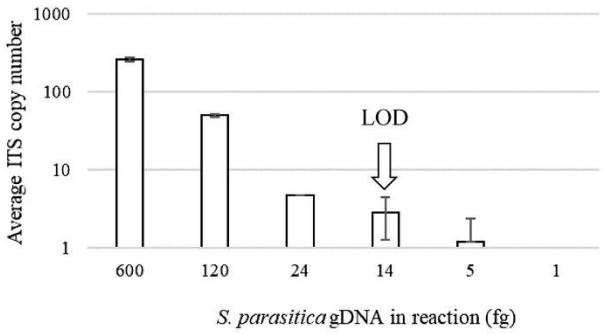

Figure 2. Relation of ITS copy number determined by ddPCR and quantity of *S. parasitica* gDNA (in fg per reaction). Limit of detection (LOD) is marked by an arrow. Error bars represent standard deviation ($n \geq 3$).

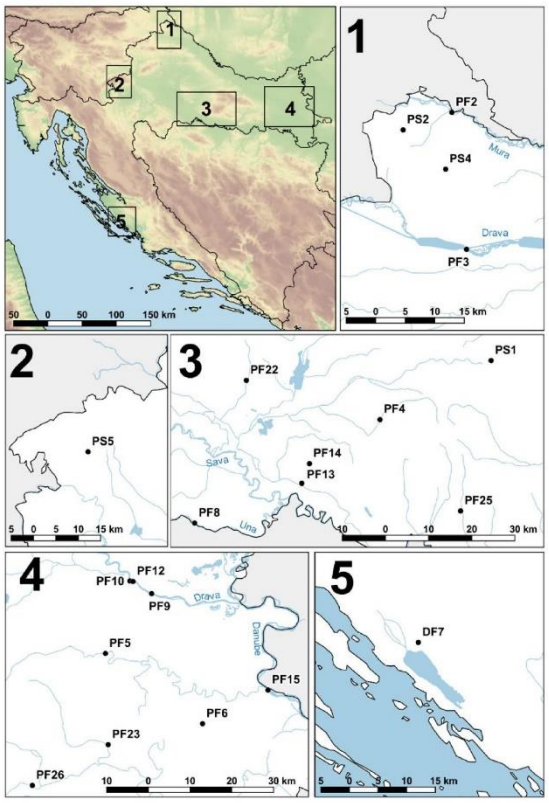

Figure 3. Positions of 21 water sampling locations in Croatia. P—Pannonian region, D—Dinaric region, F—lotic water system (flowing), S—lentic water system (stagnant). The map was generated using QGIS 3.10.7.

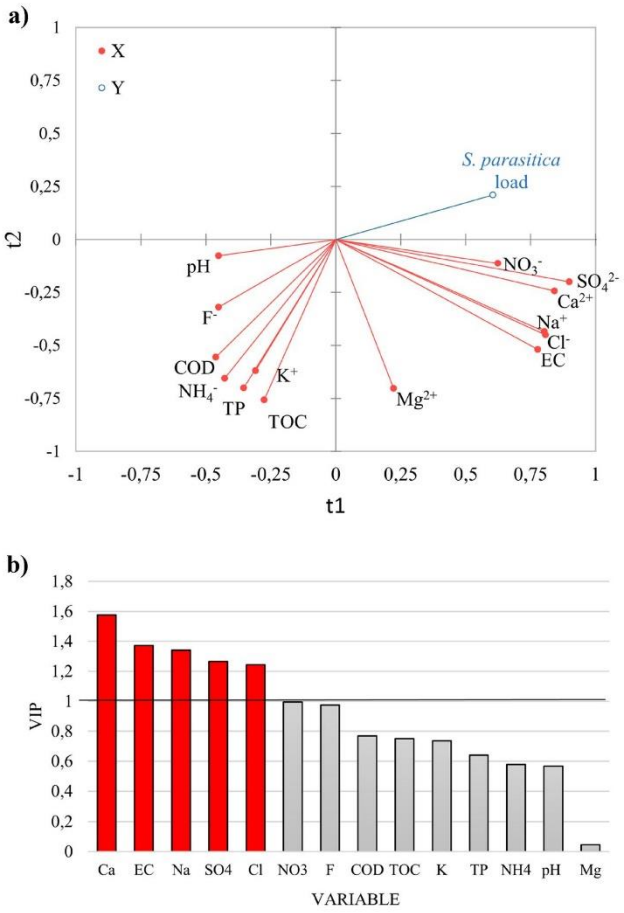

Figure 4. (**a**) Correlation radar describing the relationship between *S. parasitica* load (response variable, Y, blue line) and the physico-chemical parameters of the water (explanatory variables, X, red lines). The percentages of variances in X and Y explained by each variable are indicated on the respective axes. (**b**) The variable importance in projection (VIPs) for explanatory variables of the first component (t1). VIPs > 1 indicate the explanatory variables that contribute most to the PLS model, while VIPs < 0.8 contribute little.

Table S3). Ca^{2+}, EC, Na^{+}, SO_4^{2-} and Cl^{-} also had VIP values > 1, meaning that they are considered highly relevant in explaining the *S. parasitica* load in water and contribute significantly to the model[44,45] (Fig. 4b).

Effect of host health status on *Saprolegnia parasitica* load on the trout skin. We also inspected whether the health status of the trout host influences *S. parasitica* skin load. At the trout farms, we sampled animals (brown trout, *Salmo trutta* Linnaeus, 1758, and rainbow trout, *Oncorhynchus mykiss* Walbaum, 1792) that were apparently healthy as well as animals that had skin injuries, but without gross signs of saprolegniosis (Supplementary Table S4). No significant differences in *S. parasitica* load were found between rainbow and brown trout (data not shown), and thus data from both species were pooled together in subsequent analyses. The results have shown that the health status of the host significantly influenced *S. parasitica* skin load, and swab samples of injured trout had significantly higher *S. parasitica* load (on average 9154 ITS copies per ng of total eDNA; min = 0, max 118,094) than the apparently healthy specimens (average 1.1 ITS copies per ng of total eDNA; min = 0, max 11; Mann–Whitney U test, W = 27, $p < 0.001$, Supplementary Table S4). Noteworthy is the fact that one fish with skin lesions (B10) was *S. parasitica* negative.

Discussion

The developed *S. parasitica*-specific ddPCR assay is highly sensitive and allows absolute quantification of pathogen DNA in the environment, enabling for the first time rapid and simple monitoring of the pathogen. In this study, we have demonstrated its applicability in *S. parasitica* monitoring in the environment and in aquaculture. Most importantly, our results provide insights into the environmental factors that influence the abundance of the pathogen in different types of freshwater ecosystems.

We designed *S. parasitica*-specific primers targeting the ITS region of rDNA, a standard high copy number nuclear marker for oomycetes that is abundantly represented in DNA databases[42,46,47]. We maximised the specificity of the assay by selecting *S. parasitica*-specific primer sequences from the regions of highest divergence with closely related *Saprolegnia* species and other oomycetes, and by using touchdown PCR. This is a step forward in comparison to the previously reported qPCR assay for the detection and quantification of *S. parasitica*[37] that also targeted the ITS region, but with very low specificity: the specificity was tested with only two oomycete species, *S. diclina* and *A. astaci*, and the selected primers were not discriminatory for closely related species, i.e. the forward primer had the identical sequence to *S. australis, S. delica, S. diclina, S. ferax* and *Saprolegnia* sp. 1 among many others, while the reverse primer differed from related species by only one or two bp. Our assay showed high discrimination between *S. parasitica* (> 10,000 copies per ng gDNA) and closely related species (no amplification). Only one closely related non-target species, *Saprolegnia* sp. 1 (one bp difference along the 333F primer), was amplified but yielded only one positive droplet per reaction (or ~ 6 ITS copies per ng gDNA), which was considered a false positive. *Saprolegnia* sp. 1 was rarely detected during sampling at salmonid fish farms and hatcheries[23,24], indicating a low risk of false positives in ddPCR monitoring of *S. parasitica*. Furthermore, the sensitivity of the ddPCR method developed here, with LOD of 14 fg *S. parasitica* gDNA per ddPCR reaction (~ 3 ITS copies), is high and comparable to or more sensitive than other published qPCR/ddPCR assays for oomycetes[30,39,48]. Although the ITS copy number per *S. parasitica* genome is not known, considering the *S. parasitica* genome size of 63 Mb[49], 14 fg corresponds to about 0.2 genomic units and implies that the copy number of *S. parasitica* ITS should be about 10, at least for isolate BF1 used here. This is in line with available data for other oomycete species: the ITS copy number varies between several tens and several hundreds between different oomycete species, but also between different isolates of the same species[30,38].

We used the developed assay as a non-invasive monitoring approach and showed that *S. parasitica* is ubiquitous in the environment, as we detected it in a number of water samples (62% of analysed samples), on the surface of trout eggs and adult trout from aquaculture facilities, and on the surface of the crayfish exoskeleton in the wild (unpublished results for signal crayfish *Pacifastacus leniusculus* Dana, 1852 and narrow-clawed crayfish *Pontastacus leptodactylus* (Eschscholtz, 1823), previously reported as carriers/hosts of *S. parasitica*[50,51]). The developed method allowed us to gain insights into the ecology of the pathogen in relation to biotic and abiotic parameters in both wild populations and aquaculture. In aquaculture, for example, trout with injuries (i.e. skin lesions) had significantly higher *S. parasitica* skin loads than the healthy specimens. *Saprolegnia parasitica* was detected in 91% of swabs from injured fish, indicating that it is a dominant opportunistic skin pathogen of trout. Only one swab sample from trout with injuries (B10) was *S. parasitica*-negative, presumably because another pathogen has outcompeted *S. parasitica*. In contrast, *S. parasitica* was mostly not detected (or its load was low) in the skin swabs of apparently healthy adult trout and trout eggs. Overall, our results show that *S. parasitica* is ubiquitous in fresh waters (both in the water column and on the host surface) and can infect stressed, immunocompromised or injured host individuals at any time, as previously reported[52,53].

By combining absolute quantification of the pathogen with the data on physico-chemical properties of natural waters, we found for the first time a correlation between *S. parasitica* load and some water parameters. The positive correlation was strongest for Ca^{2+} and EC, while the parameter with the strongest negative influence was F^-. Calcium concentrations in water samples within our dataset ranged from 2.15 to 119.4 mg/L, with a median of 37.1 mg/L, which is higher than the global freshwater median of 4 mg/L[54]. Based on in vitro microbiological studies, calcium ions may positively influence the developmental stages and infection process of oomycetes[11,17,55]. For example, in *S. parasitica*, the number and length of long hooked hairs on cysts increased after the addition of 5550 mg/L $CaCl_2$, enhancing the adhesion of cysts to the host surface[11]. Our results show for the first time that higher than average Ca^{2+} concentrations in surface waters, which are common in karst areas[56], favour the growth/development of *S. parasitica*. This is in line with the above-mentioned in vitro studies showing the positive effect of (even) higher concentrations of calcium ions expected when the pathogen invades host tissues[57,58].

In addition, electrical conductivity, which is related to the total ion content of the water and mainly to the sodium, chloride and calcium ions' concentration, was found to correlate positively with *S. parasitica* load. Sodium chloride in high concentrations (> 1000 mg/L) is used as an effective and non-toxic method to control *Saprolegnia* sp. as it can reduce the vegetative growth of the pathogen and the formation, release and proliferation of sporangia[59]. In freshwater ecosystem sodium and chloride concentrations vary from country to country. For example, the range of groundwater limits set by European Union countries for chloride is between 24 and 12,300 mg/L, while limits for sodium in drinking water are between 50 and 450 mg/L[60]. In our study, water samples with *S. parasitica* load > 5 ITS copies/ng had higher average chloride (40.2 mg/L) and sodium (1.3 mg/L) concentration than the water samples with lower *S. parasitica* load (17.9 and 0.6 mg/L, respectively). Our results thus suggest that moderate salt concentrations in natural waters may have a positive effect on *S. parasitica*, which is also consistent with the fact that sodium and chloride concentrations are higher in host tissues than in water[61].

Fluoride concentration was the most important factor that negatively correlated with *S. parasitica* load in water, although it did not contribute significantly to the model (VIP < 1). It is known that excessive fluoride concentrations in the environment can affect microbial communities due to its negative effects on microbial physiology[62,63]. The World Health Organization (WHO) limit for fluoride concentrations in drinking water is 1.5 mg/L, while fluoride concentrations in unpolluted freshwaters range from 0.01 to 0.3 mg/L[64]. In our dataset,

water samples with *S. parasitica* load > 5 ITS copies/ng had an average of 0.38 mg F^-/L, compared to 0.47 mg/L in the water samples with lower *S. parasitica* loads. To our knowledge, there are no data on the toxicity of fluoride to oomycetes, but this result suggests an inhibitory effect of environmentally relevant fluoride concentrations on *S. parasitica*. However, further studies, including more extensive monitoring, are needed to confirm this since the negative correlation of fluorides and *S. parasitica* load in water was not significant in our study.

Overall, we have shown that the newly developed ddPCR method allows sensitive detection and quantification of *S. parasitica* load in various environmental DNA samples and avoids the qualitative and labour-intensive cultivation used so far. The novel method could be used in salmonid aquaculture to monitor variations in *S. parasitica* loads in both skin/egg swabs and water. Currently, toxic antioomycetic chemicals are continuously used to prevent disease outbreaks, regardless of the actual load of the pathogen. If outbreaks could be predicted in a timely manner through ddPCR-based monitoring, the use of antioomycetic chemicals could be adjusted to the current pathogen load. In this way, both chemical pollution and pathogen transmission to downstream waters could be reduced. Here, we have used the developed method to gain new insights into the ecological requirements of the pathogen and provide a basis for identifying natural habitats at increased risk for outbreaks of *S. parasitica*. For example, environmentally relevant Ca^{2+} and EC levels were found to have a positive effect on *S. parasitica* load, while F^- had a negative effect. However, more extensive field monitoring is needed to confirm and support our conclusions. In addition, laboratory experiments are needed to evaluate how the selected water parameters affect the virulence of the pathogen by examining various experimental endpoints such as sporulation efficiency, zoospore germination, host mortality, etc.

Methods

Samples used for assay development and validation. For the development of the assay (i.e. to analyse the sensitivity and specificity of the designed primers), we used mycelia from pure cultures of *S. parasitica* (positive control) and other oomycetes as well as tissue samples from known host/carrier species, namely the skin of healthy rainbow trout (*Oncorhynchus mykiss*) and the abdominal cuticle of the signal crayfish (*Pacifastacus leniusculus*)[50,53] (Table 1). Oomycete mycelia were grown in liquid glucose-yeast extract (GY) medium[65] for two days at 18 °C, then washed with sterile distilled water and centrifuged at 10,000×*g* for 15 min[6]. The resulting pellets (approx. 30 mg wet weight per sample) were stored at − 20 °C until DNA extraction[6].

For assay validation, we used environmental water samples collected at 21 different locations in Croatia (Fig. 3, Supplementary Table S1), as well as samples from aquaculture (trout farms), i.e. 45 swabs collected from trout skin and eggs (rainbow trout, *O. mykiss*, and brown trout, *S. trutta*) (Supplementary Table S4).

Water samples were collected in winter 2018/2019 (Supplementary Table S1, Fig. 3) into autoclaved polyethylene bottles washed three times with the water sample before filling and then kept in the dark and on ice during transport. Then, 500 mL of water per sampling location for determination of physico-chemical parameters of the water was freezed at − 20 °C, until the analyses of pH, electrical conductivity (EC), NH_4^+, NO_3^-, SO_4^{2-}, F^-, Cl^-, Na^+, K^+, Mg^{2+}, Ca^{2+}, total organic carbon (TOC), total phosphorus (TP) and chemical oxygen demand (COD) (Supplementary Table S2 and Supplementary Methods). For DNA extraction, 2 L of the water per sampling location was filtered immediately upon return to the laboratory through a 3-branch stainless steel manifolds (Sartorius, Germany) using hydrophilic sterile polyethersulfone filters (d = 47 mm, pore size = 0.22 µm; Millipore Express® PLUS, Germany). Filters with the microbial biomass were stored at − 20 °C until DNA extraction.

Swab samples containing the epibiotic community of *S. parasitica* hosts (including *S. parasitica*, if present) were collected from adult trout (N = 30) and trout eggs (N = 15) sampled in four selected trout farms in Croatia: Gračani (with rainbow trout *O. mykiss*), Kostanjevac (with brown trout *S. trutta*), Radovan (*O. mykiss*) and Solin (*O. mykiss*), in winter 2018/2019 (Supplementary Table S1). After capture at the farm, each animal was transported separately in a plastic bag or container and taken directly to the laboratory, where swab samples were collected as described in Pavić et al.[26].

Live, healthy, fertilised trout eggs were collected at trout farms. About 30–50 eggs were placed in a sterile 50 mL Falcon tube filled with farm water and this constituted one sample. Since no *S. parasitica*-infected eggs were available at the trout farms at the time of sampling, some of the collected egg samples were infected with *S. parasitica* in the laboratory. A total of 15 egg samples were collected: five samples of healthy eggs analysed directly after collection at the farms, five samples of healthy eggs used as negative controls in a laboratory infection trial, and five egg samples infected with *S. parasitica* in the laboratory (Supplementary Table S4). Infection of the eggs with *S. parasitica* and collection of the epibiotic community from the surface of the eggs (infected and healthy) was performed according to Liu et al.[66] with some modifications (details in Supplementary Methods).

No permissions were needed for the experimental work with trout eggs and adult trout performed within this study. Regarding the experimental infection of fertilized trout eggs with *S. parasitica*, no permission was needed since the infection experiment lasted up to 1 week post-fertilization. According to the EU Directive 2010/63/EU[67] on the protection of animals used for scientific purposes, the early life stages of vertebrates, including fish, are not protected as animals until being capable of independent feeding. For trout, the first independent feeding typically takes place about 8 weeks' post-fertilization, and our experiment was terminated much earlier. Thus, the infection experiment does not fall into the regulatory frameworks dealing with animal experimentation (including ARRIVE guidelines). Regarding the adult trout, permissions were not needed to perform the sampling as animals were already dead at the beginning of sampling and were collected as a part of routine harvesting at the fish farms.

DNA extraction. DNA from (i) pure culture oomycete mycelia (listed in Table 1), (ii) pellets of epibiotic communities from the surface of trout skin and eggs, and (iii) filters containing microbial communities from water was extracted using the NucleoSpin® Microbial DNA Kit (Macherey Nagel, Germany) according to the protocol provided by the manufacturer with minor modifications. Samples were lysed by shaking (medium

strength, 20 min) on a Vortex Mixer (Corning, USA) using Macherey Nagel Bead Tubes type B. DNA was eluted from the column using the initial 100 µL eluate for a second elution to increase DNA yield and concentration of the final sample. The NucleoSpin® Tissue Kit (Macherey Nagel, Germany) was used to extract genomic DNA from trout and crayfish, following the protocol provided. The quantity and quality of DNA samples was assessed by agarose gel electrophoresis and QuantiFluor ONE dsDNA Dye on a Quantus Fluorometer (Promega, Germany).

Design of *S. parasitica*-specific primers. ITS sequences of *S. parasitica* and a number of *Saprolegnia* spp. and other oomycetes (Supplementary Table S5) were selected based on an available study on the molecular taxonomy of *Saprolegnia*[42]. Sequences were retrieved from the National Center for Biotechnology Information (NCBI) database using the Batch Entrez tool (https://www.ncbi.nlm.nih.gov/sites/batchentrez) and aligned using MAFFT[68]. Alignment was edited in SeaView[69] and BioEdit[70]. Potential *S. parasitica*-specific primer sequences were selected after manual inspection of the alignment and positioned in the region of maximum divergence to other closely related *Saprolegnia* spp. (Fig. 1b): forward primer 333F (5' CAA ACT TGT TTC ATT TCT TGA TTG GG 3') and reverse primer 580R (5' CCT TAC GTG CCY TGT ACT TTG 3') amplifying a *S. parasitica* DNA segment of 247 bp.

Droplet digital polymerase chain reaction (ddPCR). The ddPCR assay was performed using the QX200™ Droplet Digital™ PCR System (Bio-Rad, USA). Droplet digital PCR reactions and preparations were performed in a dedicated pre-PCR room and PCR hood, separated from both the DNA extraction room and the post-PCR room. Each reaction mixture contained 10 µL of 2 × QX200™ ddPCR™ EvaGreen® Supermix, 200 nM forward and reverse primers, DNA template (1 µL gDNA, 4 µL swab DNA and 8 µL filter DNA) and DNase/RNase-free H_2O in a total volume of 20 µL. These reaction mixtures were mixed with 70 µL of droplet generation oil and droplets were generated using the QX200™ droplet generator, and then transferred to 96-well PCR plates to perform PCR amplifications using the C1000 Touch Thermal Cycler.

Amplification conditions were developed starting with the melting temperatures (Tm, salt-adjusted) of the primers predicted using the OligoCalc tool (http://biotools.nubic.northwestern.edu/OligoCalc.html): 62 °C for 333F and 60 to 61 °C for 580R. Thus, during the assay development, we have tested annealing temperatures of 58 °C, 60 °C and 63 °C. Annealing at 60 °C gave optimal results with *S. parasitica* gDNA template, but at this temperature several related species also yielded some positive droplets (data not shown). Therefore, we adopted a touchdown cycling protocol starting with annealing at 63 °C and ending at 60 °C as follows: denaturation at 95 °C for 5 min, followed by 15 cycles of 95 °C for 30 s and 63 °C for 1 min, followed by another 30 cycles of 95 °C for 30 s and 60 °C for 1 min. Finally, the signal stabilisation step was performed at 4 °C for 5 min and 90 °C for another 5 min, with a final hold at 4 °C.

After the PCR reaction, the droplets were checked for fluorescence using the QX200 Droplet Reader and the data were analysed using QuantaSoft™ version 1.7.4. Four positive controls (genomic DNA from *S. parasitica* pure culture) and one negative control (no template control, NTC) were included in the specificity and sensitivity assays, while one positive control and one NTC were used in the analyses of filters and swabs. Only samples with more than 10,000 droplets were used for the analysis. The limit of detection (LOD) of the ddPCR assay was determined using a serial fivefold dilution of *S. parasitica* gDNA, starting with a concentration of 0.06 ng/µL. Each dilution was tested in triplicate.

Next, we applied the developed *S. parasitica* ddPCR detection assay to analyse the pathogen load in environmental DNA (eDNA) extracted from 21 water samples and 45 trout skin/egg biofilm samples (Supplementary Tables S1 and S2). To obtain a comparable quantification of *S. parasitica* between eDNA samples of different origin/type, the pathogen load was expressed as the number of *S. parasitica* ITS copies per ng of total eDNA.

Data analyses. We analysed the effects of physico-chemical properties of water samples (as abiotic environmental parameters) and health status of eggs and adult fish (as biotic parameters) on *S. parasitica* load, as listed in Table 2.

Partial least squares regression (PLS-R) approach was used to examine the effects of the water composition variables (explanatory variables, X) on the *S. parasitica* load expressed as the number of *S. parasitica* ITS copies per ng of total eDNA (response variable, Y). The PLS-R analyses were performed using XLSTAT version 2021.3.1.1189 software for data analysis and visualisation (Addinsoft, Microsoft Excel).

Data on *S. parasitica* load in biofilm samples were tested for normality using the Shapiro–Wilk test. As they did not follow a normal distribution, non-parametric Mann–Whitney U test was used to examine correlations and differences in *S. parasitica* load between different sub-groups of biofilm samples. Tests were performed in R v. 3.2.0 (R Core Team, 2020), with significance level set at $p = 0.05$.

eDNA sample type for which the *S. parasitica* load was determined	Variable		Number of analysed samples per variable	Statistical analysis
Microbial community from water	Physico-chemical properties of water samples	Values for the following parameters: pH, EC, NH_4^+, NO_3^-, SO_4^{2-}, F^-, Cl^-, Na^+, K^+, Mg^{2+}, Ca^{2+}, TOC, TP and COD	21 sites	Partial least squares regression (PLS-R)
Biofilm of trout eggs	Health status	*S. parasitica* infected or healthy	15 in total 5 laboratory infected 5 healthy infection trial neg. controls 5 healthy from the hatchery	Mann–Whitney U test
Biofilm of adult trout skin collected at the fish farms	Health status	Injured or healthy	30 in total 17 injured 13 healthy	Mann–Whitney U test

Table 2. Analyses of the effect of different variables on *S. parasitica* load.

Data availability

All data generated or analysed during this study are included in this published article and its Supplementary Information files.

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Author contributions
D.P.: Investigation, Formal analysis, Writing—Original Draft, Visualization. D.G.: Software, Formal analysis, Visualization, Project administration. S.H.: Methodology, Writing—Review & Editing. U.P.Z.: Methodology, Validation, Investigation. A.M.: Investigation. R.K.: Methodology. F.V.: Software, Formal analysis, Visualization. J.Č.: Methodology, Investigation. Z.M.: Investigation. A.B.: Conceptualization, Resources, Writing—Review & Editing, Visualization, Supervision, Project administration, Funding acquisition. All authors reviewed the manuscript.

Competing interests
The authors declare no competing interests.

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2.2. Publication II: Identification and molecular characterization of oomycete isolates from trout farms in Croatia, and their upstream and downstream water environments

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Identification and molecular characterization of oomycete isolates from trout farms in Croatia, and their upstream and downstream water environments

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ITS

ABSTRACT

Oomycetes from the genus *Saprolegnia* are opportunistic pathogens that cause significant losses in salmonid aquaculture. Despite this, studies reporting dominant *Saprolegnia* species in different fish farming facilities, as well as analyses of their spreading to natural environments, are still scarce. In this study, we have for the first time identified oomycete species present in four different trout farms in Croatia. We have collected 220 oomycete isolates, both from affected tissue (46 in total: adult trout - 28, eggs - 13, and alevins - 5) and from water (174 in total: in the fish farm – 78, upstream – 50, and downstream - 46). We have used Bayesian inference to reconstruct phylogenetic relationship among the internal transcribed spacer (ITS) sequences of the collected isolates and referent strains, and determined that the isolates belonged to three different oomycete genera: *Saprolegnia* (64% of isolates), *Pythium* (35%), and *Leptolegnia* (1%). *Saprolegnia* isolates were classified into four species: *S. parasitica* with 53 isolates, *S. australis* - 52, *S. delica* – 25, and *S. ferax* – 11. *Pythium* and *Leptolegnia* isolates couldn't be identified to the species level and probably belong to so far undescribed species since their sequences didn't group with previously described species. Next, isolates from the affected tissue were mostly *S. parasitica* (32), while *S. australis, S. delica,* and *S. ferax* were less common ($\leq$ 4 isolates per species). Furthermore, we used hempseed baits to capture oomycetes from water and positioned them inside the fish farms, as well as upstream (between 55 and 155 m) and downstream (between 95 and 140 m) of the fish farms. According to correspondence analysis, *Saprolegnia* species showed a strong association with fish farms and downstream locations, while upstream locations were associated with *Pythium* species, highlighting a possible role of trout farms as a source of spreading *Saprolegnia* species into the environment.

1. Introduction

Oomycetes, commonly known as 'water molds', are fungal-like microorganisms that can be parasitic towards a large number of plant and animal host species (Beakes et al., 2012). Today, oomycete-caused disease outbreaks are threatening wild species biodiversity and food security (Fisher et al., 2012; Phillips et al., 2008). Agriculturally important plant-pathogens have traditionally been receiving much attention, but animal pathogens are understudied even though several genera, such as *Saprolegnia* and *Aphanomyces*, cause devastating diseases in freshwater ecosystems (Bruno et al., 2011; Hussein and Hatai, 2002; Kamoun et al., 2015; Phillips et al., 2008; van West, 2006).

Saprolegnia species (*S. parasitica*, *S. australis*, *S. diclina,* and others) are ubiquitous in the freshwater environment and mostly considered as opportunistic secondary pathogens that infect the host in stressful conditions (such as infection by other pathogens, injuries, or adverse environmental conditions in general) (Gozlan et al., 2014; van den Berg et al., 2013). However, some *S. parasitica* strains were reported to be highly virulent and cause primary infections (Neish and Hughes, 1980; Stueland et al., 2005; Thoen et al., 2011; Whisler, 1996; Willoughby and Pickering, 1977). Saprolegniosis is a fish disease that affects all developmental stages – from eggs to juveniles and adults. It is a major problem in many wild and farmed fish species, such as Atlantic salmon, rainbow and brown trout, and also non-salmonid species like perch, eel,

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and catfish (Bruno et al., 2011; Gozlan et al., 2014). The main symptom of the disease is circular or crescent-shaped, white or grey, cotton-like mycelium developing anywhere on the fish body (Fregeneda Grandes et al., 2001; Hussein et al., 2001; Willoughby, 1989, 1994). The disease is frequent during the winter when fish are often immunocompromised due to decreased water temperature (Bly et al., 1992; Bly and Clem, 1992).

Saprolegniosis is a serious problem in salmon and trout farms and hatcheries. Massive infections of eggs are common, and entire batches can be lost (Cao et al., 2012; Meyer, 1991; Rach et al., 2005; Thoen et al., 2011; van den Berg et al., 2013). This is a significant problem worldwide, commonly causing yearly economic losses of more than 10%, and occasionally up to 50% (Diéguez-Uribeondo et al., 2007; Rezinciuc et al., 2014; van den Berg et al., 2013; van West, 2006). Further, the primary existing disease control measure, malachite green, is banned in the European Union, due to its carcinogenicity and toxicity. The same fate is expected to befall formalin, leaving very limited control options available (Gozlan et al., 2014; Phillips et al., 2008; Tedesco et al., 2019; van den Berg et al., 2013; van West, 2006), and most likely causing an increase in saprolegniosis outbreaks.

Knowledge about the identity, distribution, and pathogenic significance of *Saprolegnia* species in aquaculture facilities is a necessary prerequisite for the development of efficient control measures. However, until recently little was known about dominant species associated with saprolegniosis outbreaks. This was probably because species were identified only based on the morphology of their sexual structures (Seymour, 1970), which was time-consuming and often unsuccessful (Diéguez-Uribeondo et al., 2007; Fregeneda-Grandes et al., 2007; van den Berg et al., 2013). Molecular diagnostic tools have been recently introduced in the identification of *Saprolegnia* spp., based on the sequence of internal transcribed spacer (ITS) region positioned between rRNA encoding genes (Cao et al., 2012; Diéguez-Uribeondo et al., 2007; Kozubíková-Balcarová et al., 2013; Rezinciuc et al., 2014; Sandoval-Sierra et al., 2014; Sarowar et al., 2019a; Tandel et al., 2020). However, the presence of many misassigned ITS sequences in DNA databases (e.g. GenBank) caused the erroneous classification of many isolates in the culture collections. A recent study on *Saprolegnia* molecular taxonomy resolved this issue and enabled the correct identification of *Saprolegnia* isolates to the species level, without the need for morphological characterization (Sandoval-Sierra et al., 2013). This allowed the recent accurate identification of *Saprolegnia* species in fish farms in Canada (Sarowar et al., 2019a), Chile (Sandoval-Sierra et al., 2014), Spain (Rezinciuc et al., 2014), and elsewhere (Paul et al., 2015; Sakaguchi et al., 2019).

It has been shown that pathogens can be transmitted from the fish farms to wild populations and vice versa (Johansen et al., 2011; Kurath and Winton, 2011; McVicar, 1997). In the context of this study, we were interested in the possible transfer of *Saprolegnia* pathogens from the fish farms to the downstream freshwater environments. Since freshwater aquaculture facilities are often connected with rivers/streams, it is possible for fish to escape or water to drain into the surrounding environment, allowing the transfer of pathogens (Andreou et al., 2012; Garseth et al., 2013; Gozlan et al., 2014; Johansen et al., 2011; Thorstad and Finstad, 2018). However, while transmission of viral and bacterial pathogens from farmed fish to wild populations has been repeatedly reported (Johansen et al., 2011; Johnsen and Jensen, 1994; Raynard et al., 2001; Wallace et al., 2008), knowledge of trout farms as points of spreading of *Saprolegnia* spp. to natural waters is limited (Galuppi et al., 2017).

The aim of this study was to perform the first survey of *Saprolegnia* species in selected trout farms in Croatia. Moreover, we have investigated the correlation between the occurrence of pathogenic *Saprolegnia* species in the fish farms and their incidence in natural waters upstream and downstream of the fish farms.

2. Materials and methods

2.1. Sampling

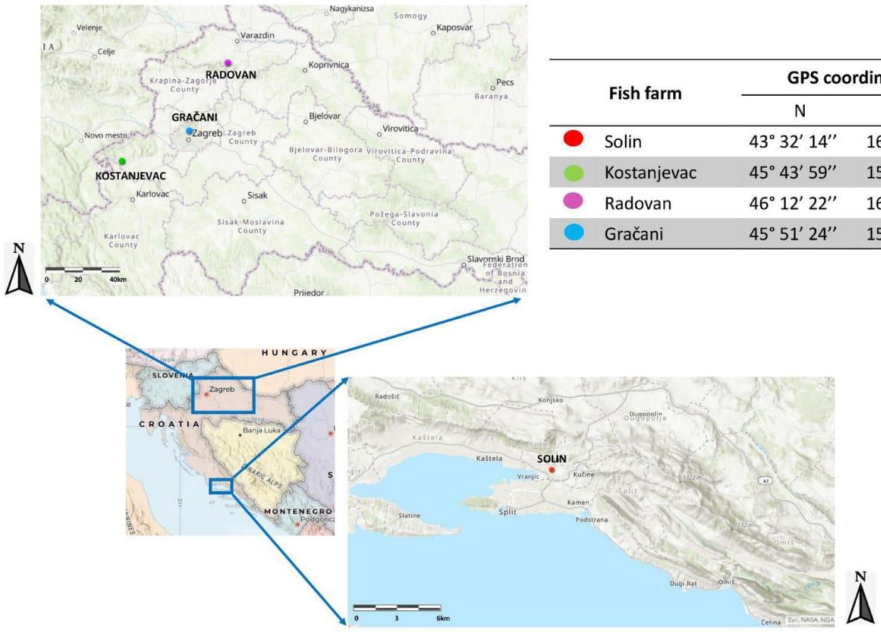
Sampling of oomycetes was carried out at four aquaculture facilities in Croatia (Fig. 1): three were located in central Croatia, at Gračani (part of Zagreb) (fish farm producing rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792)), Kostanjevac (a village near Zagreb) (fish farm producing brown trout, *Salmo trutta* (Linnaeus, 1758)), and Radovan (a village near Varaždin) (fish farm and hatchery producing *O. mykiss*), while the fourth one, Solin (a town near Split) (fish farm and hatchery producing *O. mykiss*), was located at the Adriatic coast. Sampling was carried out during winter (November, December, and January) in 2018 and 2019 (Supplementary material, Table A.1). Conditions on the fish farms at the time of sampling were favorable for trout rearing (Woynarovich et al., 2011): water temperature was between 9 and 12.5 °C, pH between 7.3 and 7.8, and dissolved O_2 between 8.5 and 10.3 mg/L, as measured by a portable multimeter (Hach® Field Case, Colorado, USA) (Table 1). Hatcheries Radovan and Solin (where alevins and eggs were sampled, respectively) were located near the adult fish rearing basins and were using the same water. The number of diseased fish in all farms was less than 1%. Oomycetes were isolated from the host (eggs, alevins, and adult trout), as well as from the water in the farm, upstream and downstream.

A total of 75 tissue samples were collected from embryonic (eggs and alevins covered in *Saprolegnia*-like mycelium) and adult specimens (having skin lesions with external signs of *Saprolegnia* spp. mycelium growth) (Supplementary material, Table A.1). Affected embryonic stages were available only in Radovan (eggs) and Solin (alevins), while affected adult trout were collected in all fish farms. Affected tissue (lesions) was excised from adult fish, while eggs and alevins with cotton-like mycelia growth, dead at the time of sampling, were taken whole. Tissue samples of adult specimens with no signs of infection (gills and skin) were also analyzed, in order to compare the oomycete isolation success and the identity of the obtained isolates with those originating from the affected samples. Samples were collected aseptically, dipped for approximately one second in 96% ethanol, and rinsed with sterile distilled water, to reduce bacterial contamination which could lead to unsuccessful oomycete isolation. Next, rinsed samples were placed onto glucose-yeast extract agar (GY, g/L: 12 g/L agar, 5 g/L glucose, 1 g/L yeast extract) (Min et al., 1998) supplemented with penicillin G and oxolinic acid in the final concentration of 6 and 10 mg/L, respectively (Alderman and Polglase, 1984). Plates were incubated at 18 °C (Galuppi et al., 2017), and pure cultures were obtained by transferring the growing mycelial tips to fresh plates every three days.

Hempseed baits (homemade 'tea balls') were used to isolate oomycetes from water. Each bait contained seven to ten halves of previously boiled hemp seeds (Seymour, 1970). Baits were placed inside of each farm and also upstream and downstream of their water system, following the protocol recently applied by Galuppi et al. (2017). The exception was fish farm Solin where *Saprolegnia* baits were not positioned upstream and downstream of the fish farm due to its specific position near the sea. The number of positioned baits and retrieved hempseeds per location is given in Supplementary Table A.1. Upstream locations were positioned 55, 155, and 60 m upstream of the Gračani, Kostanjevac, and Radovan fish farms, respectively, while downstream locations were 130, 140, and 95 m downstream. Baits were retrieved after 10 days, yielding in total 289 samples (i.e. hempseeds). Hempseeds (with attached microorganisms from water) were treated as described above for host-associated oomycetes, i.e. they were dipped in ethanol, rinsed with distilled water, and then seeded individually on GY.

2.2. DNA extraction, amplification, and sequencing

DNA extraction was carried out from mycelia grown in liquid GY medium for two days at 18 °C. Mycelia were washed with sterile distilled



Fish farm	GPS coordinates	
	N	E
Solin	43° 32′ 14″	16° 29′ 33″
Kostanjevac	45° 43′ 59″	15° 25′ 18″
Radovan	46° 12′ 22″	16° 15′ 12″
Gračani	45° 51′ 24″	15° 57′ 53″

Fig. 1. Position of studied fish farms in Croatia with coordinates (WGS84 coordinate reference system).

Table 1
Water quality parameters inside the sampled fish farms.

	Solin	Kostanjevac	Radovan	Gračani
Conductivity (μS/cm)	523	470	549	393
Dissolved oxygen (mg/L)	9.34	8.54	10.25	9.13
pH	7.65	7.84	7.94	7.35
Average temperature (°C)	8.3	8.9	12.47	12.5

water and centrifuged at 10000 ×*g* for 15 min to obtain pellets (app. 30 mg wet weight per sample) that were stored at −20 °C until DNA extraction. DNA was extracted using the NucleoSpin® Microbial DNA kit (Macherey Nagel, Germany), following the provided protocol with slight modifications. Samples were lysed by agitation (medium strength, 20 min) on a Vortex Mixer (Corning, USA), using Macherey Nagel Bead Tubes Type B. DNA was eluted from the column using the initial 100 μL eluate for a second elution to increase DNA yield and concentration.

The ITS region (ITS 1, 5.8S rDNA, and ITS 2) was amplified with universal primers for eukaryotes ITS5 (5′ GGAAGTAAAAGTCGTAACAAGG 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) (White et al., 1990) under conditions described by Sandoval-Sierra et al. (2013). Shortly, the reaction mixture contained 1 μL of the genomic DNA, 12.5 μL of EmeraldAmp® PCR 2× Master Mix (TAKARA), 0.5 μL of 10 μM primers and dH_2O to a final volume of 25 μL. Thermal cycling was performed in Alpha Cycler 1 (PCRmax) with the following conditions: 2 min at 95 °C for initial denaturation, followed by 35 cycles of 1 min at 95 °C (denaturation), 30 s at 60 °C (annealing), and 1 min at 72 °C (extension), and 10 min at 72 °C as a final extension step. *Saprolegnia parasitica* CBS 233.65 genomic DNA and distilled water were used as positive and negative control, respectively. Obtained amplicons, approximately 600 bp long for *Saprolegnia* spp. and *Leptolegnia* spp., and approximately 900 bp for *Pythium* spp. (Fig. 2 - D), were purified and then sequenced (Sanger sequencing, Microsynth, Austria) using primer ITS4. Chromatograms were analyzed and edited, including the trimming of 5′ and 3′ ends with lower quality of peaks, in GeneStudio. Obtained sequences are deposited in GenBank under accession numbers (Acc. No.) MT555787 – MT556006 (Supplementary material, Table A.1).

2.3. Alignment and phylogenetic analyses

Multiple sequence alignment (MSA) of the ITS region of all isolates and selected reference sequences was constructed in MAFFT using default settings (Katoh and Standley, 2013), and edited in SeaView (Gouy et al., 2010) and BioEdit (Hall et al., 2011). Reference sequences from genera *Achlya*, *Aphanomyces*, *Leptolegnia*, *Phytophthora*, *Pythium*, and *Saprolegnia* were selected based on the available literature on their molecular phylogeny (Lévesque and De Cock, 2004; Rocha et al., 2018; Sandoval-Sierra et al., 2013) and retrieved from NCBI database using the Batch Entrez tool (https://www.ncbi.nlm.nih.gov/sites/batchentrez) (Supplementary material, Table A.2). Two separate MSAs were constructed: one comprising the sequences from the order Saprolegniales (genera *Saprolegnia* and *Leptolegnia*) (Supplementary material, Fig. B.1), and another with sequences from the order Peronosporales (genus *Pythium*) (Supplementary material, Fig. B.2). Final MSA of *Saprolegnia* and *Leptolegnia* sequences contained in total 179 sequences (143 sequences of isolates and 36 reference sequences), while MSA of *Pythium* had 112 sequences (77 sequences of isolates and 35 reference sequences). The phylogenetic relationship among the sequences was reconstructed with the Bayesian inference method using MrBayes software 3.2.7a with 200,000 iterations (Ronquist and Huelsenbeck, 2003). Two simultaneous, independent analyses were run with four Markov chain Monte Carlo (MCMC), one cold and three heated chains with temperature set to 0.5. Every 100 generations were sampled and first 25% of the samples from the cold chain were discarded as 'burn-in'. Posterior probability was estimated for the remaining trees. Phylogenetic trees were visualized with Figtree v1.4.4. (http://tree.bio.ed.ac.uk/software/figtree/).

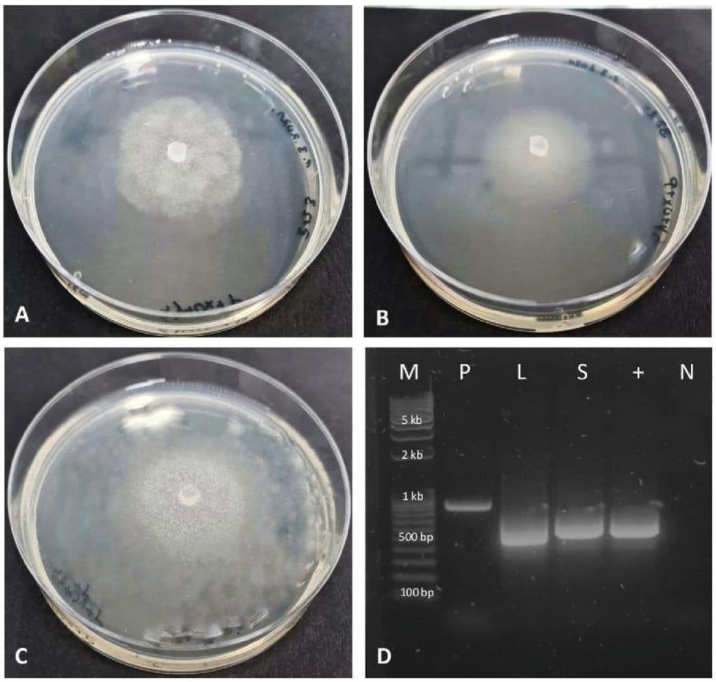


Fig. 2. Representatives of the isolates collected at the Croatian trout aquaculture facilities from the genera (A) *Pythium* (isolate Z121), (B) *Leptolegnia* (isolate BD25), and (C) *Saprolegnia* (*S. parasitica* isolate BF1). (D) PCR amplification of the ITS region of the respective isolates with universal primers ITS5 and ITS4. M – SimplyLoad™ Tandem DNA Ladder (Lonza), P – *Pythium* sp. (Z121), L – *Leptolegnia* sp. (BD25), S – *S. parasitica* (BF1), + – positive control (*S. parasitica* CBS 233.65), N - negative control (distilled water).

2.4. Species diversity and richness

Biodiversity of oomycete species isolated from tissue samples (adult and embryonic stages) and water samples (upstream - U, fish farm - F and downstream - D) was measured and estimated with Shannon (H, species diversity) and Menhinick's index (D, species richness) (Ludwig and Reynolds, 1988; Menhinick, 1964; Shannon, 1948). Species richness (D), a simple measure referring to a number of species in a sampled location, is calculated as follows:

$$D = \frac{s}{\sqrt{N}}$$

where s counts the number of different species present in a sampled location and N equals the total number of individuals in a sampled location. Diversity index (H) gives information about rarity and commonness of species in a sampled location, and is calculated as follows:

$$H = -\sum_{i=1}^{R} p_i \ln p_i$$

where proportion of species i is relative to the total number of species p_i (Ludwig and Reynolds, 1988).

2.5. Statistical analyses

Associations between oomycete species and sampling location/type of sample were analyzed by correspondence analysis (CA) which provides factor scores (coordinates) for both row and column points of the contingency table. These coordinates provide a solution for summarizing the data set in two-dimension plots, used to visualize graphically the association between the row and column elements in the contingency table (Kassambara, 2017). Dimensions 1 and 2 both indicate the percentage of association between the row and column categories. We have tested the following associations: (i) tissue-associated isolates (from all four fish farms) vs. trout developmental stage (egg, alevin, adult), (ii) tissue-associated isolates (from all tissue types) vs. fish farm (all four fish farms included); (iii) farm water-associated isolates (captured by hempseed baits in the fish farms) vs. fish farm (all four fish farms included), and (iv) water-associated isolates (captured by hempseed baits) vs. sampling location (upstream, fish farm, and downstream, from fish farms Kostanjevac, Radovan and Gračani). Noteworthy, fish farm Solin was excluded from the last analysis, since in this case the hempseed baits could not be positioned upstream and downstream of the fish farm due to its specific position near the sea.

CA was obtained and plotted using R v. 3.2.0. To compute and interpret CA two R packages were used: i) FactoMineR for the analysis, and ii) factoextra for data visualization. The observed associations were tested using Pearson's χ2-test.

3. Results

3.1. Molecular identification of oomycete isolates from Croatian trout farms

A total of 220 oomycete isolates were cultured, 46 originating from tissue samples, and 174 from hempseed baits (Fig. 2 A - C; Tables 2 and 3; Supplementary material, Table A.1). Oomycete detection frequency for both sample types/sampling methods was similar: 61% for tissue samples (46 samples with oomycete growth out of the total number of 75 samples), and 60% for hempseed halves (174 out of 289 hempseeds resulted in oomycete growth) (Supplementary material, Table A.1). From the gill and tissue samples showing no clinical signs of saprolegniosis, the isolation of oomycetes was less successful than from skin lesions, 50% (i.e. 7 samples out of 14 resulted in oomycete growth) and

Table 2
Overview of oomycete isolates obtained from the surface of eggs, alevins, and adult fish with signs of disease at the trout farms in Croatia.

Species	Adult stage				Embryonic stage		Total no. (%)
	Solin	Kostanjevac	Radovan	Gračani	Radovan (eggs)	Solin (alevins)	
S. australis	0	4	0	0	0	0	**4 (9)**
S. delica	0	2	0	0	0	1	**3 (6)**
S. ferax	0	0	0	0	0	1	**1 (2)**
S. parasitica	2	2	0	13	12	3	**32 (70)**
Leptolegnia sp.	0	0	1	0	0	0	**1 (2)**
Pythium sp.	2	2	0	0	1	0	**5 (11)**
No. of isolates	**4**	**10**	**1**	**13**	**13**	**5**	**46 (100)**

Table 3
Overview of oomycete isolates obtained by hempseed baits from the water in the fish farms (F), as well as upstream (U) and downstream (D) locations.

Species	Solin[a]	Kostanjevac			Radovan			Gračani			Total no. (%)
	F	U	F	D	U	F	D	U	F	D	
S. australis	8	1	12	13	0	0	0	2	12	0	**48 (27)**
S. delica	3	1	10	8	0	0	0	0	0	0	**22 (13)**
S. ferax	1	1	0	0	0	3	5	0	0	0	**10 (6)**
S. parasitica	12	0	1	1	0	3	2	0	2	0	**21 (12)**
Leptolegnia sp.	0	0	0	0	0	0	1	0	0	0	**1 (1)**
Pythium sp.	0	29	0	1	5	10	6	11	1	9	**72 (41)**
No. of isolates	**24**	**32**	**23**	**23**	**5**	**16**	**14**	**13**	**15**	**9**	**174 (100)**

[a] Hempseed baits were not positioned upstream and downstream of the fish farm Solin due to its specific position near the sea.

60% (21/35) respectively. Furthermore, only 57% of the isolates collected from the healthy gills and skin were identified as *Saprolegnia* sp., while 90% of isolates collected from the skin lesions belonged to the genus *Saprolegnia*.

Morphologically, the isolates could be divided into three groups, as depicted in Fig. 2 A-C. PCR amplification of the ITS region of the isolates yielded DNA fragments of 600–900 bp (Fig. 2 - D) that were sequenced and used for species identification. Due to the large number of isolates, two separate phylogenetic trees were constructed, one for *Saprolegnia* and *Leptolegnia* isolates (order Saprolegniales), and another for *Pythium* isolates (order Peronosporales) (Figs. 3 and 4). The obtained grouping of the Saprolegniales sequences showed that the *Saprolegnia* isolates (64% of the total number of isolates) were mostly *S. parasitica* (53; 24%) and *S. australis* (52; 24%), followed by *S. delica* (25; 11%) and *S. ferax* (11; 5%) (Fig. 3). Additionally, two *Leptolegnia* isolates (B11L3 and BD25, 1% of the isolates) were identified, but could not be classified to the species level, since they grouped with another unidentified (*Leptolegnia* sp.) sequence. Thus, these isolates probably belong to a so-far undescribed *Leptolegnia* species.

All collected *Pythium* isolates, comprising 77 isolates or 35% of the total isolates, grouped within the previously described *Pythium* group B (Lévesque and De Cock, 2004) (Fig. 4). Among them, a majority of 75 isolates formed a well-supported clade within the B2 group, most probably a novel *Pythium* species. Isolate B3S1 showed the highest identity with *P. lutarium* (78.28%), *P. diclinum* (78.15%), and *P. marinum* (78.03%), all from the B2 group, while Z111 belonged to the B1 group and showed the highest identity with *P. vanterpoolii* (71.18%).

3.2. Oomycete isolates from diseased adult and embryonic trout

Diseased adult fish were collected from all farms yielding a total of 28 isolates, while infected eggs and alevins were available only at Radovan and Solin, yielding 13 and 5 isolates, respectively (Table 2; Supplementary material, Table A.1).

The most frequent species was *S. parasitica* (70%, 32 isolates), while other *Saprolegnia* species (*S. australis*, *S. delica*, and *S. ferax*) were less common ($\leq$ 4 isolates per species). *Saprolegnia parasitica* was isolated both from adult and embryonic samples and was found as dominant species in Radovan, Gračani, and Solin fish farms. Besides genus *Saprolegnia*, two more oomycete genera were isolated, *Leptolegnia* sp. from the adult stage and *Pythium* sp. from both adult and embryonic stages.

Correspondence analysis (CA) was performed to analyze the associations between collected oomycete species and trout developmental stage, as well as between oomycete species and fish farms. No significant association ($p = 0.1$, Table 4) was found between oomycete species and trout developmental stage (Supplementary material, Fig. A.1-A). However, significant differences were observed ($p = 0.002$, Table 4) between oomycete species isolated from tissue samples and different fish farms (Supplementary material, Fig. A.1-B). Mainly, Kostanjevac differed from the other fish farms with *S. australis*, and not *S. parasitica*, being the dominant detected species.

Diversity of tissue-associated oomycete isolates, as estimated by the Shannon index (H), was greatest in Kostanjevac (1.33), followed by Solin (1.15) and Radovan (0.51), while in Gračani only *S. parasitica* was detected (H = 0). However, Solin had greater species richness (1.33) than Kostanjevac (1.27), Radovan (0.8), and Gračani (0.28).

3.3. Oomycete isolates from water

The most prevalent *Saprolegnia* species found in water samples was *S. australis*, followed by *S. delica*, *S. parasitica*, and *S. ferax* (Table 3). Additionally, besides being isolated from tissue samples, one *Leptolegnia* sp. isolate was also found downstream of Radovan. *Pythium* sp. isolates were also found, mostly in upstream locations.

The oomycete species collected from water varied according to the fish farm (Solin, Kostanjevac, Radovan or Gračani; Table 3, Supplementary material, Fig. A.1-C) and the sampling location (U, F and D) (Tables 3 and 5; Fig. 5). Generally, dominant *Saprolegnia* species captured from water in different fish farms were in accordance with *Saprolegnia* species detected in diseased animal tissues (Tables 2 and 3, Supplementary material, Fig. A.1-B and C). For instance, *S. parasitica* dominated in Solin and *S. australis* in Kostanjevac (Tables 2 and 3). However, in one farm (Gračani), *S. parasitica* was dominantly isolated from tissue samples (Table 2), while *S. australis* was most prevalent among isolates from water (Table 3). Further, the correspondence analysis showed strong association ($p = 2.73 \times 10^{-9}$, Table 4) between the sampling location (U, F, or D) and collected oomycete species (Fig. 5). In this analysis, the first two dimensions explained the 100% of association that exists between oomycete species and sampling locations, where the first dimension explained 91.9%, and the second

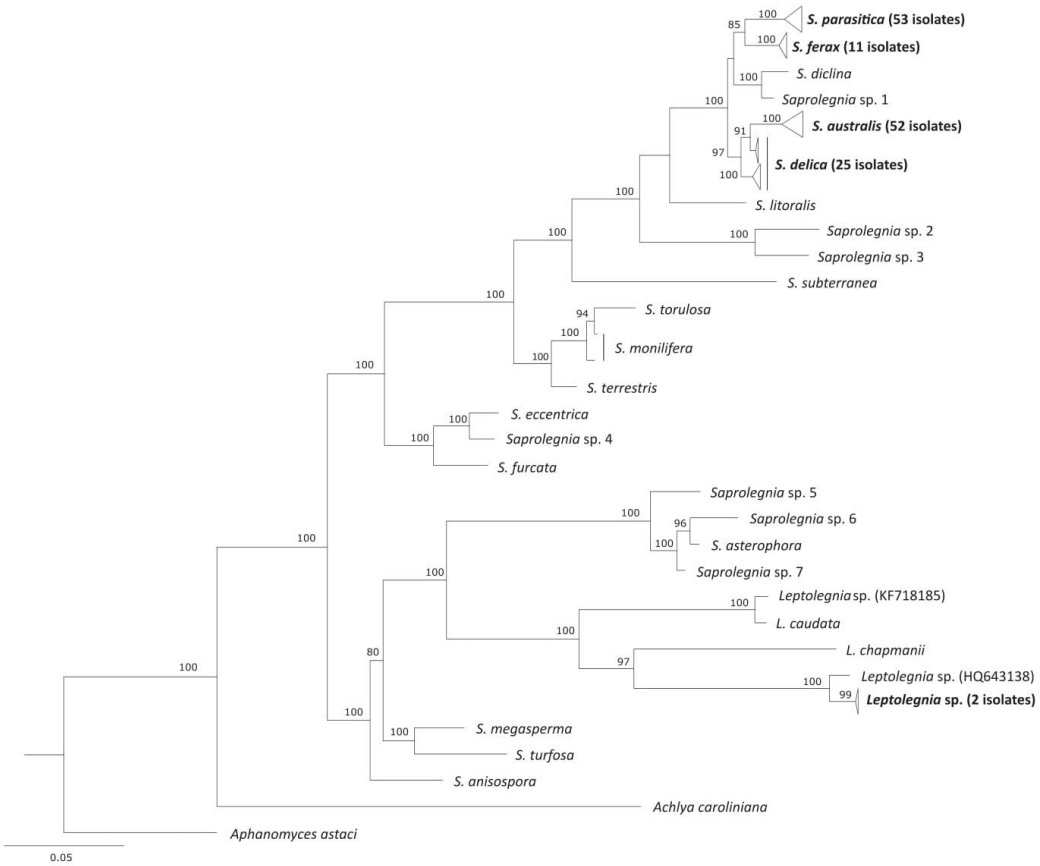


Fig. 3. Phylogenetic analysis of *Saprolegnia* and *Leptolegnia* isolates (in bold) from Croatian trout farms and their upstream and downstream water environments. The phylogenetic tree is based on Bayesian inference analysis of ITS sequences. Bayesian posterior probabilities ≥80% are shown at the nodes. GenBank accession numbers of reference sequences are given in Supplementary Table A.2, except for two *Leptolegnia* sp. sequences (accession numbers shown in the tree).

dimension explained 8.1% of the association. Namely, the presence of pathogenic *Saprolegnia* species was associated with fish farms and downstream locations, while *Pythium* sp. was typically dominant in upstream locations (Fig. 5, Table 5).

Oomycete species richness and diversity were highest downstream of fish farms (0.88 and 1.53, respectively). Upstream and inside the fish farm richness was approximately the same (0.56 and 0.57), but diversity was higher inside the fish farms (1.43) than upstream (0.42).

4. Discussion

We report on the oomycete species identified in selected trout farms in Croatia, with emphasis on *Saprolegnia* spp. that cause saprolegniosis and significant economic losses in aquaculture worldwide (van den Berg et al., 2013; van West, 2006). Importantly, we discuss the possible role of trout farms as points of spreading pathogenic *Saprolegnia* species into the environment.

4.1. Pathogenic oomycete sampling approaches in freshwater ecosystems

We have combined two sampling approaches (tissue and water samples) to get the most insight into pathogenic oomycete species present in selected fish farms and the natural environment. Both methods have advantages and disadvantages and can be complementary when used in combination, as was demonstrated in several previous studies (Galuppi et al., 2017; Rahman and Sarowar, 2016; Rezinciuc et al., 2014; Sarowar et al., 2019b; Sarowar et al., 2013; Thoen et al., 2015). For instance, since hempseeds attract zoospores/cysts in the water, some non-zoosporic species can go undetected. Also, bacteria in the water can sometimes disable zoospore attachment and germination on the baits (Sarowar et al., 2019b). On the other hand, personnel in the fish farms are often reluctant to provide affected animals (as was also the case during this study). Also, it is sometimes difficult to obtain infected, but still living embryonic stages, since they quickly succumb to the disease. It is therefore hard to know whether the isolated oomycete species was the primary pathogen, or if the initial pathogen was overgrown by a secondary, opportunistic species. In our case, the fact that *S. diclina*, well known for egg infections (Fregeneda-Grandes et al., 2007; Sandoval-Sierra et al., 2014; Thoen et al., 2011; van den Berg et al., 2013), was not isolated from dead eggs, might indicate that sometimes the opportunistic species were cultivated (e.g. when *Pythium* sp. was isolated from the infected egg).

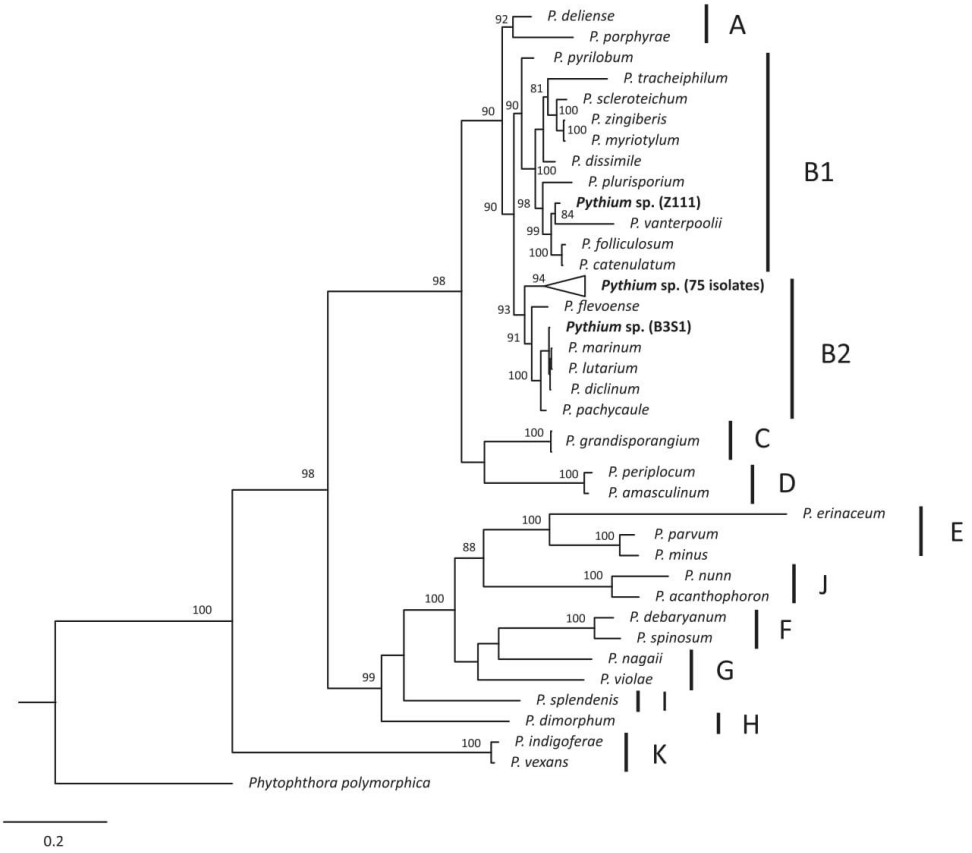


Fig. 4. Phylogenetic analysis of *Pythium* isolates (in bold) from Croatian trout farms and their upstream and downstream water environments. The phylogenetic tree is based on Bayesian inference analysis of ITS sequences. Bayesian posterior probabilities ≥80% are shown at nodes. Clades A – K are labeled according to the available molecular phylogeny and taxonomy of the genus *Pythium* (Lévesque and De Cock, 2004). GenBank accession numbers of reference sequences are given in Supplementary Table A.2.

Table 4
Chi-square test displaying dependence between row and column categories. *P* values falling below the critical α = 0.05 are in boldface. Location: U – upstream locations; F – fish farm; D – downstream locations. Fish farms: S – Solin; K – Kostanjevac; R – Radovan; G – Gračani.

Pearson's Chi-squared test	*X*-squared	df	*p*-Value
Tissue-associated isolates vs. trout developmental stage	15.984	10	0.1001
Tissue-associated isolates vs. fish farm (S, K, R, G)	56.798	30	**0.002**
Farm water-associated isolates (F) vs. fish farm (S, K, R, G)	107.69	15	**4.48×10^{-16}**
Water-associated isolates vs. sampling location (U, F, D)	60.653	10	**2.73×10^{-9}**

Table 5
A contingency table displaying the number of oomycete species isolated from water (hempseed baits) at different sampling locations (upstream, fish farm or downstream, from fish farms Kostanjevac, Radovan and Gračani).

Oomycete species	Upstream	Fish farm	Downstream	Total
Leptolegnia sp.	0	0	1	**1**
Pythium sp.	45	11	16	**72**
Saprolegnia australis	3	24	13	**40**
Saprolegnia delica	1	10	8	**19**
Saprolegnia ferax	1	3	5	**9**
Saprolegnia parasitica	0	6	3	**9**
Total	**50**	**54**	**46**	**150**

In overall, oomycete detection frequency in our study was equal for both sampling methods, approximately 60%. However, we have observed some differences in *Saprolegnia* isolates collected from the surface of affected animals (mostly *S. parasitica*) and from farm water (most often *S. australis*). This could be explained by the higher pathogenicity of *S. parasitica* (Gozlan et al., 2014; van den Berg et al., 2013; van West, 2006).

4.2. Diversity of oomycete species associated with trout farms in Croatia

In this study, three genera of oomycetes were identified: *Saprolegnia*, *Leptolegnia* and *Pythium*. The dominant species and the only one that was isolated from all fish farms and all trout developmental stages was

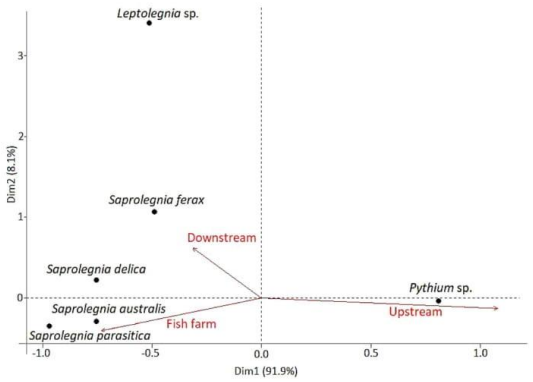


Fig. 5. Correspondence analysis biplot displaying the associations of oomycete species isolated from water (hempseed baits) with the sampling location (upstream, fish farm, or downstream). Oomycete species are represented by black points and sampling locations by red arrows. The distance between any species points or sampling location points gives a measure of their similarity (or dissimilarity). Points with a similar profile are closer on the factor map. Dimensions (Dim) 1 and 2 both indicate the percentage of association between the row and column categories. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

S. parasitica. Thus, our results confirm its dominance over other *Saprolegnia* species in aquaculture facilities (Hussein and Hatai, 2002; Noga, 1993; Sandoval-Sierra et al., 2014; Sarowar et al., 2019a; van den Berg et al., 2013; van West, 2006). Previous infection trials demonstrated pathogenicity of *S. parasitica* towards eggs (Kitancharoen and Hatai, 1996), fingerlings (Yuasa and Hatai, 1995), and adult salmonids (Stueland et al., 2005). In contrast to our findings, in Chilean salmonid farms *S. parasitica* was detected in adult *Salmo salar* and *O. mykiss*, but not in eggs and alevins (Sandoval-Sierra et al., 2014). This could be explained by the known variations in pathogenicity of *S. parasitica* isolates towards different developmental stages of the host (Stueland et al., 2005; Thoen et al., 2011; Yuasa and Hatai, 1995).

Other *Saprolegnia* species isolated from adult and embryonic stage (alevins) in Croatian trout farms as well as from water, were *S. australis*, *S. delica*, and *S. ferax*. Regarding *S. australis,* this species was only isolated from adult *S. trutta* individuals at fish farm Kostanjevac (two isolates from skin lesions and two from healthy skin), while it was not found on *O. mykiss* in other fish farms (that were dominated by *S. parasitica*). Although *Saprolegnia australis* is mostly regarded as pathogenic towards fish embryonic stages (Fregeneda-Grandes et al., 2007; Rezinciuc et al., 2014; Sandoval-Sierra et al., 2014; Tandel et al., 2020; Thoen et al., 2011), in our study it wasn't isolated from this sample type. This result may be caused by the small sample size (i.e. overall low number of isolates collected from eggs and alevins - 18). An earlier analysis of fish farms in Chile, with higher number of isolates from embryonic stage (122) showed an association between different *Saprolegnia* species and salmonid developmental stage, i.e. *S. australis* was associated with alevins (Sandoval-Sierra et al., 2014). Interestingly, *S. australis* was the most prevalent species found in water, especially in fish farms Kostanjevac and Gračani. This might be explained by the fact that, although *S. australis* zoospores might have been present in the water in high number (and thus easily captured by hempseed baits), infection with *S. australis* rarely occurred because the fish and eggs were healthy (and thus the animals were more often infected by *S. parasitica*, as a more virulent pathogen) (van den Berg et al., 2013; van West, 2006).

Lastly, *S. delica* was the second most isolated *Saprolegnia* species in water samples, and *S. ferax* was also occasionally captured by hempseed baits, while these two species were rarely obtained from tissue samples. Both species have previously been associated with embryonic mortality of fish and amphibians (Blaustein et al., 1994; Cao et al., 2012; Fregeneda-Grandes et al., 2007; Kiesecker et al., 2001) and were also often isolated from water (Rezinciuc et al., 2014; Sarowar et al., 2013). Our results are similar to a recent study done in Chilean salmonid farms, where *S. ferax* and *S. delica* were found both on adult and embryonic stages of salmonid fish without a clear preference for any particular developmental stage (Sandoval-Sierra et al., 2014).

Besides *Saprolegnia* species, two isolates from fish farm Radovan were identified as *Leptolegnia* sp., one from a tissue sample (adult stage - lesion) and one from water downstream of the fish farm. It is possible that these two isolates represent new *Leptolegnia* species, since they were not grouped with any of the known species, *L. caudata* or *L. chapmanii*, parasites of mosquitoes (Bisht et al., 1996; Lastra et al., 2004; Montalva et al., 2016; Schimmel and Noblet, 1985). *Leptolegnia* sp. have also been isolated from cladocerans, fish, and amphibian eggs and larvae (Petrisko et al., 2008; Rezinciuc et al., 2014; Wolinska et al., 2009), but so far their pathogenicity has only been proven towards amphibian eggs (Ruthig, 2009). Our results might indicate that some *Leptolegnia* species could be opportunistic fish pathogens, but infection trials are needed to confirm this.

Furthermore, *Pythium* sp. isolates were also found in this study, mostly from water upstream of the fish farms (B2 isolates, and one B1 isolate - Z111 from Radovan), while a small number of B2 isolates were also found on diseased fish and only one B2 isolate on an egg sample. Known *Pythium* species are mostly plant pathogens or saprotrophs, mainly associated with natural and agricultural soils (Rahman and Sarowar, 2016; Robideau et al., 2011; Schroeder et al., 2013). However, *Pythium* sp., including B1 and B2 clades, were also isolated from natural and aquacultural freshwater environments (Nechwatal et al., 2008; Rahman and Sarowar, 2016; Schroeder et al., 2013), and some were suggested to be pathogenic towards freshwater animals (Miura et al., 2010). *Pythium* spp., including members of the clade B, were isolated from the carapace of dead crustaceans (Czeczuga et al., 2002b) and from dead or alive fishes and eggs (Czeczuga, 1996; Czeczuga et al., 2002a). *Pythium flevoense* (belonging to clade B2 and most closely related to isolate B3S1 found on *O. mykiss* from Solin) was reported to be responsible for mass mortality of freshwater fish (ayu larvae), but pathogenicity of isolates was not confirmed by infection trials (Miura et al., 2010). Taking all this into account, most of our isolates are probably plant pathogens or soil saprotrophs that arrived to the fish farms by water routes, as can be presumed from their phylogenetic grouping with saprotrophs and plant pathogens, and the fact that the majority were captured in the water upstream of the fish farms. For instance, isolate Z111 was most closely related to a known plant pathogen *P. vanterpoolii* (clade B1) (Asano et al., 2010; Ichitani et al., 1989; Muse et al., 1974). Also in concordance with this hypothesis, most of the studies that isolated *Pythium* spp. from fish tissue or water samples were conducted in the fish farms or ponds surrounded by agricultural fields, grassland, or forests (Czeczuga et al., 2005; Czeczuga et al., 2002a; Naznin et al., 2017; Rahman and Sarowar, 2016; Sarowar et al., 2019b). This was also the case for three out of four fish farms sampled here (Gračani, Kostanjevac, Radovan).

Finally, our *Pythium* isolates probably belong to a novel, so far undescribed species. To confirm this, a detailed morphological description of the isolates is needed (Tambong et al., 2006), coupled with the analysis of additional molecular markers, besides ITS. Cytochrome c oxidase subunit I (COI) is a mitochondrially encoded gene that is more discriminative at the species level than the ITS region (Schroeder et al., 2013). Using both ITS and COI, rather than only one of them, is recommended for taxonomic identification of *Pythium* species (Bala et al., 2010; Robideau et al., 2011), and should be applied in the future studies of *Pythium* isolates associated with fish farms.

4.3. Trout farms enrich the pathogenic oomycetes in the downstream freshwater environment

To elucidate whether trout farms act as reservoirs of pathogenic *Saprolegnia* species that can spread to natural environments, we have collected oomycete isolates upstream, downstream, and inside the farms. Our study is the first one highlighting the spread of *Saprolegnia* species from Croatian fish farms to downstream locations. All *Saprolegnia* species captured by hempseed baits (*S. australis*, *S. delica*, *S. ferax*, and *S. parasitica*) were more abundant in the fish farms (43 isolates) and downstream locations (29) than upstream (5) of the fish farms (Supplementary material, Table A.1; Table 5). In comparison, upstream locations were strongly associated with *Pythium* species (45), which were less often captured in the fish farms (11) and downstream (16). Noteworthy, *Saprolegnia* species were not found downstream from one fish farm (Gračani) which indicates that this farm had a smaller negative impact on the downstream environment than Kostanjevac and Radovan, probably due to a well maintained settler tank.

Salmonid farms have been previously pinpointed as 'hot spots' of infections for nearby wild populations (Johansen et al., 2011). Fish escaping or water draining from fish farms often leads to the transfer of pathogens to the natural environment, as demonstrated for salmon lice, infectious pancreatic necrosis virus (IPNV), betanodavirus (NV), *Aeromonas salmonicida* subsp. *salmonicida* and other pathogens (Andreou et al., 2012; Garant et al., 2003; Johansen et al., 2011; Munday et al., 2002; Raynard et al., 2001; Thorstad and Finstad, 2018; Wallace et al., 2008). Our study illustrates this effect also for trout farms and *Saprolegnia* pathogens, which is relevant since saprolegniosis causes high annual economic losses in salmonid aquaculture (Hussein and Hatai, 2002; Phillips et al., 2008; van den Berg et al., 2013; van West, 2006) and has a negative impact on wild populations of salmonids and other freshwater fish, as well as other aquatic animals (Blaustein et al., 1994; Fregeneda Grandes et al., 2000; Kiesecker et al., 2001; Neitzel et al., 2004; Pickering and Willoughby, 1982; van West, 2006). For instance, *Saprolegnia* spp. can infect and kill crayfish specimens and it can be pathogenic towards amphibians (adult salamander and frog eggs) (Dieguez-Uribeondo et al., 1994; Gil-Turnes et al., 1989; Hirsch et al., 2008; Kiesecker and Blaustein, 1995; Kozubíková-Balcarová et al., 2013; Krugner-Higby et al., 2010; Lefcort et al., 1997). *Saprolegnia* species that have been introduced to the natural environment via fish restocking caused amphibian mortality (Blaustein et al., 1994; Kiesecker et al., 2001). In the last two decades, many diseases have increased in prevalence and distribution (emerging infectious diseases) (Fisher et al., 2012; Gozlan et al., 2014; van den Berg et al., 2013). Due to negative anthropogenic impacts on the natural environment (e.g. climate change, pollution, the introduction of new species, habitat alteration and degradation) the host species are becoming more vulnerable to various pathogens leading to disease outbreaks and sometimes even to the extinction of whole populations (Fisher et al., 2012; Gozlan et al., 2014). In this context, the transfer of *Saprolegnia* spp. from fish farms to the surrounding environment could lead to increased mortalities in natural populations, and it is crucial to undertake detailed surveys to follow pathogenic *Saprolegnia* spreading and distribution from fish farms to the natural environment, such as this one.

5. Conclusions

Our study highlights the role of trout farms as potential points of release of *Saprolegnia* pathogens to downstream freshwater ecosystems. Further studies are needed to assess the real impact of such pathogen spread, for instance, by sampling multiple points downstream from aquaculture sites coupled with pathogen quantification via molecular techniques, such as quantitative PCR or droplet digital PCR. This could be done by the combination of hempseed baiting (as applied here) and isolation of environmental DNA (eDNA) directly from water, an approach that has been widely used in recent years for detection and monitoring of species of interest (Dougherty et al., 2016; Strand et al., 2011). Also, the knowledge on the *Saprolegnia* spp. pathogenicity for free-living animal species is scarce, and further studies should be performed to assess *Saprolegnia* virulence, especially in combination with other stressors, such as elevated water temperature due to climate change, anthropogenic pollution, and pressure of invasive competing species.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2021.736652.

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2.3. Publication III: Variations in the sporulation efficiency of pathogenic freshwater oomycetes in relation to the physico-chemical properties of natural waters

microorganisms

MDPI

Article

Variations in the Sporulation Efficiency of Pathogenic Freshwater Oomycetes in Relation to the Physico-Chemical Properties of Natural Waters

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Abstract: Oomycete pathogens in freshwaters, such as *Saprolegnia parasitica* and *Aphanomyces astaci*, are responsible for fish/crayfish population declines in the wild and disease outbreaks in aquaculture. Although the formation of infectious zoospores in the laboratory can be triggered by washing their mycelium with natural water samples, the physico-chemical properties of the water that might promote sporulation are still unexplored. We washed the mycelia of *A. astaci* and *S. parasitica* with a range of natural water samples and observed differences in sporulation efficiency. The results of Partial Least Squares Regression (PLS-R) multivariate analysis showed that SAC (spectral absorption coefficient measured at 254 nm), DOC (dissolved organic carbon), ammonium-N and fluoride had the strongest positive effect on sporulation of *S. parasitica*, while sporulation of *A. astaci* was not significantly correlated with any of the analyzed parameters. In agreement with this, the addition of environmentally relevant concentrations of humic acid, an important contributor to SAC and DOC, to the water induced sporulation of *S. parasitica* but not of *A. astaci*. Overall, our results point to the differences in ecological requirements of these pathogens, but also present a starting point for optimizing laboratory protocols for the induction of sporulation.

Keywords: *Aphanomyces astaci*; crayfish plague; dissolved organic carbon (DOC); humic acid (HA); *Saprolegnia parasitica*; saprolegniosis; spectral absorption coefficient (SAC); zoospores

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1. Introduction

Animal pathogenic oomycetes are a cause of serious diseases worldwide [1–3], and in freshwater ecosystems species from the genera *Aphanomyces* and *Saprolegnia* are the most significant, since they cause severe disease outbreaks both in aquaculture and in the wild [4–7]. Crayfish plague, a disease caused by *Aphanomyces astaci* is responsible for decimating the populations of indigenous crayfish species in Europe [8] and elsewhere (e.g., Asia and South America) [9–12]. The pathogen was introduced into Europe along with North American non-indigenous invasive crayfish species which now act as its carriers, being mostly resistant to infection due to a long co-evolution with the pathogen [13,14]. Members of the genus *Saprolegnia* cause saprolegniosis, which is responsible for significant economic losses in salmonid farms and hatcheries [15–18]. Among them, *Saprolegnia parasitica* is highly virulent and widespread [4,7,19,20], and recent studies suggest that aquaculture facilities can act as the sources of its spread into the natural environment [21,22].

The complete life cycle of oomycetes involves asexual and sexual stages, although in some species, including *A. astaci*, the sexual stage has not been documented [6,23,24]. The asexual stage is considered crucial for pathogen's dispersal, since it includes the production of motile zoospores, the major infectious stage of the life cycle [23,24]. Zoospores are released into the surrounding water from the hyphal tips (i.e., sporangia) after colonizing

the infected host tissue [23,25], and the new infection is established when they locate the suitable host by chemotaxis [26,27]. Zoospores encyst on the surface of the host, such as on the crayfish cuticle or fish skin, and then germinate into hyphae that penetrate the body of the host [6,28–30]. In some oomycete species, including *A. astaci* and *S. parasitica*, if zoospores are unable to find a suitable host, they encyst and then release a new zoospore generation (repeated zoospore emergence), thereby increasing the possibility of finding a suitable host [31,32]. Dispersal of zoospores from one host to another by water and by contaminated items (like fishing gear) present the main way of spreading the oomycete diseases in freshwater systems [25,33,34].

The formation of zoospores can be triggered under laboratory conditions, for example, by the lack of nutrients or after a sudden drop in temperature [28,35,36]. Most protocols for sporulation of oomycetes are therefore based on washing the mycelium with stream or lake water, but without defining the components present in the water [32,37,38]. During our work with *A. astaci* and *S. parasitica*, we noticed that sporulation efficiency varied depending on the water used to wash the mycelium. This suggested that the water composition might influence the sporulation process, but there is little data on this in the literature. Several in vitro studies have shown that some salts, such as KCl, NaCl, $MgCl_2$, $CaCl_2$ or K_2SO_4, can affect the formation, motility and germination of oomycete zoospores [31,39,40], but the salt concentrations used were significantly higher than in natural freshwaters [41]. However, a recent study used environmentally relevant concentrations of a sea salt mixture ranging from 0 to 45 g/L and found that survival, growth and infectivity of the plant oomycete pathogen *Phytophthora ramorum* were negatively correlated with salt concentration [42]. At concentrations > 20 g/L, no zoospores were released, but infection could still occur via mycelial growth.

We hypothesized that physico-chemical characteristics of natural waters can affect the intensity of sporulation of freshwater pathogenic oomycetes, and the aim of this study was to test for the first time the sporulation efficiency of two oomycete pathogens, *A. astaci* and *S. parasitica*, upon washing their mycelia with natural surface waters of varying physico-chemical characteristics. The subsequent statistical analysis allowed us to identify the specific parameters of natural waters that could stimulate or inhibit the zoospore formation in freshwater oomycetes.

2. Materials and Methods

2.1. Water Sampling

Water sampling was performed during winter 2018/2019 in different freshwater bodies in Croatia (Figure 1, Table S1). Water sampling locations (n = 36) were selected to vary in terms of geological composition of the substrate, ecosystem type (lentic or lotic) and ecoregion (Dinaric with continental or Mediterranean climate, or Pannonic with continental climate) (Table S1). For some of the locations, data on the presence/absence of the model pathogens *A. astaci* and *S. parasitica* were also available. Water was sampled into autoclaved 1 L polyethylene bottles previously washed three times with the sample and kept in the dark and on ice during transport. Upon arrival to the laboratory, the water samples were immediately autoclaved and then placed in a dark room at +18 °C until physico-chemical analyses (see Section 2.2) and sporulation experiments (see Section 2.3). Noteworthy, water had to be autoclaved before the sporulation experiments to exclude the activity of aquatic microbial communities as a factor that could influence sporulation intensity. Since autoclaving was expected to change the water composition compared to the original sample at the sampling site (e.g., phosphates may form insoluble salts with bivalent metals and precipitate, CO_2 may be lost due to heating, etc.), all physico-chemical analyses of the water were performed after autoclaving.

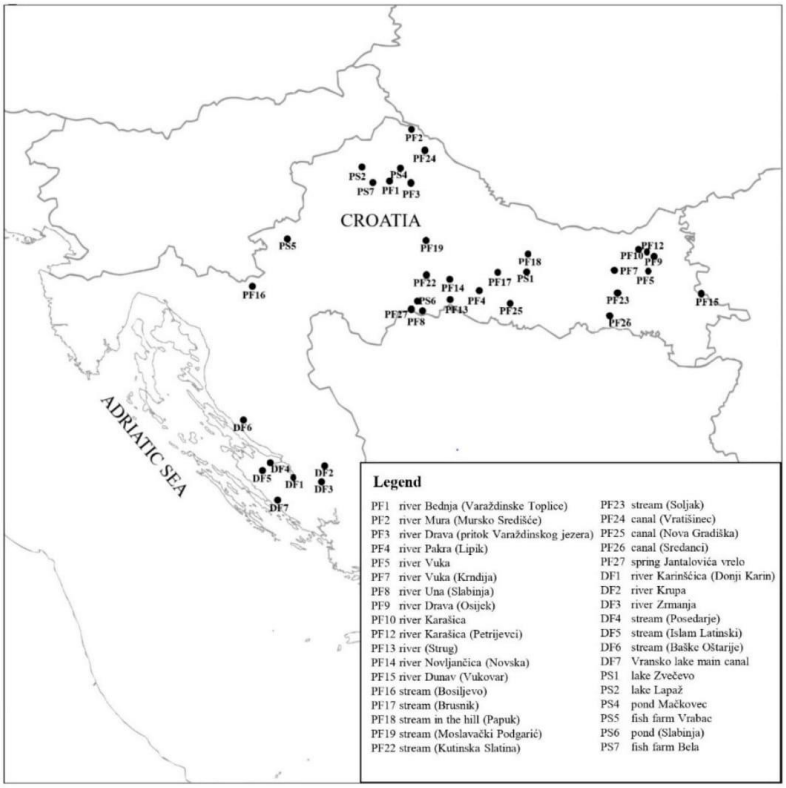

Figure 1. Locations of water sampling (n = 36) in Croatia. P—Pannonian ecoregion, D—Dinaric ecoregion, F—flowing, S—stagnant.

2.2. Physico-Chemical Analyses of Water

Physico-chemical parameters of the autoclaved water samples, i.e., spectral absorption coefficient (SAC), dissolved organic carbon (DOC), pH, electrical conductivity (EC), ammonium nitrogen (NH_4-N), nitrate nitrogen (NO_3-N), SO_4^{2-}, F^-, Cl^-, Mg^{2+}, Ca^{2+}, Br^-, total nitrogen (TN) and total phosphorus (TP), are listed in Table S2. Electrical conductivity and pH were measured using a pH/Cond—meter inoLab 720 instrument (WTW, Xylem Analytics, Weilheim, Germany). The concentration of TP in water samples was determined according to ISO 6878_2004 (DIN EN 6878/D11), while TN was determined according to EN ISO 11905-1. Ammonium nitrogen (NH_4-N) was determined according to ISO 7150-1 (DIN 38406 E5-1, UNI 11669:2017). SAC was measured at 254 nm using a DR 6000 Spectrophotometer (Hach, Düsseldorf, Germany) according to DIN 38404 Part 3 (C3). DOC was determined using TOC—LCPH FA E200 (Shimadzu, Kyoto, Japan). Dissolved anions (fluorides, chlorides, nitrates, sulfates) and cations (calcium, magnesium) were determined according to HRN EN ISO 10304-1: 2009/cor.1:2012 and HRN EN ISO 14911:2001 on the ion chromatograph DIONEX DX-500 with a conductometric detector (CD20) in combination with electrochemical suppressor. An anion column Dionex IonPac AS9-HC and a cation column Dionex IonPac CS12A were used. In addition, 9 mmol/L Na_2CO_3 was used to elute the anions and 20 mmol/L methanesulfonic acid was used to elute the cations. The eluent flow rate through the column was 1 mL/min.

2.3. Sporulation

For the in vitro sporulation experiments, we used *Aphanomyces astaci* isolate PEC 8 (haplogroup B), provided by F. Grandjean (University of Poitiers, Poitiers, France), and *Saprolegnia parasitica* isolate A1, collected from the surface of eggs of rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) [21]. Both isolates were cultured and maintained in the laboratory at 18 °C on the appropriate solid growth media: peptone-glucose (PG1) medium [43] for *A. astaci*, and glucose-yeast (GY) medium [44] for *S. parasitica*.

To test for the differences in sporulation efficiency after washing the mycelia with different samples of natural waters, previously described sporulation protocols were used [32,37], with some modifications. Namely, uniform pieces of agar (4 mm^2) containing the fresh mycelial tips of pure cultures were cut and placed into wells of 12-well plates filled with 3 mL of liquid PG1 medium for *A. astaci* or liquid GY medium for *S. parasitica*. Cultures were incubated at 18 °C for four days for *A. astaci* and two days for *S. parasitica*. In the case of *A. astaci*, after three days of incubation the agar plug was removed, and grown hyphae were placed back in the same well filled with liquid PG1 medium and then left to grow for one more day. After incubation of both pathogens, the liquid medium was removed, and hyphal biomass was washed three times with 2 mL of autoclaved natural water samples using sterile Pasteur pipette and then left in 4 mL of the same autoclaved water for 24 h at 18 °C. Then, the mycelia were removed, and the produced motile zoospores were counted in the Thoma chamber using a light microscope LCD MICRO 5MP (Bresser, Rhede, Germany). Three biological replicates (i.e., mycelial inoculums originating from three independently grown cultures) were made for each water sample (i.e., location) and oomycete species, and the number of zoospores of each replicate was counted three times. The average number of zoospores/mL for each location is given in Table S2.

To test whether the addition of humic acid (HA), as an ubiquitous substance that contributes to both SAC and DOC, can induce sporulation of *A. astaci* and *S. parasitica*, a range of environmentally relevant HA concentrations were added to the water samples. Two types of water were selected for this experiment: artificial water (AW, ISO 7346-1 and 7346-2, ISO 1996) and one of the natural water samples from our dataset (PF4, Table S2). Artificial water (AW) was prepared by adding salts to Milli-Q water according to ISO 7346-1 and 7346-2 (ISO 1996), i.e., 294.0 mg/L $CaCl_2 \times 2\ H_2O$, 123.3 mg/L $MgSO_4 \times 2\ H_2O$, 63.0 mg/L $NaHCO_3$ and 5.5 mg/L KCl, and autoclaved. HA stock solution (160 mg/L in 0.1M NaOH, DOC = 48.0 mg/L, SAC = 379.9 m^{-1}) was filtered through a syringe polyester filter (pore size = 0.45 µm, d = 25 mm, CHROMAFIL® Xtra PET-45/25, Macherey-Nagel, Düren, Germany). Water samples with different concentrations of HA, i.e., 4, 8 and 16 mg/L, were prepared by adding 10 mL of HA stock solution, or stock solution diluted with 0.1M NaOH, to 90 mL of AW or PF4. Since the addition of HA, dissolved in 0.1M NaOH, to the water increased the pH, the samples were titrated with 6M HCl to reduce the pH to the initial values. Two negative controls were used, one with the addition of 0.1 NaOH instead of HA (solvent control) and another with the addition of Milli-Q water. In vitro sporulation efficiency was then determined after washing *A. astaci* and *S. parasitica* mycelia with HA-supplemented water samples using the 12-well plate protocol described above. Three biological replicates were performed for each oomycete species, water sample and HA concentration.

2.4. Data Analysis

To analyze the effects of different physico-chemical properties of water samples (explanatory variables or predictors, X: SAC, DOC, pH, EC, NH_4-N, NO_3-N, SO_4^{2-}, F^-, Cl^-, Mg^{2+}, Ca^{2+}, Br^-, TN, TP) on *A. astaci* and *S. parasitica* sporulation efficiency (response variables, Y), Partial Least Squares Regression (PLS-R) analysis was performed using the XLSTAT version 2021.3.1.1189 software provided by Microsoft Excel by Addinsoft.

The non-parametric Mann–Whitney U-test was used to determine the significance of differences in sporulation efficiency between HA-supplemented AW and PF4 water samples. The Kruskal–Wallis test, followed by Dunn's post hoc test, was used to estimate the significance of differences in sporulation efficiency between the different concentrations

of HA and the negative controls. In all cases, the significance level was set at $p < 0.05$. The tests were performed in R v. 3.2.0 (R Core Team, 2020).

3. Results

Physico-chemical analyses showed an overall good quality of the water samples collected at 36 sites, covering different water types and biogeographical regions in Croatia (Figures 1 and 2, Table S2). However, some parameters in some samples exceeded the thresholds set by national legislation [45], particularly those related to nitrogen content. Nitrate-N and total nitrogen were elevated in 15 out of 36 samples (41%), ammonium-N in 3/36 (8%), pH (>9) in 2/36 (6%) and total phosphorus in 1/36 (3%).

We used the collected water samples to wash the mycelia of oomycete pathogens *A. astaci* and *S. parasitica* and induce sporulation. The average sporulation efficiency was similar for both pathogens, i.e., 4444 zoospores/mL for *A. astaci* (min = 0, max = 42,222) and 4537 zoospores/mL for *S. parasitica* (min = 0, max = 37,778), but we obtained variable zoospore numbers upon using different water samples, as listed in Table S2.

Using PLS-R multivariate analysis we examined the relationship between the sporulation intensity of *A. astaci* and *S. parasitica* (response variables, Y) and the physico-chemical parameters of water (explanatory variables, X). Quality indices Q^2(cum), R^2Y(cum) and R^2X(cum) of the obtained model were 0.09, 0.15 and 0.21, respectively, for the first component and −0.03, 0.22 and 0.36, respectively, for the second component. The PLS-R modelled relationship between blocks of response and explanatory variables is visually presented as a radar of correlation (Figure 3A). In the radar, positively correlated variables are presented close to each other, and those negatively correlated are located far apart, while the strength of the correlation between any two variables is predicted by their respective r values (listed in correlation matrix, Table S3). Based on this, SAC (r = 0.476), DOC (r = 0.375), NH_4-N (r = 0.356) and F^- (r = 0.353) most strongly positively affected *S. parasitica* sporulation, while *A. astaci* sporulation was not significantly affected, neither positively nor negatively, by any of the analyzed water parameters (Figure 3A, Table S3). Moreover, SAC, DOC, F^-, NH_4-N and TN also had VIP values > 1 (Figure 3B), indicating that these parameters are relevant for explaining the sporulation efficiency and contribute significantly to the PLS model [46,47].

Based on the positive effect of SAC and DOC on the sporulation intensity of *S. parasitica*, we experimentally tested whether the addition of environmentally relevant concentrations of humic acid (HA), a widespread substance that contributes to both SAC and DOC, to water can stimulate sporulation of freshwater oomycete pathogens. The water samples used, artificial water (AW) and one of the natural water samples from our dataset (PF4, Table S2), were selected on the basis that they did not induce sporulation when previously used to wash the oomycete mycelia. AW contains no organic matter (see Materials and Methods), while the SAC and DOC values of PF4 were below average: 3.39 m^{-1} and 2.73 mg/L, respectively (average SAC in our dataset was 7.09 m^{-1}, while the average DOC was 5.13 mg/L). The results obtained were in agreement with the PLS model and showed that the sporulation efficiency of *S. parasitica* increased with increasing concentration of HA (4, 8 and 16 mg/L) for both AW and PF4 (Figure 4). In contrast, the addition of HA to the water had no effect on the sporulation of *A. astaci*, i.e., sporulation failed when washing the mycelia with both control samples and HA-supplemented water (data not shown). The sporulation efficiency of *S. parasitica* was significantly higher after washing the mycelium with PF4 than after washing with AW (Mann–Whitney U-test; W = 639.5; $p = 0.004$), probably due to baseline presence of organic matter in the natural water sample. In addition, the sporulation efficiency of *S. parasitica* was significantly higher in HA-supplemented water than in the negative controls, for both AW and PF4 (Kruskal–Wallis test; $p < 0.001$) (Figure 4).

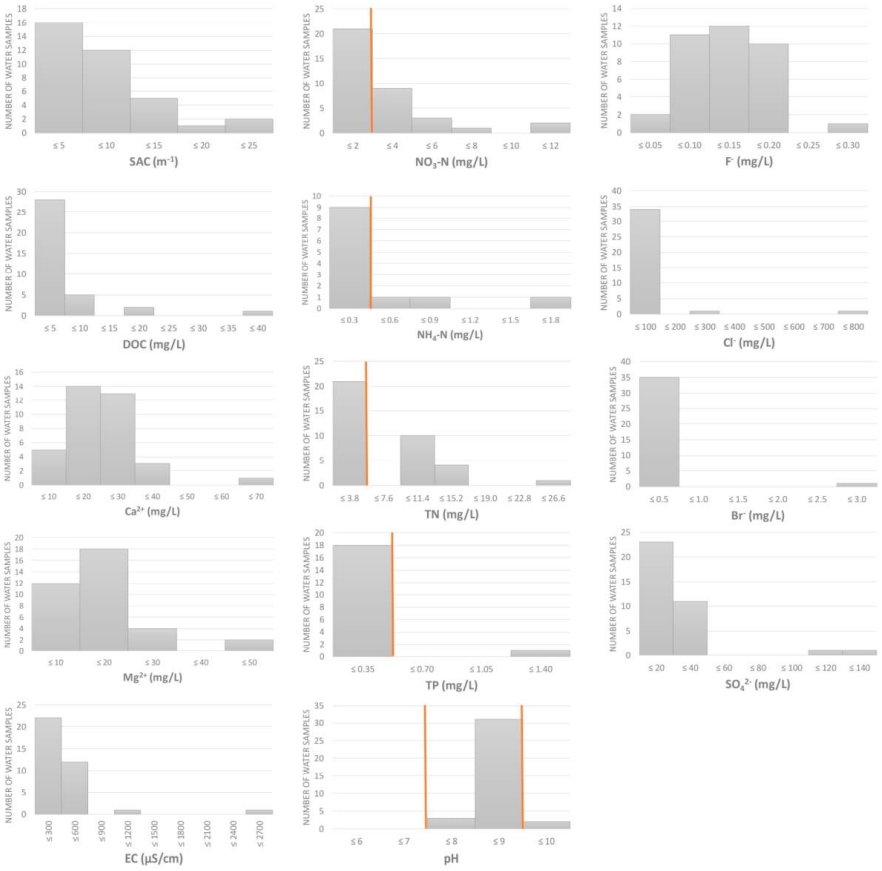

Figure 2. Physico-chemical properties of water samples (n = 36). Orange lines represent the thresholds set by national legislation, available for some of the measured parameters [45], i.e., the upper threshold for NO_3-N, NH_4-N, TN and TP, and lower and upper thresholds for pH. SAC = spectral absorption coefficient, DOC = dissolved organic carbon, EC = electrical conductivity, TN = total nitrogen, TP = total phosphorus.

A)

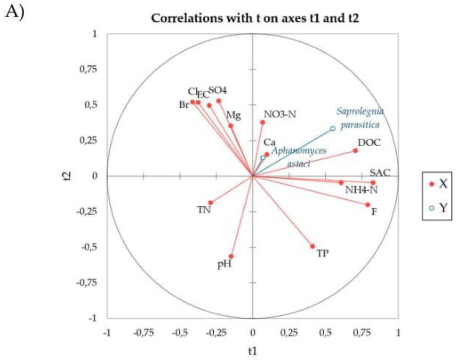

Figure 3. *Cont*.

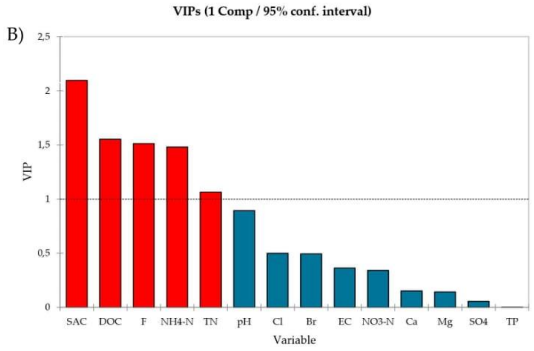

Figure 3. (**A**) Radar of correlation describing the relationship between the sporulation intensity of *A. astaci* and *S. parasitica* (response variable, Y, blue lines) and physico-chemical parameters of water (explanatory variables, X, red lines). The percentages of the variances in X and Y explained by each variable are indicated on the respective axes. (**B**) The Variable Importance in the Projection (VIPs) for explanatory variables of the first component (t1). VIPs > 1 indicate the explanatory variables that contribute the most to the PLS model.

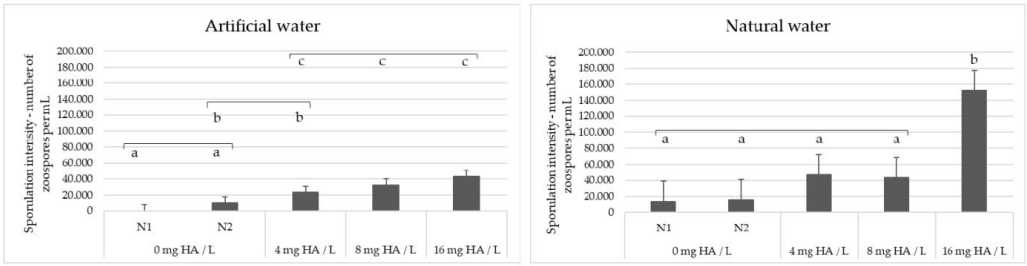

Figure 4. *Saprolegnia parasitica* sporulation intensity after washing the mycelium with artificial water (AW) or natural water (PF4) supplemented with increasing concentrations of humic acid (HA). In control experiments N1 and N2, water and 0.1 M NaOH were mixed with water samples instead of HA solution in 0.1 NaOH, respectively. Bars marked with the same letter (a, b or c) within each panel are not statistically different from one another.

4. Discussion

We present for the first time the differences in sporulation intensity of the freshwater oomycete pathogens *A. astaci* and *S. parasitica* in natural water samples with different physicochemical properties and show that the addition of humic acid, a widespread substance contributing to SAC and DOC levels in freshwater, can trigger sporulation of *S. parasitica*. These results are important both in terms of extending the knowledge on the ecological requirements of these pathogens and as a starting point for the optimization of laboratory protocols for the induction of sporulation.

The parameters related to organic matter in water, especially its aromatic part, were the most important factors positively affecting the sporulation intensity of *S. parasitica*. In contrast, sporulation of *A. astaci* was not significantly affected by any of the water quality parameters studied. Moreover, the sporulation intensity of *A. astaci* and *S. parasitica* was not correlated. These results are consistent with the contrasting life strategies of these two pathogens. *Aphanomyces astaci* is a specialized animal parasite with a narrow host range, whereas *S. parasitica* has a much wider host range and is usually considered an opportunistic secondary pathogen that alternates between a saprophytic and pathogenic

lifestyle [4,48], although some *S. parasitica* strains have been reported to be highly virulent and cause primary infections [16,49–52]. It has already been shown that the survival of *A. astaci* depends mainly on the presence of host crayfish species [6,23], while our unpublished results on the monitoring of *S. parasitica* in freshwater systems have shown that its load is correlated with some parameters of water composition, such as electrical conductivity and calcium. We hypothesize that host-specific factors might favor sporulation of *A. astaci*, while in the case of *S. parasitica,* water composition may be more important, but further studies are needed to clarify these possible differences.

The PLS-R results showed that the sporulation intensity of *S. parasitica* was positively correlated with the SAC and DOC content of the water samples in our dataset. The DOC levels in freshwater environments vary widely from 0.1 to hundreds of mg/L, but their average values were reported to be about 5 mg/L [53,54], similar to the values measured in our dataset. Absorbance at 254 nm (SAC) usually refers to the presence of organic compounds in water, especially those containing aromatic rings or unsaturated carbon bonds (double or triple) in their molecular structure. Thus, the PLS-R results indicate that the presence of aromatic organic matter in water, such as humic acids, can promote sporulation of *S. parasitica*. This was confirmed experimentally, as washing *S. parasitica* mycelia with a series of HA-supplemented water samples stimulated sporulation compared to negative controls. In contrast, the addition of HA had no effect on the sporulation of *A. astaci*. Interestingly, humic substances were previously shown to inhibit mycelial growth of *S. parasitica* and a number of related oomycetes from the order Saprolegniales (genera *Saprolegnia*, *Achlya*, *Leptolegnia*, *Pythium*), but without concomitant inhibition of sporangia development [55,56]. This is consistent with our results and suggests that in the presence of humic substances, mycelial growth is impaired and oomycetes therefore switch to the production of sporangia and motile zoospores that can spread to new, more favorable environments.

Furthermore, the PLS-R modelling results suggest that increased fluoride and ammonium concentration could also act as a sporulation trigger, at least for *S. parasitica*. The range of fluoride concentrations in freshwater is between 0.01 and 0.3 mg/L [57], which is in accordance with the average fluoride concentration of 0.1 mg/L in our dataset. The induction of sporulation by environmentally relevant fluoride concentrations could be explained as a response to unfavorable environmental conditions, as in the case of the humic substances mentioned above. High fluoride concentrations have been shown to have negative effects on microbial physiology [58–61], but there are no data yet on the toxicity of fluoride to oomycetes. Furthermore, in some of our samples, ammonium-N concentrations were above the threshold of 0.3 mg/L set by national legislation [45]. Environmentally relevant ammonium concentrations (0.05 and 0.5 mg/L) were related to an increased susceptibility of rainbow trout (*Oncorhynchus mykiss*) to saprolegniosis [62,63]. This was explained by the host stress response and specific impairments of the defense mechanisms against saprolegniosis, but, considering our results, might also partly be due to the ammonium-induced sporulation increase and thereby the increased virulence of the pathogen. However, it was shown that in the presence of high ammonium-N concentrations (8 and 16 mg/L, not found in our dataset) the relative abundance of oomycetes in freshwater habitat can decrease [64]. Similarly, our analysis of *S. parasitica* load in water samples by droplet digital PCR (ddPCR) showed a negative correlation with ammonium and fluoride concentration (unpublished results). Additional experiments, using a series of increasing ammonium concentrations in the water, should be performed to determine the ammonium concentration range that promotes sporulation, and to compare the effects of ammonium towards mycelium and zoospores of pathogenic oomycetes.

Altogether, our results suggest that some substances might suppress the mycelial growth of the pathogen in a certain range of environmentally relevant concentrations and at the same time promote sporulation, thus facilitating the spread of the pathogens into more favorable environment. Thus, the effects of these compounds, such as ammonium and fluoride, on the sporulation intensity and virulence of freshwater oomycete pathogens

should be tested, as it was tested here with HA. Furthermore, one of the limitations of this study is that we used only a single isolate from each species. This is unlikely to be representative at the species level, as significant within-species differences in mycelial growth rate, sporulation temperature, zoospore motility, and other parameters were observed for both *A. astaci* [65] and *S. parasitica* [66–68]. Therefore, a wider range of isolates/genotypes should be included in in vitro sporulation intensity tests in the future.

The knowledge obtained would enable, from an ecological standpoint, the prediction of the water conditions that might promote the pathogen spreading in natural environments and aquaculture facilities, and thereby aid in the development of preventive measures. On the other hand, the research community working on pathogens from the order Saprolegniales would greatly benefit if the composition of water used for sporulation would be standardized. A range of artificial water samples, with defined composition, could be designed and tested to provide optimal and reproducible oomycete sporulation in laboratory conditions.

Supplementary Materials: The following are available at https://www.mdpi.com/article/10.3390/microorganisms10030520/s1, Table S1: Water samples collected at different locations in Croatia; Table S2: Physico-chemical parameters of the collected water samples and the average number of zoospores obtained; Table S3: Correlation matrix describing the relationship between sporulation efficiency of *A. astaci* and *S. parasitica* (response variable) and physico-chemical parameters of water (explanatory variables).

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3. DISCUSSION

The results of this thesis provide new insights into the ecological requirements of the pathogenic freshwater Oomycota *Aphanomyces astaci* and *Saprolegnia* spp. A highly sensitive *Saprolegnia parasitica*-specific ddPCR assay was developed to detect and quantify the pathogen directly from eDNA isolated from host biofilm and water (Publication **I**). Furthermore, the diversity of Oomycota isolates in selected Croatian trout farms was determined for the first time and it was shown that trout/fish farms could act as sources from which *Saprolegnia* spp. can spread into natural waters (Publication **II**). Finally, specific parameters of natural waters have been identified, including physico-chemical parameters and microbial community composition that may influence the spread of freshwater Oomycota pathogens and serve as indicators of their presence (Publications **I** and **III**, Appendices 1 to 3).

3.1. Detection and quantification of *Saprolegnia parasitica* by droplet digital PCR (ddPCR)

A highly sensitive *S. parasitica*-specific ddPCR assay has been developed that allows absolute quantification of pathogen DNA, enabling the rapid and simple monitoring of *S. parasitica* in the environment (Publication **I**). ddPCR is a relatively new and advanced technology capable of quantifying small amounts of target DNA in the sample by fractionating a PCR reaction into more than 20,000 droplets using an oil emulsion (Pinheiro et al., 2012). After the completion of thermal cycling protocol, positive (target DNA amplified) and negative (no amplification of target DNA) droplets are counted individually by measuring the fluorescence with the droplet reader. The number of copies of the target DNA is estimated from the ratio of the positive and negative droplets. Compared to conventional qPCR, ddPCR allows absolute and direct quantification of the target DNA without the need for a standard curve (Cao et al., 2016). In addition, ddPCR is more sensitive and less susceptible to inhibitors than qPCR (Hindson et al., 2013; Hoshino and Inagaki, 2012), making it particularly suitable for eDNA-based applications.

Saprolegnia parasitica-specific primers were designed to target the ITS region of rDNA, a standard high copy number nuclear marker for Oomycota species abundantly represented in sequence databases (Montalva et al., 2016; Robideau et al., 2011; Sandoval-Sierra et al., 2014b). The specificity of the assay was maximised by selecting primer sequences from the regions with the highest sequence variability between closely related *Saprolegnia* species and other Oomycota, and by using touchdown PCR. Specificity was tested several *Saprolegnia* species (*S. parasitica, S. australis, S. delica, S. diclina, S. ferax, S. litoralis* and *Saprolegnia* sp. 1) and other Oomycota (*A.*

astaci and *Pythium* sp.) and showed reliable discrimination between *S. parasitica* (> 10,000 copies per ng gDNA) and closely related species (no amplification). Only one closely related non-target species, *Saprolegnia* sp. 1 (one bp difference in 333F primer), was amplified but yielded only one positive droplet per reaction (or ~ 6 ITS copies per ng gDNA), which was considered a false positive. *Saprolegnia* sp. 1 was rarely detected during sampling at salmonid fish farms and hatcheries (Sakaguchi et al., 2019; Sandoval-Sierra et al., 2014a), indicating a low risk of false positive results in ddPCR monitoring of *S. parasitica*. The specificity of the developed ddPCR assay is comparable to or better than other *S. parasitica* assays (Chanu et al., 2022; Korkea et al., 2022; Rocchi et al., 2017) for which specificity was confirmed, but some closely related *Saprolegnia* species, such as *Saprolegnia* sp. 1 (Chanu et al., 2022; Korkea et al., 2022) or *S. ferax* (Chanu et al., 2022), were not tested.

Regarding the sensitivity of the developed ddPCR method (Publication **I**), the LOD of 14 fg *S. parasitica* gDNA per ddPCR reaction (~ 3 ITS copies) demonstrates a high sensitivity, which is about 500-fold higher than that of the recently published multiplex PCR assay for the detection of *S. parasitica* (Chanu et al., 2022). However, the qPCR assay for *S. parasitica* had a slightly higher sensitivity (LOD = 1.8 fg) (Korkea et al., 2022; Rocchi et al., 2017). This could be explained by the known variation in ITS copy number per genome: several tens to several hundreds of ITS copies have been estimated in the genomes of different Oomycota species, and ITS copy number also varies between different isolates of the same species (Blaya et al., 2016; Gibert et al., 2021; McGowan et al., 2019). Korkea et al. (2022) used the *S. parasitica* isolate VH28 (isolated from brown trout in Vuoksi, Finland) for sensitivity testing, while in this dissertation the *S. parasitica* isolate BF1 (collected from a trout farm in Solin, Croatia, Publication **II**) was used. With the developed ddPCR assay, the ITS copy number of *S. parasitica* BF1 could be approximated: considering the *S. parasitica* genome size of 63 Mb (Jiang et al., 2013), 14 fg corresponds to about 0.2 genomic units and implies that the copy number of *S. parasitica* BF1 ITS copy number should be about 10. Isolate VH28 could have a significantly higher ITS copy number, but this assumption still needs to be confirmed experimentally.

Overall, the markedly different sensitivity of PCR-based (Chanu et al., 2022) versus ddPCR/qPCR-based (Publication **I**, Korkea et al., 2022, Rocchi et al., 2017) molecular *S. parasitica* detection assays indicates that the latter should be the method of choice for eDNA-based monitoring protocols. However, the main problem when detecting pathogen nucleic acids in

environmental samples (e.g. water samples) are the PCR inhibitors (Hunter et al., 2019). To reduce the presence of potential inhibitory substances, Korkea et al. (2022) used a small volume of water per sample (15 mL), but usually filtering larger volumes of water is preferable for more accurate detection and identification of the specific pathogen (up to 7L, in the case of this dissertation 2L per site) (Strand et al., 2019, 2014). Therefore, ddPCR is a better choice than qPCR for eDNA-based monitoring as it is less susceptible to inhibitors arising from the partitioning of the sample into thousands of droplets (Hoshino and Inagaki, 2012; Pinheiro et al., 2012).

In conclusion, the newly developed ddPCR method could be used in salmonid aquaculture to monitor variations in *S. parasitica* loads in both skin/egg swabs and water. Currently, toxic antioomycetic chemicals are continuously used to prevent disease outbreaks, regardless of the actual load of the pathogen. If outbreaks could be predicted in time through ddPCR-based monitoring, the use of antioomycetic chemicals could be adjusted to the current pathogen load. In this way, both chemical pollution and pathogen transmission to downstream waters could be reduced. In addition, until now *S. parasitica* was only assumed to be ubiquitous in natural waters, without being possible to monitor its exact distribution or abundance. In the future, with the help of recently developed highly sensitive molecular assays, such as the ddPCR assay developed here, it will be possible to understand the dynamics of the *S. parasitica* pathogen in natural waters. For example, the omnipresence of *S. parasitica* was demonstrated by ddPCR in natural waters in Croatia, where the pathogen was detected at 13 out of 21 sites (62%) (Publication **I**).

3.2. First molecular identification of Oomycota in Croatian trout farms and their incidence in the natural waters upstream and downstream of the fish farms

Saprolegniosis is a serious problem in salmonid farms and hatcheries worldwide (Cao et al., 2012; Meyer, 1991; Thoen et al., 2011; van den Berg et al., 2013), and aquaculture facilities may represent reservoirs for the spread of *Saprolegnia* spp. into the natural environment, as has been demonstrated for several other pathogens (Johansen et al., 2011; Kurath and Winton, 2011; McVicar, 1997). Therefore, it is important to analyse the identity, distribution and pathogenic significance of *Saprolegnia* species in aquaculture facilities and adjacent natural habitats to assess the risks of disease outbreaks in both fish farms and associated natural areas. In this dissertation, Oomycota isolates were collected and identified in selected trout farms in Croatia, focusing on

Saprolegnia species. In addition, the possible role of trout farms as sites for the spread of pathogenic *Saprolegnia* species into the environment was analysed (Publication **II**).

3.2.1. Oomycota species from trout farms in Croatia are dominated by *Saprolegnia parasitica*

Using a cultivation-based approach, various Oomycota species were detected in selected Croatian trout farms and their upstream and downstream environments. To gain the most comprehensive insight into the diversity of pathogenic Oomycota, two sampling methods were combined: tissue sampling (i.e. inoculation of infected skin pieces on glucose-yeast extract agar) and water sampling (i.e. hemp seed baits were left in water for 10 days to capture the Oomycota and then cultivated on glucose-yeast extract agar). Overall, the frequency of detection of Oomycota was 60% for both sampling methods (samples with Oomycota growth / total number of samples placed on the solid agar medium), but still the methods have different advantages and disadvantages. For example, hemp seed baits attract zoospores or cysts in the water, but the Oomycota that don't sporulate under certain environmental conditions might remain undetected. In addition, bacteria in the water can sometimes prevent zoospores from attaching to the hemp seeds and germinating (Sarowar et al., 2019b). Furthermore, a tendency to isolate more saprophytic (less pathogenic) Oomycota species from hemp seed baits has been observed here (Publication **II**) and in other studies (Tedesco et al., 2021). For example, when hemp seed baits were used, the predominant Oomycota species isolated from fish farm water was *S. ferax* in Italian salmonid farms and *Pythium* sp. in Scottish ones, although *S. parasitica* was most frequently detected in animal samples (Tedesco et al., 2021). Similarly, *Pythium* spp. and *S. australis* were most frequently isolated from hemp seed baits in Croatian trout farms, rather than *S. parasitica* (Publication **II**). On the other hand, one of the obstacles in collecting tissue samples is that fish farm workers are sometimes unwilling to provide infected animals. Furthermore, it is sometimes difficult to obtain infected but still living embryonic stages, as they succumb to the disease quickly. It is therefore hard to know whether the isolated Oomycota species was the primary pathogen or the original pathogen was overgrown by a secondary, opportunistic species. In line with this, *S. diclina* well known for egg infections (Fregeneda-Grandes et al., 2007; Sandoval-Sierra et al., 2014a; Thoen et al., 2011; van den Berg et al., 2013) was not isolated from dead eggs, but rather the opportunistic species such as *Pythium* sp. were cultivated (Publication **II**).

Sampling in Croatian fish farms identified three genera of Oomycota: *Saprolegnia*, *Leptolegnia* and *Pythium*. The only species isolated in all fish farms, in both sampled trout species (*Salmo trutta* and *O. mykiss*) and in all developmental stages of the trout was *S. parasitica*. Such dominance of *S. parasitica* over other Oomycota species has also been observed in salmonid farms in Italy, Scotland and elsewhere (Hussein and Hatai, 2002; Noga, 1993; Sandoval-Sierra et al., 2014a; Sarowar et al., 2019a; Tedesco et al., 2021; van den Berg et al., 2013; van West, 2006). Previous infection trials have demonstrated the pathogenicity of *S. parasitica* to eggs (Kitancharoen and Hatai, 1996), fingerlings (Yuasa and Hatai, 1995) and adult salmonids (Stueland et al., 2005). In contrast to the results of this dissertation, in Chilean salmonid farms *S. parasitica* was detected in adult *Salmo salar* and *O. mykiss*, but not in eggs and alevins (Sandoval-Sierra et al., 2014a). This could be explained by the known differences in pathogenicity of *S. parasitica* isolates towards different developmental stages of the host (Stueland et al., 2005; Thoen et al., 2011; Yuasa and Hatai, 1995).

Other *Saprolegnia* species isolated from adult and embryonic stages (alevins) in Croatian trout farms as well as from the water were *S. australis*, *S. delica* and *S. ferax*. As for *S. australis*, this species was isolated only from adult *S. trutta* in the Kostanjevac fish farm (two isolates from skin lesions and two from healthy skin), while it was not found on *O. mykiss* in other fish farms (where *S. parasitica* isolates were more prevalent). Although *S. australis* is usually considered pathogenic to fish embryos (Fregeneda-Grandes et al., 2007; Rezinciuc et al., 2014b; Sandoval-Sierra et al., 2014a; Tandel et al., 2021), it was not isolated from that sample type in this study. This result could be due to the small sample size (i.e. the overall low number of isolates from eggs and alevins identified – 18 in total). A previous analysis of fish farms in Chile with a higher number of isolates from the embryonic stage (122) showed an association between different *Saprolegnia* species and the developmental stage of salmonids, i.e. *S. australis* was associated with alevins (Sandoval-Sierra et al., 2014a). Here, *S. australis* was the most common species found in the water, especially in the fish farms Kostanjevac and Gračani. This could be because, while *S. australis* zoospores were present in large numbers in the water (and therefore easily caught with hemp seed baits), infection with *S. australis* was relatively rare because the fish and eggs were healthy (and therefore the animals were more often infected with *S. parasitica*, a more virulent pathogen) (van den Berg et al., 2013; van West, 2006).

Finally, the second most frequently isolated *Saprolegnia* species from water samples was *S. delica*. *Saprolegnia ferax* was also occasionally caught by hemp seed baits. Both species were rarely isolated from tissue samples. *Saprolegnia delica* and *S. ferax* have previously been associated with embryonic mortality in fish and amphibians (Blaustein et al., 1994; Cao et al., 2012; Fregeneda-Grandes et al., 2007; Kiesecker et al., 2001) and have also been frequently isolated from water (Rezinciuc et al., 2014b; Sarowar et al., 2013; Tedesco et al., 2021). These results are similar to a recent study conducted in Chilean salmonid farms, where *S. ferax* and *S. delica* were found in both adult and embryonic stages of salmonid fish, with no clear preference for a particular developmental stage (Sandoval-Sierra et al., 2014a).

In addition to the *Saprolegnia* species, two isolates from the Radovan fish farm were identified as *Leptolegnia* sp., one from a tissue sample (adult stage - lesion) and one from the water downstream of the fish farm. It is possible that these two isolates represent new *Leptolegnia* species, as in the phylogenetic analyses they were not grouped with any of the known species, *L. caudata* or *L. chapmanii*, parasites of mosquitoes (Bisht et al., 1996; Lastra et al., 2004; Montalva et al., 2016; Schimmel and Noblet, 1985). *Leptolegnia* spp. have also been isolated from cladocerans, fish and amphibian eggs and larvae (Petrisko et al., 2008; Rezinciuc et al., 2014b; Tedesco et al., 2021; Wolinska et al., 2009), but so far, their pathogenicity has only been demonstrated towards amphibian eggs (Ruthig, 2009). The results of this dissertation suggest that some *Leptolegnia* species may be opportunistic fish pathogens, but infection trials are needed to confirm this.

Isolates of *Pythium* spp. were also obtained. Most of them belonged to clade B2 (according to the available molecular phylogeny and taxonomy of the genus *Pythium*, described in Lévesque and De Cock (2004)) - 76 isolates, while one isolate belonged to the related clade B1. *Pythium* spp. isolates were mainly from water upstream of fish farms (majority of B2 isolates and the B1 isolate - Z111 from Radovan), while a small number of B2 isolates were also found on diseased fish and only one B2 isolate on an egg sample. The known *Pythium* species are mostly plant pathogens or saprotrophs associated with natural and agricultural soils (Rahman and Sarowar, 2016; Robideau et al., 2011; Schroeder et al., 2013). However, *Pythium* spp. including clades B1 and B2 have also been isolated from natural and aquaculture freshwater environments (Nechwatal et al., 2008; Rahman and Sarowar, 2016; Schroeder et al., 2013), and some of them have been considered pathogenic to freshwater animals (Miura et al., 2010). *Pythium* spp., including group B members,

have been isolated from the carapace of dead crustaceans (Czeczuga et al., 2002b) and from both dead or alive fish and eggs (Czeczuga et al., 2002a; Czeczuga and Muszynska, 1996). *Pythium flevoense* (which belongs to clade B2 and is most closely related to isolate B3S1 found on *O. mykiss* from Solin) has been suspected as a causative agent of mass mortality of freshwater fish (ayu larvae), but its pathogenicity has not been confirmed by infection trials (Miura et al., 2010). Considering all this, most of the isolates from Croatian trout farms are probably plant pathogens or soil saprotrophs that entered the fish farms by water, as suggested by their phylogenetic grouping with saprotrophs and plant pathogens and the fact that most of them were captured in the water upstream of the fish farms. For example, isolate Z111 was most closely related to the known plant pathogen *P. vanterpoolii* (clade B1) (Asano et al., 2010; Ichitani et al., 1989; Muse et al., 1974). Consistent with this hypothesis, most studies in which *Pythium* spp. were isolated from fish tissue or water samples were conducted in fish farms or ponds surrounded by agricultural fields, grassland, or forests (Czeczuga et al., 2005, 2002a; Naznin et al., 2017; Rahman and Sarowar, 2016; Sarowar et al., 2019b). This was also the case for three of the four fish farms sampled here (Gračani, Kostanjevac, Radovan). Finally, the Croatian *Pythium* isolates possibly belong to a new, previously undescribed species. To confirm this, a detailed morphological description of the isolates is required (Tambong et al., 2006), coupled with the analysis of additional molecular markers, besides ITS. For example, COX1 is a mitochondrially encoded gene that is more discriminatory at the species level than the ITS region (Schroeder et al., 2013). It is recommended that both ITS and COX be used for taxonomic identification of *Pythium* species, rather than just one (Bala et al., 2010; Robideau et al., 2011), and thus both should be used in future studies of *Pythium* isolates associated with fish farms.

3.2.2. Trout farms as potential points of release of *Saprolegnia* pathogens to the downstream freshwater environment

To determine whether trout farms serve as reservoirs of pathogenic *Saprolegnia* species that can spread into the natural environment, Oomycota isolates were collected upstream, downstream and within the farms. The results of the correspondence analysis showed the spread of *Saprolegnia* species from the fish farms to downstream locations. All *Saprolegnia* species successfully isolated from hemp seed baits (*S. australis*, *S. delica*, *S. ferax* and *S. parasitica*) were more abundant in the fish farms (43 isolates in total for the fish farms Kostanjevac, Radovan and

Gračani) and downstream (29 in total) than upstream (5 in total). In comparison, upstream sites were strongly associated with *Pythium* species (45 isolates in total), which were less abundant in fish farms (11) and downstream (16). Remarkably, no *Saprolegnia* species were found downstream from one fish farm (Gračani), suggesting that this farm had a lower negative impact on the downstream environment than Kostanjevac and Radovan, probably due to a well-maintained settler tank at this fish farm.

Salmonid farms have already been identified as 'hotspots' for the release of pathogens into nearby wild populations (Johansen et al., 2011). Fish escape or water run-off from fish farms often results in the transfer of pathogens to the free-living hosts, as has been demonstrated for salmon lice, infectious pancreatic necrosis virus (IPNV), betanodavirus (NV), *Aeromonas salmonicida* subsp. *salmonicida* and other infectious agents (Andreou et al., 2012; Garant et al., 2003; Johansen et al., 2011; Munday et al., 2002; Raynard et al., 2001; Thorstad and Finstad, 2018; Wallace et al., 2008). The results of this dissertation also illustrate this effect for trout farms and *Saprolegnia* pathogens, which is relevant as *Saprolegnia* causes high annual economic losses in salmonid aquaculture (Hussein and Hatai, 2002; Phillips et al., 2008; van den Berg et al., 2013; van West, 2006) and has negative impacts on wild populations of salmonids and other freshwater fish, as well as on other aquatic species (Blaustein et al., 1994; Fregeneda Grandes et al., 2000; Kiesecker et al., 2001; Neitzel et al., 2004; Pickering and Willoughby, 1982; van West, 2006). However, in a similarly designed study in Italian and Scottish salmonid farms (i.e. Oomycota isolates collected from tissue and water samples in the farms, upstream and downstream from the fish farms), this effect on the downstream areas was not confirmed, although the occurrence of *S. parasitica* was still higher in the Italian farms and downstream than upstream, but these differences were not statistically significant (Tedesco et al., 2021). Such weaker effect of salmonid farms in Italy and Scotland on the spread of saprolegniosis pathogens could be because the occurrence of *S. parasitica* is mainly related to the presence of susceptible host individuals. If the wild hosts living downstream of the fish farms were in good condition, they might have been resistant to the development of the disease, although zoospores of the pathogen spread from the farms into the natural environment. Conversely, any disturbance affecting wild populations could lead to an increase in saprolegniosis outbreaks downstream of salmonid farms. Due to negative anthropogenic impacts on the natural environment (e.g. climate change, pollution, introduction of new species, habitat modification and degradation), host species are becoming more susceptible to various pathogens, leading to disease

outbreaks and sometimes even extinction of entire populations (Fisher et al., 2012; Gozlan et al., 2014). In this context, the transfer of *Saprolegnia* spp. from fish farms into the environment could lead to increased mortality in natural populations in the future. Therefore, it is crucial to conduct detailed surveys to track the spread and distribution of pathogenic *Saprolegnia* spp. from fish farms into the natural environment, such as this one.

3.3. Water characteristics influence the sporulation and spreading of *Aphanomyces astaci* and *Saprolegnia parasitica*

As explained in the Introduction (Section 1.3.), various abiotic and biotic factors can influence the occurrence and distribution of animal Oomycota pathogens in freshwater ecosystems, but most of these are not sufficiently studied. To better understand the ecology of Oomycota pathogens, some of these factors were investigated in this dissertation, namely:

- the effect of water composition on the production of *A. astaci* and *S. parasitica* zoospores as the major infectious stage of their life cycle (subsection 3.3.1., Publication **III**)
- the effect of water composition on *S. parasitica* occurrence in freshwaters in Croatia (subsection 3.3.2., Publication **I**, Appendix 1)
- the effect of specific microbial taxa in the water on *S. parasitica* occurrence in freshwaters in Croatia (subsection 3.3.3., Appendices 2 and 3).

3.3.1. Effect of water composition on the sporulation intensity of *Aphanomyces astaci* and *Saprolegnia parasitica*

To analyse the influence of physico-chemical properties of water on zoospore formation, different natural water samples were used to wash the mycelium of *A. astaci* and *S. parasitica* (Publication **III**). Differences in sporulation efficiency were found and the sporulation intensity of the pathogens was then correlated with the physico-chemical properties of the water by Partial Least Squares Regression (PLS-R) multivariate analysis. The results showed that the parameters related to the organic matter in the water, especially the content of aromatics, were the most important factors that positively influenced the sporulation intensity of *S. parasitica*. In contrast, the sporulation of *A. astaci* was not significantly influenced by any of the water quality parameters

studied. Furthermore, the sporulation intensity of the two pathogens was not correlated. These results are consistent with their contrasting life strategies. *Aphanomyces astaci* is a specialised animal parasite with a narrow host range, whereas *S. parasitica* has a much wider host range and is usually considered an opportunistic secondary pathogen that alternates between a saprophytic and pathogenic lifestyle (de la Bastide et al., 2015; van den Berg et al., 2013). It has already been shown that the survival of *A. astaci* depends mainly on the presence of host crayfish species (Diéguez-Uribeondo, 2009; Söderhäll and Cerenius, 1999). Based on this, it can be hypothesised that the presence of host-specific factors favours the sporulation of *A. astaci*, whereas in the case of *S. parasitica*, water composition might be more important. However, further studies are needed to clarify these possible differences.

The PLS-R results showed that the sporulation intensity of *S. parasitica* was positively correlated with the spectral absorption coefficient measured at 254 nm (SAC) and the dissolved organic carbon (DOC) in water. The DOC levels in freshwater vary widely from 0.1 to hundreds of mg/L, but their average values have been reported to be around 5 mg/L (Bittar et al., 2015; Sobek et al., 2007), similar to the values measured in the studied dataset. SAC usually reflects the presence of organic compounds in water, especially those containing aromatic rings or unsaturated carbon bonds. The PLS-R results thus suggest that the presence of aromatic organic compounds in water, such as humic acids (HA), can promote the sporulation of *S. parasitica*. This was confirmed experimentally, as washing *S. parasitica* mycelia with a series of HA-supplemented water samples resulted in a concentration-dependent increase in sporulation. In contrast, the addition of HA did not affect the sporulation of *A. astaci*. Interestingly, humic substances have previously been shown to inhibit mycelial growth of *S. parasitica* and several related oomycetes from the order Saprolegniales (genera *Saprolegnia*, *Achlya*, *Leptolegnia*, *Pythium*), but without concomitant inhibition of sporangia development (Masigol et al., 2019; Meinelt et al., 2007). This is consistent with the results of this dissertation and suggests that in the presence of humic substances, mycelial growth is impaired and, therefore, Oomycota switch to the production of sporangia and motile zoospores that can spread to new, more favourable environments.

In addition, the PLS-R modelling results indicated that elevated fluoride and ammonium concentrations could also act as sporulation triggers, at least for *S. parasitica*. The range of fluoride concentrations in freshwater is between 0.01 and 0.3 mg/L (Camargo, 2003), which is consistent with the average fluoride concentration of 0.1 mg/L in the studied dataset. The induction of

sporulation by environmentally relevant fluoride concentrations could be explained as a response to unfavourable environmental conditions, as in the case of the humic substances discussed above. High fluoride concentrations have been shown to have negative effects on microbial physiology (Marquis et al., 2003; Mendes et al., 2014; Montagnolli et al., 2017; Zhang et al., 2019), but there are no data yet on the toxicity of fluorides to Oomycota. Further, ammonium-N concentrations of 3/36 (8%) samples were above the limit of 0.3 mg/L set by national legislation (OG 96/19). The exposure of juvenile or adult rainbow trout to environmentally relevant sublethal ammonium concentrations (0.05 and 0.5 mg/L, respectively) caused an increased susceptibility to saprolegniosis (Carballo et al., 1995; Carballo and Munoz, 1991). Also, the individuals that developed the disease had increased cortisol levels, leading to the conclusion that the susceptibility to saprolegniosis might be correlated with stress response and specific impairments in host defense mechanisms. However, in view of the results obtained here, the increased susceptibility to the pathogen could also be partly due to ammonium-induced zoospore proliferation and thus an increase in zoospore concentration. However, it has been shown that at high ammonium-N concentrations (8 and 16 mg/L, which were not found in the acquired dataset), the relative abundance of Oomycota in freshwater habitats can decrease (Yan et al., 2019). Further experiments should be conducted with a range of increasing ammonium concentrations in water to determine the range of ammonium concentrations that promote sporulation and to compare the effects of ammonium on mycelium and zoospores of pathogenic Oomycota.

Overall, the results obtained suggest that some compounds might suppress the mycelial growth of the pathogen in a certain range of environmentally relevant concentrations and at the same time promote sporulation, facilitating the spread of the pathogen to a more favorable environment. Therefore, the effects of these compounds, such as ammonium and fluorides, on the sporulation intensity and virulence of freshwater Oomycota pathogens should be tested experimentally, as was done in the Publication **III** with the addition of a range of HA concentrations to the water used to induce sporulation. Furthermore, one of the limitations of this study is that only one isolate from each species was used. This might not be representative at the species level, as significant within-species variability in mycelial growth rate, sporulation temperature, zoospore motility and other parameters were observed in both *A. astaci* (Diéguez-Uribeondo et al., 1995) and *S. parasitica* (Bangyeekhun et al., 2001; Diéguez-Uribeondo et al.,

2007; Masigol et al., 2020). Therefore, a wider range of isolates/genotypes should be included in the *in vitro* sporulation intensity tests in the future.

Finally, in addition to new insights into the physico-chemical parameters of water that promote zoospore formation, the results obtained could also be a starting point for the development of artificial water that could be used for optimal and reproducible Oomycota sporulation under laboratory conditions, which is known to be highly variable and poorly reproducible. The research community working on these pathogens would benefit from standardising the composition of the water used for sporulation induction. Therefore, it would be interesting for future studies to develop a series of artificial water samples with defined composition and test their effects on sporulation intensity.

3.3.2. Effect of water composition on *Saprolegnia parasitica* occurrence in freshwaters in Croatia

The developed *S. parasitica*-specific ddPCR assay was used to correlate for the first time the *S. parasitica* load in water with the physico-chemical parameters of water, including trace elements determined by high-resolution inductively coupled plasma mass spectrometry (HR-ICP-MS) (Publication **I**, Appendix 1). Among the basic physico-chemical parameters, the positive correlation was strongest for Ca^{2+} and electrical conductivity (EC), while the parameter with the strongest negative influence was F^-. Calcium concentrations in water samples within the studied dataset ranged from 2.15 to 119.4 mg/L, with a median of 37.1 mg/L, which is significantly higher than the global freshwater median of 4 mg/L (Weyhenmeyer et al., 2019), but common in karst areas which are abundant in Croatia (Ford and Williams, 2007). In *in vitro* studies, calcium ions have been shown to positively influence the developmental stages and infection process of Oomycota (Burr and Beakes, 1994; Deacon and Donaldson, 1993; Rezinciuc et al., 2018). For example, in *S. parasitica*, the number and length of long hooked hairs on the cysts increased after the addition of 5,550 mg/L $CaCl_2$, which improved the adhesion of the cysts to the host surface (Rezinciuc et al., 2018). The results obtained here show for the first time that higher than average Ca^{2+} concentrations in surface waters favour the growth/development of *S. parasitica*. This is in line with the above-mentioned *in vitro* studies showing the positive effect of (even) higher concentrations of calcium ions expected when the pathogen invades host tissues (Baldisserotto et al., 2005; Barszcz et al., 2014). In addition, EC, which is related to the total ion content of water and mainly to the concentration of sodium, chloride and calcium ions, has been found to correlate

positively with *S. parasitica* load. In freshwater ecosystems, sodium and chloride concentrations vary from country to country. For example, the range of groundwater limits for chloride set by European Union countries is between 24 and 12,300 mg/L, while the upper limits for sodium in drinking water are between 50 and 450 mg/L. Sodium chloride in high concentrations (> 1,000 mg/L) is used as an effective and non-toxic method to control *Saprolegnia* spp. because it can reduce the vegetative growth of the pathogen as well as the formation, release and multiplication of sporangia (Ali, 2005). The results of this dissertation showed that the water samples with *S. parasitica* load > 5 ITS copies/ng had higher average chloride (40.2 mg/L) and sodium (1.3 mg/L) concentrations than the water samples with lower *S. parasitica* load (17.9 and 0.6 mg/L, respectively). This suggests that moderate salt concentrations in natural waters (neither too low, nor too high) may have a positive effect on *S. parasitica*, which is consistent with the fact that sodium and chloride concentrations are higher in host tissues than in water (Boisen et al., 2003). Finally, fluoride concentration was the most important factor negatively correlated with *S. parasitica* load in the water, although it did not contribute significantly to the PLS-R model. In the studied dataset, water samples with *S. parasitica* load > 5 ITS copies/ng had an average of 0.38 mg F^-/L compared to 0.47 mg/L in the water samples with lower *S. parasitica* load. This indicates an inhibitory effect of environmentally relevant fluoride concentrations on *S. parasitica* that should be further experimentally confirmed in the future.

Overall, the tendency was observed that *S. parasitica* load in the water samples (determined by ddPCR in Publication **I**) was negatively correlated with some water parameters that have been shown to promote sporulation (Publication **III**), such as organic matter, fluoride and ammonium content. This supports the assumption that environmentally relevant concentrations of certain water parameters can have a negative impact on the survival of the pathogen and at the same time promote sporulation and thus the migration of the pathogen via zoospores to more suitable environments.

Absolute ddPCR-based quantification of *S. parasitica* was also correlated with concentrations of trace elements in natural waters. PLS-R modelling results are shown in Appendix 1, Figures S1 and S2. The relationship between blocks of predictor and response variables is visually represented in the form of a correlation radar, with positively correlated variables close together and negatively correlated ones far apart (Figures S1a and S2a, Appendix 1). The strength of the correlation can also be seen from the correlation coefficients (r), where the closer the values are to 1, the stronger the positive correlation. The results showed that U (r_{sol} = 0.50, r_{tot} = 0.53), Li

(r_{sol} = 0.48, r_{tot} = 0.45) and V (r_{sol} = 0.39, r_{tot} = 0.36) were the most important soluble and total trace elements, respectively, that positively influenced the *S. parasitica* load in the water samples. Moreover, these trace elements had VIP values > 1 (Figures S1b and S2b, Appendix 1), which means that they are considered highly relevant in explaining the *S. parasitica* load in water and contribute significantly to the PLS-R model.

Uranium, lithium and vanadium concentrations at the investigated sites (Appendix 2) were within the acceptable ranges (soluble concentration: 1.12 ± 1.27, 1.92 ± 1.95 and 0.34 ± 0.21; total concentration: 1.02 ± 1.15, 2.21 ± 1.78 and 0.72 ± 0.42, respectively (mean ± standard deviation)), as expected for freshwater habitats without significant pollution with these elements (Bleise et al., 2003; Chen et al., 2022; Kavanagh et al., 2017). Thus, the obtained results suggest that low, environmentally relevant concentrations of these elements could act stimulatory towards *S. parasitica* pathogen. It was shown that some bacteria *Pseudomonas putida*, *Halomonas mono* and *Spirulina plantensis* can be used for biosorption or bioreduction of U or V from contaminated waters (Safonov et al., 2018), while some soil fungi are able to transform metallic U into more thermodynamically stable minerals for long-term U retention (Fomina et al., 2008). Currently, knowledge about the effects of U, V and Li on different Oomycota species is mostly lacking. In general, metals are known to be toxic towards Oomycota species at elevated concentrations and can be used as inhibitory compounds in Oomycota disease management. For instance, fungicides that contain Cu are often applied to control saprolegniosis (e.g. copper sulfate at a concentration ≥ 0.5 mg/L) (Sun et al., 2014). However, at low concentrations, metals may benefit the pathogen (Gajewska et al., 2022 and references within) and might even promote its virulence (Bakti et al., 2018; Gajewska et al., 2020; Li et al., 2018). For example, disease symptoms of *P. infestans* develop faster if the pathogen is exposed to a low concentration of Cd (i.e. 5 mg/L) (Gajewska et al., 2020). However, additional research is required to determine the precise impact that specific metals, like U, V and Li, at different environmentally relevant concentrations have on virulence, pathogenicity and abundance of *S. parasitica* and other relevant Oomycota pathogens.

In conclusion, by combining physico-chemical analysis of water with the results of sensitive *S. parasitica* quantification by the ddPCR method, some chemical parameters of water, such as environmentally relevant concentrations of Ca^{2+}, U, V and Li, were found to be positively correlated with the pathogen load in the water. Such results could provide a basis for the prediction

of the water conditions that might promote the pathogen spreading in natural environments and aquaculture facilities, and thereby aid in the development of preventive measures.

3.3.3. Microbial communities as indicators of *Saprolegnia parasitica* presence in freshwater ecosystems

Freshwater ecosystems are a habitat of diverse microbial communities that can have a major impact on their' health and function (Karner et al., 2001; Sagova-Mareckova et al., 2021). Besides having an important role in the decomposition of organic matter, the biogeochemical cycling of elements and the biodegradation and biotransformation of pollutants (Bai et al., 2014; Fuhrman, 2009; Wang et al., 2018), freshwater microbes can have a positive or a negative impact on the reproduction and survival of co-habiting freshwater Oomycota, such as *Saprolegnia parasitica*. In the scope of this dissertation PLS-R modelling was applied to correlate:

(i) physico-chemical properties of water (including the concentration of trace elements) with the relative abundance of different aquatic microbial taxa determined by high-throughput sequencing of the *16S* rRNA marker gene (Appendix 2);

(ii) *Saprolegnia parasitica* load in ITS copies per ng of total eDNA determined by ddPCR and the relative abundance of different aquatic microbial taxa (Appendix 3).

The first approach revealed that a series of bacterial taxa were positively correlated with some of the water parameters (Appendix 2, Tables S1 and S2), confirming that the physico-chemical characteristics of water quality affect the composition and diversity of aquatic microbial communities (Niu et al., 2019; Tiquia, 2010), including the Oomycota pathogens (Publications **I** and **III**). The highest number of microbial taxa (belonging to phyla Proteobacteria, Actinobacteria and Bacteroidetes) detected at sampled locations, showed a positive correlation with ion content (i.e. the concentration of SO_4^{2-}, Ca^{2+}, Cl^- and Na^+ and the values of EC) (Appendix 2, Table S1). Regarding the trace elements, most of the microbial taxa (e.g. families Comamonadaceae, Erythrobacteraceae and Rhodobacteraceae) were positively correlated with the U and V (Appendix 2, Table S1). Similarly, a significant positive correlation was established between *S. parasitica* load in the water and Ca^{2+} and EC (Publication **I**), and with U, V and Li (Appendix 1). Thus, the locations where bacterial indicators of ion content/U/V/Li are found, might also present a suitable habitat for *S. parasitica*, with an increased risk of disease outbreaks. On the other hand, *S.*

parasitica load was negatively correlated with fluoride concentration in the water and with organic nutrients such as ammonium, total phosphorous (TP) and total organic carbon (TOC), suggesting the inhibitory effect of these water parameters on the pathogen (Publication **I**). Thus, locations, where these parameters are elevated, might be unfavourable for disease development. In parallel, PLS-R results yielded a series of bacterial taxa with significant positive correlation with fluorides (e.g. bacteria from the family Methylobacteriaceae or genus *Leptolyngbya*) or organic nutrients (such as Firmicutes genera *Clostridium* and *Enterococcus*, known faecal indicators) (Appendix 2, Table S1). Thus, such bacteria might indicate freshwater locations with reduced probability of saprolegniosis outbreaks.

Further, *S. parasitica* load in the water was positively and significantly correlated with the relative abundance of some specific microbial taxa (Appendix 3, Table S1). For instance, a positive correlation with *S. parasitica* was obtained for the genera *Nitrospira* and *Nitrosopumilus* which are known as the nitrite-oxidizing bacteria and ammonia-oxidizing archaea, respectively (Tian et al., 2016). Some *Nitrospira* spp. were also found to be capable of complete oxidation of ammonia to nitrate (i.e. comammox bacteria) (Mehrani et al., 2020). On the other hand, *S. parasitica* load was negatively correlated with ammonium (Publication **I**). Thus, a hypothesis can be raised that these microbial taxa efficiently remove ammonium making the habitat more suitable for *S. parasitica*. Further, members of the family Flavobacteriaceae, positively correlated with *S. parasitica* load here, were also the most dominant bacterial genera detected in the chum salmon eggs infected with Oomycota from the genus *Pythium* (Sakaguchi et al., 2022). Based on this, the possibility of bacteria-oomycete co-infection was discussed.

In conclusion, the results of this dissertation indicate that specific microbial taxa could serve as indicators of the habitat suitability for freshwater pathogenic Oomycota, such as *S. parasitica*. Also, aquatic microbial taxa that were positively correlated with *S. parasitica* load in the water were identified, opening the questions on the nature of their interactions to be solved in the future.

4. CONCLUSION

The main findings of this dissertation can be summarized in the following conclusions:

- Dominant cultivable Oomycota genus in the Croatian trout farms and the adjacent natural environments was *Saprolegnia* (64%), while other detected genera were *Pythium* (35%) and *Leptolegnia* (1%). Dominant *Saprolegnia* species at the trout farms was *S. parasitica*.
- *Saprolegnia* species showed a strong association with fish farms and downstream locations, while upstream locations were associated with *Pythium* species. Thus, trout farms can act as points of release of *Saprolegnia* pathogens to downstream freshwater ecosystems.
- *Saprolegnia parasitica*-specific ddPCR assay was developed, allowing sensitive detection and absolute quantification of the pathogen load in various environmental DNA samples (e.g. water and/or swabs). The method could be applied in salmonid aquaculture as well as in the wild to monitor variations in *S. parasitica* load over space and time and to timely predict possible disease outbreaks.
- Combining ddPCR-based absolute quantification of *S. parasitica* with the data on the water parameters made it possible to gain new insights into the ecological requirements of this pathogen. Electrical conductivity (EC), calcium, and trace elements uranium, vanadium and lithium were positively correlated with *S. parasitica* load in the water, while for fluorides a negative correlation was demonstrated.
- The water parameters that had the most significant positive effect on *S. parasitica* sporulation intensity were related to the organic content of the water, while sporulation of *A. astaci* was not significantly correlated with any of the analysed parameters and is presumably more dependent on the host-specific factors. In agreement with this, the addition of environmentally relevant concentrations of humic acid to the water induced sporulation of *S. parasitica* but not of *A. astaci*.
- Various microbial taxa can be used as bioindicators of specific disturbances of water quality and at the same time can serve as indicators of the ecological conditions that are favourable for freshwater pathogenic Oomycota such as *S. parasitica*.

5. LITERATURE

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Curriculum Vitae

Dora Pavić, mag. oecol., was born on 11 October 1993 in Bjelovar (Croatia). In 2015, she graduated from the Faculty of Forestry with a Bachelor's degree and in the same year enrolled in a Master's degree programme in Environmental Science at the Faculty of Science, University of Zagreb, where she has graduated in 2018. In the same year, she has started working as a research assistant at the Faculty of Food Technology and Biotechnology in the Laboratory for Biology and Microbial Genetics, and enrolled in a PhD programme in Biology at the Faculty of Science, University of Zagreb. Her PhD research was carried out within the framework of the project "Interactions of the freshwater pathogenic oomycetes and their environment" (InteractOomyc) of the Croatian Science Foundation under the supervision of A. Bielen.

Dora is first author or co-author of nine scientific publications indexed in the SCI-expanded database. She participated in eight international and national congresses with three oral presentations. She was a recipient of FEMS Research and Training Grant to stay at Department of Veterinary Medical Science, Alma Mater Studiorum, University of Bologna (Italy), an University of Zagreb Academic Mobility Grant for a stay at Molecular Biology Laboratory of Labena d.o.o., BIA Separation CRO, Ljubljana (Slovenia) and an Erasmus+ Traineeship Grant for a stay at Faculty of Agriculture, University of South Bohemia, České Budějovice (Czech Republic). She teaches basic courses in Biology and Human physiology at the Faculty of Food Technology and Biotechnology. She has participated in the supervision of bachelor (1) and master (2) theses as well as student research projects, which were awarded with two Rector's awards.

Proširenisažetak

Oomicetni patogeni slatkovodnih životinja uzročnici su brojnih bolesti i smatraju se emergentnim patogenima na globalnoj razini. Među njima, *Aphanomyces astaci* uzročnik je bolesti račje kuge. Ova je bolest uzrok smanjenja brojnih populacija zavičajnih vrsta slatkovodnih rakova u Europi i svijetu te danas predstavlja jedan od glavnih problema u zaštiti slatkovodnih rakova. Vrsta *Saprolegnia parasitica* i druge vrste iz tog roda uzrokuju bolest saprolegniozu kod širokog raspona domaćina, većinom riba. Saprolegnioza uzrokuje značajne gubitke u akvakulturi, kako u ribogojilištima tako i u mrijestilištima.

Unatoč tome što slatkovodni oomicetni patogeni predstavljaju značajnu prijetnju u prirodnim i akvakulturnim sustavima, mnogi aspekti njihove ekologije nedovoljno su istraženi. Čimbenici koji potiču njihovo širenje, poput utjecaja akvakulturnih objekata na širenje patogena u nizvodna prirodna vodena staništa, još uvijek su najvećim dijelom nepoznati. Stoga su ciljevi ove doktorske disertacije bili: (i) razviti droplet digital PCR (ddPCR) test za visoko osjetljivu detekciju i kvantifikaciju patogena *S. parasitica* u uzorcima okolišne DNA (engl. *environmental DNA*, eDNA) (Publikacija **I**); (ii) identificirati oomicetne vrste u hrvatskim pastrvskim ribnjacima te ih usporediti s izolatima sakupljenim uzvodno i nizvodno od njih (Publikacija **II**); (iii) odrediti kemijska i mikrobiološka svojstva prirodnih voda koja potiču širenje/sporulaciju patogena *A. astaci* i *S. parasitica* (Publikacije **I** i **III**, dodaci 1 – 3).

U prvom koraku razvijen je brzi i osjetljivi ddPCR test za detekciju vrste *S. parasitica* (Publikacija **I**). Razvijeni test je visoko specifičan odnosno omogućuje diskriminaciju između ciljnog patogena *S. parasitica* i blisko srodnih vrsta kao što su *S. australis, S. delica, S. diclina, S. ferax* i *S. litoralis*. Osjetljivost testa je također visoka, s donjom granicom detekcije od 14 fg genomske DNA *S. parasitica*. Novorazvijeni test po prvi je puta omogućio praćenje patogena izravno iz okolišnih uzoraka (npr. vode) izbjegavajući dugotrajnu izolaciju patogena u čistoj kulturi.

U hrvatskim je ribnjacima po prvi puta određena prisutnost vrste *S. parasitica* te drugih vrsta iz skupine vodenih plijesni. Izolati su uzgojeni iz inficiranog tkiva domaćina i iz uzoraka vode (Publikacija **II**). Izolati su identificirani na temelju sekvenci ITS (engl. *internal transcribed species*) regije i većina je pripadala rodu *Saprolegnia* (*S. parasitica* – 24 %, *S. australis* – 24 %, *S. delica* – 11 % i *S. ferax* – 5 %), a ostatak rodovima *Pythium* i *Leptolegnia*. Prisutnost izolata iz roda *Saprolegnia* bila je povećana u ribnjacima i nizvodnim prirodnim staništima, dok su na

uzvodnim staništima većinom detektirane vrste roda *Pythium*. Ovi rezultati ukazuju na moguću ulogu pastrvskih ribnjaka u širenju vrsta iz roda *Saprolegnia* u okolna prirodna staništa.

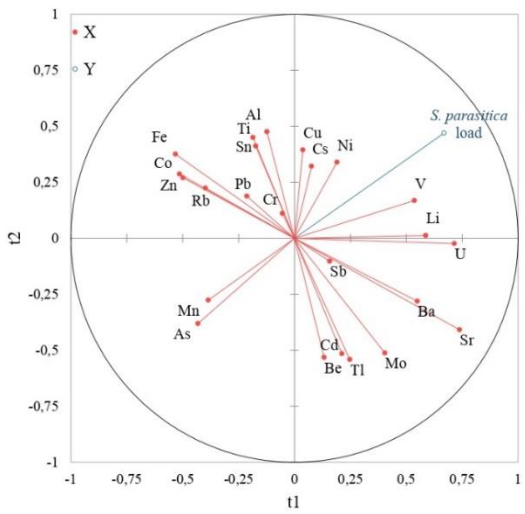
Konačno, identificirane su fizikalno-kemijske i mikrobiološke značajke prirodnih voda koje potiču širenje/sporulaciju vrsta *A. astaci* i *S. parasitica* (Publikacije **I** i **III**, dodaci 1 – 3). Kako bi se istražio utjecaj sastava vode na intenzitet sporulacije, micelij je ispiran s uzorcima prirodnih voda sakupljenih na različitim lokacijama u Hrvatskoj. Rrezultati PLS-R analize pokazali su da spektralni apsorpcijski koeficijent (engl. *spectral absorption coefficient*, SAC), otopljeni organski ugljik (engl. *dissolved organic carbon*, DOC), amonijak i fluor imaju najveći pozitivni učinak na sporulaciju patogena *S. parasitica*, dok ni jedan od analiziranih parametara nije značajno utjecao na sporulaciju patogena *A. astaci*. U skladu s time, dodatak huminske kiseline (koja doprinosi SAC i DOC vrijednostima) u okolišno relevantnim koncentracijama u vodu za ispiranje micelija potaknuo je sporulaciju vrste *S. parasitica*, ali ne i *A. astaci* (Publikacija **III**). Nadalje, zastupljenost vrste *S. parasitica* u uzorcima vode (Publikacija **I**) bila je pozitivno korelirana s električnom provodljivosti (engl. *electrical conductivity*, EC) te koncentracijom kalcija i nekih drugih elemenata poput topivog i netopivog uranija, vanadija i litija (dodatak 1). Dodatno, identificiran je niz mikrobnih taksona koji bi mogli predstavljati indikatore ekoloških uvjeta pogodnih za patogene slatkovodne oomicete poput vrste *S. parasitica* (dodaci 2 i 3).

Zaključno, rezultati ove disertacije donose nove podatke o uvjetima okoliša koji pogoduju patogenim slatkovodnim oomicetima. Dobiveni rezultati bi u budućnosti mogli pomoći u pravovremenom predviđanju proliferacije patogena, tj. identifikaciji "vrućih točaka" u slatkovodnim ekosustavima koje imaju povećani rizik od izbijanja bolesti uzrokovanih patogenima *A. astaci* i *S. parasitica*.

Appendix 1

Correlation of trace elements concentration in the water samples and *S. parasitica* load (expressed as the number of *S. parasitica* ITS copies per ng of total eDNA), analysed using PLS-R modelling

a)



b)

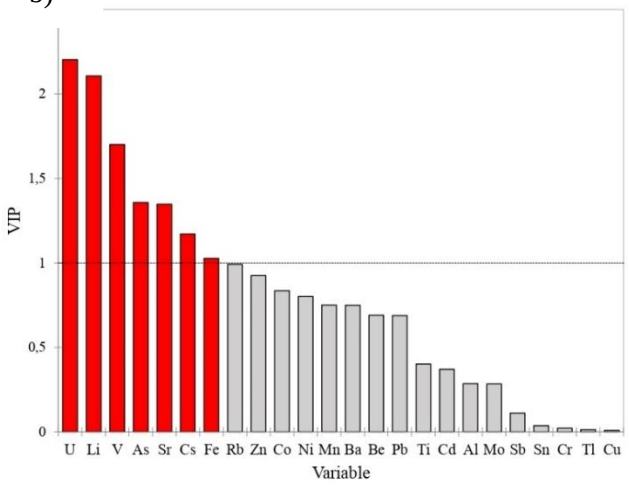


Figure S1. (a) Correlation radar describing the relationship between *S. parasitica* load in ITS copies per ng of total eDNA (response variable, Y, blue line) and concentration of soluble trace elements in mg/L in the water (explanatory variables, X, red lines). The precentages of variances in X and Y explained by each variable are indicated on the respective axes. **(b)** The variable importance in projection (VIPs) for explanatory variables of the first component (t1). VIPs > 1 indicate the explanatory variables that contribute most to the PLS model, while VIPs < 0.8 contribute little.

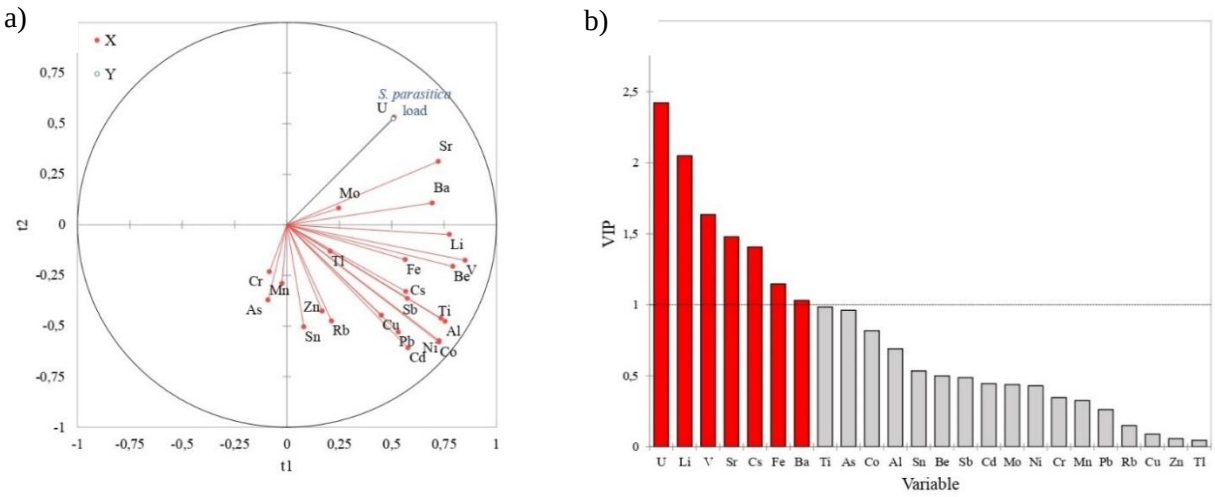


Figure S2. (a) Correlation radar describing the relationship between *S. parasitica* load in ITS copies per ng of total eDNA (response variable, Y, blue line) and concentration of total trace elements in mg/L in the water (explanatory variables, X, red lines). The precentages of variances in X and Y explained by each variable are indicated on the respective axes. **(b)** The variable importance in projection (VIPs) for explanatory variables of the first component (t1). VIPs > 1 indicate the explanatory variables that contribute most to the PLS model, while VIPs < 0.8 contribute little.

Appendix 2

Determining the impact of water chemistry on freshwater microbial communities

Materials and Methods

Water sampling

Water was sampled at 35 locations in Croatia in winter 2018/2019: (i) 500 mL per location for the physico-chemical analyses (pH, EC, NH_4^+, NO_3^-, SO_4^{2-}, F^-, Cl^-, Na^+, K^+, Mg^{2+}, Ca^{2+}, TOC, TP and chemical oxygen demand (COD)), (ii) 50 mL for highly sensitive analysis of trace elements in the water by high resolution inductively coupled plasma mass spectrometry (HR-ICP-MS), and (iii) 2L for DNA extraction and microbial community analyses. The geological makeup of the substrate, the kind of ecosystem (lentic or lotic), and the ecoregion were all taken into consideration while choosing sampling locations to include variable water samples with variable composition in the dataset. Samples were transported to the laboratory at 4°C in the dark. Protocols used for basic physico-chemical analyses of water and the extraction of DNA from water are described in Publication **I**.

High resolution inductively coupled plasma mass spectrometry (HR-ICP-MS)

At each sampling location 50 mL of surface water was collected in Falcon tubes washed three times with the water sample before filling. Upon the arrival at the laboratory, each water sample was divided into two aliquots: one was filtered through a 0.45 μm syringe filter to analyse the soluble portion of trace elements in water samples, while the unfiltered aliquots were used to determine the total trace elements (soluble + insoluble). Samples were stored at 4°C until further analysis.

Multielement analysis of the prepared water samples was performed by HR-ICP-MS using an Element 2 instrument (Thermo, Bremen, Germany), as described in detail previously (Fiket et al., 2015). External calibration was used for the quantification. All consumables (e.g. Falcon tubes and syringes) used in HR-ICP-MS analyses were soaked in 10% HNO_3 solution for two weeks and rinsed with Milli-Q water before use. Prior to analysis, samples were acidified with 2% (v/v) HNO_3

(65%, supra pur, Fluka, Stein-heim, Switzerland) and In (1 µg L^{-1}) was added as an internal standard. Samples were analysed for the total and dissolved concentrations of 24 elements (Al, As, Ba, Be, Cd, Co, Cr, Cs, Cu, Fe, Li, Mn, Mo, Ni, Rb, Pb, Sb, Sn, Sr, Ti, Tl, U, V and Zn). High purity Milli-Q water (resistivity less than 18 MΩ/cm) obtained with a Millipore purification system was used to prepare the standard and blank solutions. Standards for multielement analysis were prepared by appropriate dilution of a multielement reference standard (Analytika, Prague, Czech Republic) containing Al, As, Ba, Be, Cd, Co, Cr, Cs, Cu, Fe, Li, Mn, Mo, Ni, Rb, Pb, Sr, Ti, Tl, V and Zn, in which single-element standard solutions of U (Aldrich, Milwaukee, WI, USA), Sn (Analytika, Prague, Czech Republic), and Sb (Analytika, Prague, Czech Republic) were added. The working standards as well as the blank solutions were prepared by adding 1% high purity HNO_3 (Fluka, Steinheim, Switzerland) and 1% high purity HCl (Merck, Darmstadt, Germany). Quality control of the analytical procedure was performed by simultaneous analysis of the blank sample and the certified reference material for water (SLRS-4, NRC, Canada). For all elements, good agreement was obtained between the analysed and the certified concentrations within their analytical uncertainties.

Microbial community analysis by 16S rRNA gene sequencing

The bacterial community composition in eDNA extracted from water was analysed by sequencing of 16S rRNA gene amplicon performed at Microsynth, Switzerland. The target V4 region (~ 300 bp) of the 16S rRNA gene was amplified using forward primer 515F (5' GTGCCAGCMGCCGCGGTAA 3') and reverse primer 806R (5' GGACTACHVGGGTWTCTAAT 3') (Bates et al., 2011) using 16S Nextera twostep PCR (including purification and pooling), while sequencing was done on Illumina MiSeq using the MiSeq Reagent kit v3 (2 x 300 bp paired-end reads). The sequences were analysed in Quantitative Insights Into Microbial Ecology 2 (QIIME2) software (Bolyen et al., 2019), release 2021.2. Upon preliminary check-up, reverse sequences were shown to be of insufficient quality and length in some samples, therefore the reverse reads were discarded and only single end forward reads were used to identify the bacterial taxa present in water samples. Raw sequences were first demultiplexed and trimmed of Illumina adaptor residuals and then imported into the QIIME2 using the sample manifest format. In the next step the quality of raw sequence data was checked by

DADA2 (q2-dada2) plugin (Callahan et al., 2016). During this step reads were denoised, the primer sequences trimmed off (first 19 bp) and each sequence truncated at the position of median Phred quality score crashing (273 bp). Additionally, predicted chimeric sequences were removed and assigned into amplicon sequence variants (ASVs). The DADA2-generated feature table was filtered to remove ASVs at a frequency of less than 0.1 % per sample and appearing in less than two samples. The q2-feature-classifier/QIIME2 feature classifier plugin (Bokulich et al., 2018) was used for taxonomic classification of ASVs with the classifier being trained on the Greengenes 13_9 99% Operational Taxonomic Unit (OTU) reference sequences (McDonald et al., 2012) targeting the V4 region of the rRNA gene. Next, sequences classified as chloroplast or mitochondria were filtered out from the analysis via q2-taxa and the phylogenetic tree was generated using fasttree2 based on MAFFT alignment of ASVs as implemented in the q2-phylogeny plugin. To have an even number of reads across all samples, ASV table was rarefied at 2 000 reads, and two water samples (PF7 and PS7) were excluded from the subsequent analyses due to having < 2 000 reads. Alpha diversity, analysed by q2-diversity plugin, was evaluated using the three indices: (i) Pielou's evenness index that ranges between 1 (all ASVs equally represented) and close to zero (one ASV strongly dominates); (ii) observed species index (number of different ASVs in the sample, i.e. species richness); and (iii) Shannon's index, that ranges from one (in case of a single dominant species) to the total number of all species (in case of all species having equal abundance), and thus is a combined measure for both richness and diversity.

Data analysis

To analyse the relationship of different physico-chemical parameters of water samples (explanatory variables or predictors, X: pH, EC, NH_4^+, NO_3^-, SO_4^{2-}, F^-, Cl^-, Na^+, K^+, Mg^{2+}, Ca^{2+}, TOC, TP, COD, Al, As, Ba, Be, Cd, Co, Cr, Cs, Cu, Fe, Li, Mn, Mo, Ni, Rb, Pb, Sb, Sn, Sr, Ti, Tl, U, V and Zn) and the bacterial communities at different taxonomic levels (response variables, Y), Partial Least Squares Regression (PLS-R) analysis was performed using XLSTAT version 2021.3.1.1189 software provided by Microsoft Excel by Addinsoft.

Results

Physico-chemical properties of the water samples

The results have showed the overall good quality of the 35 collected natural water samples. Values for some of the physico-chemical parameters of water were higher than the legal limits (Official Gazette, 96/2019): nitrates were higher than 1.2 mg/L 13 out of 35 samples (37%), ammonium than 0.4 mg/L in 6/35 (17%) and TP than 0.35 mg/L in 2/35 (6%) of samples. Further, acceptable fluoride concentration in unpolluted freshwater is usually between 0.01 and 0.3 mg F^- /L (Camargo, 2003), while in obtained dataset all samples had fluoride levels > 0.3 mg F^-/L (in average 0.43 mg/L, min. 0.35 mg/L, max. 0.88 mg/L).

Further, the soluble concentrations of the majority of trace elements did not exceed the environmental quality standard (EQS) set by Directive 2008/105/EC and amended by Directive 2013/39/EU. For example, concentration of soluble Cd and Ni was below EQS in all water samples ($\leq$ 0.45 µg/L and $\leq$ 34 µg/L, respectively). However, the soluble Pb concentration was higher than the EQS limit (0.07 µg/L) in 20/35 samples (in average 0.09 µg /L, min. 0.02 mg/L, max. 0.19 mg/L). Further, concentration of some soluble trace elements exceeded the Croatian legal limits (Official Gazette, 96/2019): As was higher than 7.5 µg/L in 1 out of 35 samples, Cr than 9 µg/L in 2 samples, Cu than 1.1 in 6 samples and Zn than 7.8 µg/L in 8 samples. All water samples had soluble concentrations of Cd, Cr, Cu, Ni and Pb below the limits set by WHO (2011) for drinking water (3, 50, 2000, 70 and 10 µg/L, respectively). Moreover, for some trace elements such as Al, Fe, Mn and Ti, the average total concentration (including soluble and insoluble fraction: 119, 466, 69 and 3.9 µg/L, respectively) was higher than their soluble concentration (4.3, 21, 45 and 0.1 µg/L, respectively) as they are mainly bound to the particulate fraction.

Composition of aquatic bacterial communities

A total of 2,365,353 reads was obtained from high-throughput sequencing of the V4 region of 16S rRNA genes from the 35 collected water samples. After the DADA2 processing and filtering of the resulting feature table, 2,014,103 reads remained, containing in total 20,143 different amplicon sequence variants (ASVs). Alpha diversity indices were calculated for each sample. In average, Pielou's evenness index for the microbial communities in Croatian water samples was

0.75 ± 0.1, total observed ASVs were 273 ± 114, and Shannon's diversity index was 6.04 ± 1.3 (mean ± standard deviation).

Further, dominant bacterial phyla in most of the samples were Proteobacteria with average abundance of 58%, followed by Bacteroidetes (14%), Actinobacteria (9%) and Firmicutes (4%) (Figure S1). Dominant bacterial families were Comamonadaceae (22%, phylum Proteobacteria), followed by Flavobacteriaceae (10%, phylum Bacteroidetes) and Oxalobacteraceae (6%, phylum Proteobacteria) (Figure S2). At the genus level, *Flavobacterium* (10%, family Flavobacteriaceae), *Rhodoferax* and *Acidovorax* (8% and 6%, respectively, both from family Comamonadaceae) were more prevalent than other genera.

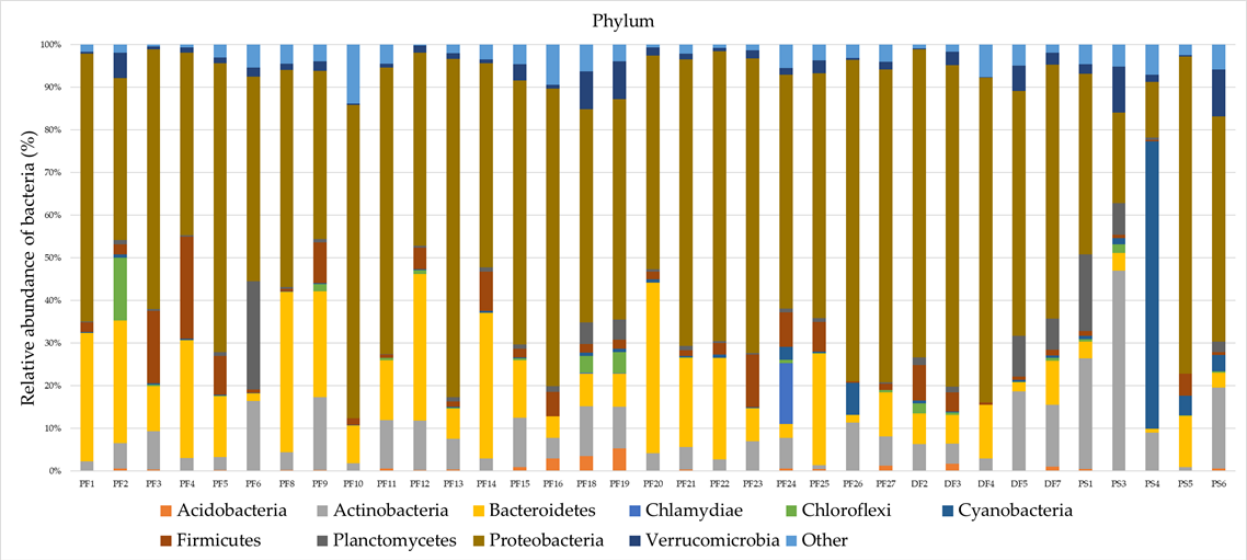


Figure S1. Relative abundance (%) of the bacterial phyla in the collected water samples (n = 35). Bacterial phyla and families with relative abundance > 5 % are shown, while the remaining were pooled and indicated as "other".

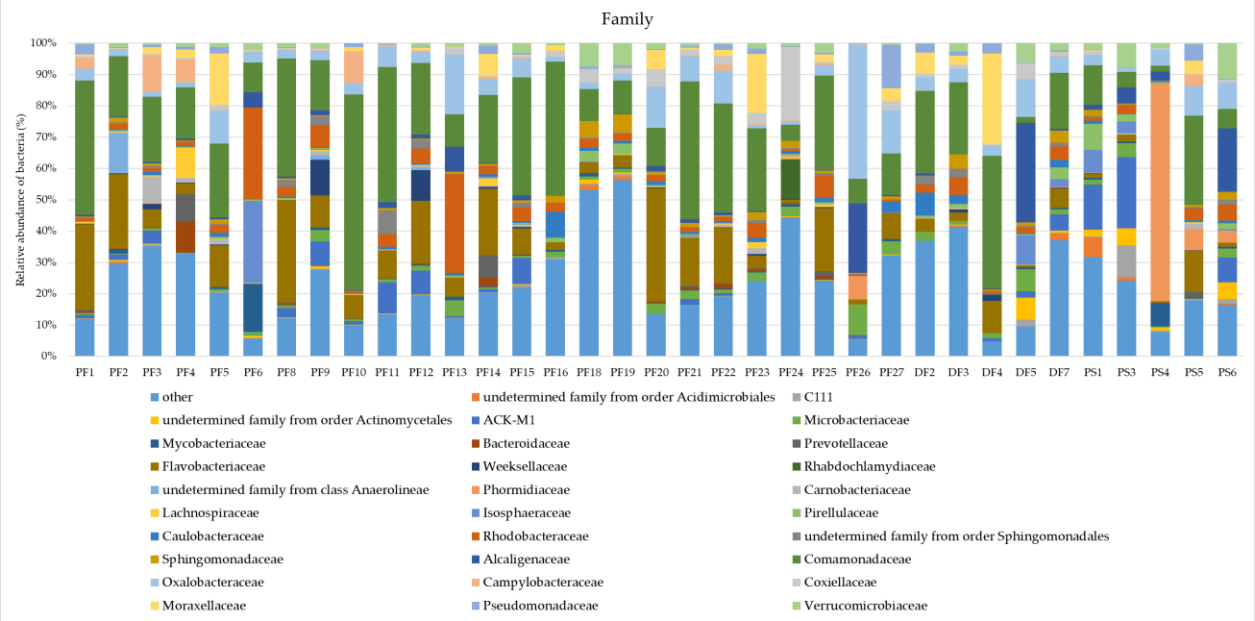


Figure S2. Relative abundance (%) of the bacterial families in the collected water samples (n = 35). Bacterial phyla and families with relative abundance > 5 % are shown, while the remaining were pooled and indicated as "other".

Correlation between water chemistry and bacterial communities

PLS-R multivariate analysis was used to analyse the relationship between bacterial communities (response variable, Y) and water parameters (explanatory variables, X). Bacterial communities were represented by microbial taxa at different taxonomic levels (phylum, class, order, family and genus) or by alpha diversity indices (Pielou's evenness index, total ASV number, Shannon's index). Water parameters were represented by physico-chemical parameters, total trace elements (soluble + insoluble) or soluble trace elements.

For each pair of Y and X variables, the strength of the correlation was predicted by r value, where the correlation is stronger with values getting closer to 1. Within this study, r values ≥ 0.66 were regarded as strong correlation. With this cut-off, no significant correlation was found between the alpha diversity indices that represent the richness and diversity of microbial communities and any of the analysed water paratmeters (data not shown). However, a series of microbial taxa were identified to be positively correlated with some of the water parameters, as overviewed in Tables S1 and S2. Firstly, a series of bacterial taxa (64 in total) belonging to phyla Proteobacteria (48%),

Actinobacteria (30%) and Bacteroidetes (8%) was positively affected by ion content in the water (i.e. concentration of SO_4^{2-}, Ca^{2+}, Cl^- and Na^+ as well as EC values). Examples of such taxa are genera *Modestobacter* and *Cellulomonas* from phylum Actinobacteria, genera *Winogradskyella* and *Spirosoma* (Bacteroidetes) and families Rhodobacteraceae, Alteromonadaceae and Erythrobacteraceae (Proteobacteria) (Table S1). Further, positive correlation with ammonium and TP content was found for 15 taxa, mostly from phyla Actinobacteria (like the genus *Bifidobacterium*) and Firmicutes (like genera *Clostridium* and *Enterococcus*) (Table S1). Lastly, a total of 17 bacterial taxa was positively correlated with fluoride content, mostly from phyla Proteobacteria, Planctomycetes and Cyanobacteria (e.g. *Planctomycetes* family Pirellulaceae and Cyanobacteria genus *Leptolyngbya*).

Table S1. Positive correlation (r values ≥ 0.66) of physico-chemical parameters of water and microbial taxa.

Water parameters		Phylum	Examples of bioindicator taxa	No. of taxa
Physico-chemical parameters	NH_4^+, TP	Actinobacteria	Coriobacteriaceae, *Bifidobacterium*	6
		Bacteroidetes	Saprospiraceae	1
		Euryarchaeota	*Methanobrevibacter*	1
		Firmicutes	*Enterococcus, Clostridium*	4
		Proteobacteria	*Rubrivivax*	1
		TM7	Blgi18	1
		Verrucomicrobia	*Akkermansia*	1
	SO_4^{2-}, Ca^{2+}, Cl^-, Na^+, EC	Acidobacteria	Ellin6075	1
		Actinobacteria	*Modestobacter, Cellulomonas*	19
		Armatimonadetes	Armatimonadaceae	1
		Bacteroidetes	*Spirosoma, Winogradskyella*	5
		Chloroflexi	A4b	1
		Firmicutes	*Planococcus*	1
		NKB19	TSBW08	2
		Planctomycetes	planctomycete	2
		Proteobacteria	Alteromonadaceae, Rhodobacteraceae, Erythrobacteraceae	31
		Thermi	*Truepera*	1
		WPS-2	ND	1
	F^-	Actinobacteria	Acidimicrobiales	1
		Bacteroidetes	*Hymenobacter*	1
		Cyanobacteria	*Leptolyngbya*	3
		Planctomycetes	Pirellulaceae	4
		Proteobacteria	Methylobacteriaceae	8

Regarding correlation of aquatic microbial communities with the trace elements, majority of the positive associations was found between microbial taxa and U and/or V (119 taxa in total). Soluble

U and V, as well as total U, correlated mostly with genera from Proteobacteria, Actinobacteria and Bacteroidetes phyla (Table S2). Examples of U/V bioindicator taxa are Proteobacteria families Calulobacteraceae, Comamonadaceae, Erythrobacteraceae, Methylobacteraceae, Rhodobacteraceae, Sphingomoadaceae and Xanthomonadaceae. Further, soluble Cs showed positive association with 15 different taxa, most of them from the phylum Proteobacteria, such as family Rhodospirillaceae. Finally, a total of six taxa belonging to four different phyla (Bacteroidetes, unnamed phylum OP3, Proteobacteria and Verrucomicrobia) showed correlation with soluble Mn and As, while only three taxa from Proteobacteria were associated with total Mn and As. An example of bacteral taxon positively associated with both soluble and total Mn/As is genus *Simplicispira* from the family Comamonadaceae.

Finally, the values for the physico-chemical parameters of TOC, pH and Ca, soluble elements Zn, Ba, Li, Cr, Sr, Cn, Sn, Ti and Cu and total elements Be, Al, Tl, Cr and Li, were positively correlated with the abundance of low number of microbial taxa (≤ 3).

Table S2. Positive correlation (r values ≥ 0.66) of trace elements (soluble and total) in the water and microbial taxa

Water parameters		Phylum	Examples of bioindicator taxa	No. of taxa
Soluble trace elements	U, V	Actinobacteria	*Strepomyces*	15
		Armatimonadetes	Armatimonadaceae	1
		Bacteroidetes	Saprospiraceae	4
		Chloroflexi	A4b	1
		Firmicutes	*Planococcus*	1
		NKB19	TSBW08	1
		Planctomycetes	planctomycete	1
		Proteobacteria	Calulobacteraceae, Comamonadaceae, Erythrobacteraceae, Methylobacteraceae, Rhodobacteraceae, Sphingomonadaceae, Xanthomonadaceae	35
		Thermi	Truepera	1
		WPS-2	ND	1
	Cs	Actinobacteria	*Rhodococcus*	2
		Bacteroidetes	Sphingobacteriales	1
		Chloroflexi	SHA-20	2
		Cyanobacteria	MLE1-12	1
		Proteobacteria	Rhodospirillaceae	6
		TM7	Rs-045	2
		Verrucomicrobia	*Candidatus Xiphinematobacter*	1
	Mn, As	Bacteroidetes	ND	1
		OP3	PBS-25	1
		Proteobacteria	*Simplicispira*	3
		Verrucomicrobia	Opitutaceae	1
Total trace elements	U	Actinobacteria	*Streptomyces*	14
		Armatimonadetes	Armatimonadaceae	1
		Bacteroidetes	Saprospiraceae	3
		Chloroflexi	SBR1031	1
		Firmicutes	*Planococcus*	1
		NKB19	TSBW08	1
		Planctomycetes	planctomycete	1
		Proteobacteria	Calulobacteraceae, Comamonadaceae, Erythrobacteraceae, Methylobacteraceae, Rhodobacteraceae, Sphingomonadaceae, Xanthomonadaceae	34
		Thermi	*Truepera*	1
		WPS-2	ND	1
	Mn, As	Proteobacteria	*Simplicispira*	3

Appendix 3

Effect of specific microbial taxa present in the water on the *S. parasitica* load analysed with the PLS-R modelling

Table S1. Positive correlation of *Saprolegnia parasitica* load and microbial taxa

Phylum	Class	Order	Family	Genus	Positive correlation (r)
Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	*Zhouia*	0,52
Acidobacteria	iii1-8	DS-18	ND	*ND*	0,55
Crenarchaeota	Thaumarchaeota	Cenarchaeales	Cenarchaeaceae	*Nitrosopumilus*	0,58
Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	*ND*	0,63
Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	*Nitrospira*	0,64
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Perlucidibaca*	0,66