Doctoral Thesis

Host-parasite-herbicide interactions in lake phytoplankton
an eco-evolutionary approach

Author(s):
Wyngaert, Silke van den

Publication Date:
2012

Permanent Link:
https://doi.org/10.3929/ethz-a-007612714

Rights / License:
In Copyright - Non-Commercial Use Permitted

This page was generated automatically upon download from the ETH Zurich Research Collection. For more information please consult the Terms of use.
Host - Parasite - Herbicide Interactions in Lake Phytoplankton: 
an Eco-Evolutionary Approach

A dissertation submitted to
ETH ZURICH
for the degree of
Doctor of Sciences

presented by
Silke Van den Wyngaert
Master of Science in Biology, University of Zürich
born 23.11.1981
from Belgium

accepted on the recommendation of
PD Dr. Piet Spaak
Dr. Bas W. Ibelings
Prof. Bruce McDonald
Prof. Luc De Meester

2012
# Table of content

Summary ............................................................................................................................................... 5
Résumé .................................................................................................................................................. 7
General introduction ............................................................................................................................ 11
Chapter 1 .............................................................................................................................................. 23
   *Herbicides in the environment alter infection dynamics in a microbial host-parasite system* ........ 23
Chapter 2 .............................................................................................................................................. 47
   *Parasite fitness traits under environmental variation: the case of a diatom infecting chytrid fungus* ................................................................................................................................. 47
Chapter 3 .............................................................................................................................................. 71
   *Hidden diversity in the freshwater planktonic diatom Asterionella formosa.* ............................... 71
Summary discussion and outlook ......................................................................................................... 105
Curriculum vitae .................................................................................................................................. 115
Acknowledgements .............................................................................................................................. 119
Summary

Infectious diseases are a natural process that reflects a long evolutionary history between host and parasites. Therefore a sustainable balance between host and parasites in nature is presumably an indication for a “normal” functioning and “healthy” ecosystem. There is increasing evidence that anthropogenic induced stresses may disrupt this natural balance thereby increasing the impact of disease on host populations. Host genetic diversity can play an important role in buffering populations against environmental stresses and disease spread.

In my doctoral research, I explored how an anthropogenic pollutant (herbicide) affects the strength and direction of host-parasite interactions. I used a host-parasite system, consisting of the diatom *Asterionella formosa* Hassall and its obligatory host-specific chytrid parasite *Zygorhizidium planktonicum*. I also characterized the genetic diversity and population structure of the host *A. formosa* on different spatial scales.

In chapter 1 I assessed the independent and combined effects of the widely used herbicide diuron and the chytrid parasite on the fitness of genetically different monoclonal *A. formosa* populations. Furthermore, I evaluated how herbicide exposure influenced infection dynamics and the impact of infectious disease on host populations. This study showed that phenotypic traits (host cell volume) can play a significant role in response to pollution and parasite spread and may obscure clear genotypic differences. They should be considered when studying strain sensitivity to toxicants or host-parasite genotype interactions. It also showed that herbicides modify the spread of disease in *A. formosa*. Whereas herbicide exposure initially posed a constraint on parasite transmission it enhanced the spread of disease over time. This resulted at first in an antagonistic interaction between herbicide and parasite on exponential host growth rate. It ended however in more severe epidemics.

In chapter 2 I conducted experiments and developed an individual based model (ABM) to unravel mechanisms that caused the opposing patterns of parasite transmission. This study revealed that herbicide exposure negatively interfered with parasite transmission, however, only at low host densities. At high host densities, herbicide exposure enhanced transmission. With an ABM model we simulated this interference of herbicide exposure by removing chemotaxis mechanisms. The model produced patterns similar to those observed in our experiment. Hence, it supported the hypothesis that chemotaxis is an important process in this host-parasite system but also that the
importance of chemotaxis in locating hosts is host density dependent. However, it could not completely reproduce the significant higher infection success in the presence of the herbicide at high host density. Thus in chapter 2 the infection dynamic observed in chapter 1 could be reproduced solely through the interaction effect between herbicide exposure and host density on parasite transmission success. Interference of the herbicide with host finding mechanisms explained the initial parasite transmission inhibition. Why parasite transmission success is enhanced by herbicide exposure at high host density remains unresolved at present.

The studies in both chapter 1 and 2 indicate that herbicide exposure will only increase the spread of disease when sufficient high host densities are present to outweigh the negative effects of herbicide exposure on host finding. However, the low host density conditions in our experiments were already very high and comparable with peak bloom densities of A. formosa in the field. The lack of evidence that herbicide stress increases host susceptibility in A. formosa together with its negative effect on chytrid host finding mechanisms (transmission efficiency) imply that development of chytrid epidemics will require higher host densities in the presence of the herbicide diuron.

In chapter 3 I studied the genetic diversity and population structure of the host A. formosa on different spatial scales. An unexpected pattern, revealed much higher genetic differentiation within lakes than among them. Two genetically distinct subpopulations were present in all the studied Swiss lakes. This study indicates the potential presence of cryptic species within the cosmopolitan freshwater diatom A. formosa. It also creates interesting opportunities for further research on coevolutionary dynamics between A. formosa and its chytrid parasites. An intriguing hypothesis to test is whether chytrid parasites are a driving force of A. formosa population subdivision which could give potential for Red Queen coevolutionary dynamics in this host-parasite system.
Résumé

Les maladies infectieuses sont un processus naturel qui révèle une longue histoire évolutionnaire entre hôtes et parasites. De ce fait, un équilibre durable entre hôtes et parasites dans la nature doit être l’indicateur d’un écosystème "en bonne santé" qui fonctionne "normalement". Il est de plus en plus évident que les divers stress anthropiques peuvent créer un déséquilibre et ainsi accroître l’impact d’une épidémie sur les populations d’hôtes. La diversité génétique au sein des hôtes peut aussi jouer un rôle tampon important contre les stress environnementaux et la propagation des infections.

Au cours de mes recherches doctorales, j’ai cherché à savoir de quelle façon un polluant anthropique (un herbicide) affectait la force et la direction des interactions entre hôtes et parasites. Pour cela, j’ai utilisé un système hôte-parasite composé de la diatomée Asterionella formosa et de son parasite obligatoire spécifique, le chytride Zygorhizidium planctonicum. J’ai également caractérisé la diversité génétique et la structure de la population de l’hôte A. formosa à différentes échelles spatiales.

Dans le deuxième chapitre j’ai conduit des expériences et développé une simulation multi-agents pour révéler les mécanismes sous-jacents aux observations contradictoires quant à la propagation des parasites. Cette étude a révélé que l'exposition à l'herbicide interfère négativement avec la propagation du parasite, mais ceci uniquement quand les densités d'hôte sont faibles. Pour de grande densité d'hôte, l'exposition à l'herbicide augmente la propagation. Grâce au modèle multi-agents, nous avons pu simuler, en supprimant le mécanisme de chimiotaxie, cette interférence due à l'exposition à l'herbicide. La simulation a reproduit les motifs observés dans nos expériences. Cela soutient donc l'hypothèse que la chimiotaxie joue un rôle important dans les systèmes hôte-parasite mais aussi que l'importance de la chimiotaxie pour la localisation d'un hôte est dépendante de la densité de l'hôte. Malgré cela, le modèle ne reproduit pas complètement le motif dans le cas où l'herbicide est présent et la densité de l'hôte élevée et pour lequel on a un plus grand succès de transmission dans les expériences. Donc dans le chapitre 2, la dynamique de l'infection observée dans le chapitre 1 pourrait être reproduite uniquement par l'effet d'interaction entre l'exposition aux herbicides et la densité des hôtes sur le succès de la transmission du parasite. L'interférence de l'herbicide sur les mécanismes de localisation des hôtes explique l'inhibition de la transmission initiale des parasites. Pourquoi, pour des densités élevées d'hôte, le succès de la transmission des parasites est augmenté par l'exposition à l'herbicide reste inconnu.

Les études des deux premiers chapitres indiquent que l'exposition à l'herbicide augmentera seulement la propagation de la maladie quand la densité d'hôte est suffisamment haute pour contrebalancer l'effet négatif de l'exposition à l'herbicide sur les mécanismes de localisation des hôtes. Néanmoins, dans nos expériences, les conditions de faible densités seraient déjà de très fortes densités dans la nature, comparables aux densités présentes lors de piques de croissance. L'absence d'évidence que le stress par l'herbicide augmente la susceptibilité de l'hôte chez _A. formosa_ combiné avec ses effets négatifs sur les mécanismes de localisation chez le chytride (efficacité de transmission) implique que le développement des épidémies de chytride exige un seuil de densité d'hôte plus élevé en présence de l'herbicide Diuron.

Dans le troisième chapitre, j’ai étudié la diversité génétique et la structure des populations chez l'hôte _A. formosa_ sur différentes échelles spatiales. Un motif surprenant a révélé une plus grande différentiation génétique au sein d'un même lac qu'entre des lacs différents. Deux sous-populations génétiquement distinctes étaient présentes dans tous les lacs suisses étudiés. Cette
étude soulève la question de savoir si des espèces cryptiques existent pour *A. formosa*. Cela ouvre également des voies de recherche intéressantes sur les dynamiques co-évolutionnaires entre *A. formosa* et son chytride parasite. Une intéressante hypothèse à tester serait de savoir si les chytrides parasites sont une force motrice pour la subdivision des populations chez *A. formosa* donnant ainsi l'opportunité d'étudier une dynamique type Reine Rouge sur le terrain.
General introduction

Why are parasites important?
Parasitism is recognized as the most common life style on earth (Lafferty et al. 2006) and has evolved in virtually all groups of species (Schmid-Hemperl 2011). Considering the high abundance and diversity of parasites we can fairly say that every organism is affected by parasites in one way or another (Windsor 1998). Parasites are a heterogeneous group of organisms with a huge variety in life history and host exploitation strategies. They are mostly classified according to their life cycle (simple versus complex) and type of interactions with their host(s), which may vary from specialist versus generalist, obligate versus facultative or low versus high virulence (in extreme cases killing the host). Since the first discovery of microscopic parasitic protozoa by Antonie van Leeuwenhoek (17th century), much research has been (and still is) devoted to understanding the complex interactions between hosts and parasites. Moreover, the study of parasites is not the sole domain of the parasitologist and immunologist anymore. Ecologists, conservationists and evolutionary biologists are increasingly aware of the important role that parasites play in host ecology and evolution (Altizer et al. 2003; Decaestecker et al. 2005; Ebert et al. 2000; Hamilton 1980; Jaenike 1978). It has been demonstrated that parasites have the potential to regulate the density of their host populations and alter their genetic structure and diversity (Decaestecker et al. 2007; Duncan and Little 2007; Jokela et al. 2009; Jokela et al. 2003). Antagonistic coevolution, caused by the reciprocal selection pressures that host and parasite exert on each other, is believed to be an important underlying mechanism for shaping host and parasite population dynamics (Thompson and Nuismer 2000). It may result in so called “Red Queen dynamics”, i.e. oscillation of host and parasite genotype frequencies through frequency dependent selection where parasites track the most common host genotype giving rare genotypes an advantage (Bell 1982). Such coevolutionary arms races are predicted to maintain sex and genetic variation within populations (Jaenike 1978). A prerequisite for coevolution is that both host susceptibility and parasite infectivity have a genetic basis, which is commonly found in host parasite systems (Carius et al. 2001; Jokela et al. 2009; Lambrechts et al. 2005).

Such population level effects can mediate changes up to the community level through alteration of interspecific competition or through changes in host behaviour (Mouritsen and Poulin 2010; Wood et al. 2007). Finally, one can expect that these effects of parasites on community structure may be
propagated through the whole ecosystem by changing food web interactions, thereby influencing energy flow (Hudson et al. 2006).

**Emerging disease in freshwater ecosystems**

Freshwater ecosystems are amongst the most degraded and altered ecosystems on earth and in many cases humans are the cause of it (Davis et al 2010). Yet freshwater systems are also among the most biodiverse ecosystems (e.g. Dudgeon et al. 2007). Traditionally humans preferred to settle near freshwater bodies, using it for drinking water, irrigation, transporting people and goods etc. Increasing population growth led to increasing urbanisation, industrialisation and agricultural land use, which became major drivers of climate change and freshwater pollution (Moss et al. 2009). Especially pesticides and other chemical contaminants are a pervasive problem in freshwaters because rivers and lakes act as conduits or sinks that transport, accumulate and concentrate chemical pollutants (Relyea 2009).

Infectious diseases are a natural process that reflects a long evolutionary history between host and parasites. Therefore a sustainable balance between host and parasites in nature is presumably an indication for a “normal” functioning and “healthy” ecosystem (Hudson et al. 2006). There is increasing evidence that climate change and other anthropogenic induced stresses, including pollution may disrupt this natural balance thereby increasing the impact of disease on host populations (Daszak et al. 2001; Garrett et al. 2006; Johnson and Paull; Patz et al. 2000). A possible explanation for this may be that host organisms exposed to environmental stress lack the energy for mounting an efficient defence against parasite attack and therefore become more susceptible to infection (Holmes 1996; Marcogliese and Pietrock 2011). Several studies on vertebrate host parasite model systems provide evidence for this line of reasoning: e.g. an amphibian-trematode system (Koprivnikar et al. 2007; Rohr et al. 2008a; Rohr et al. 2008b), or fish-pathogen systems (Kelly et al. 2010; Kreutz et al. 2010). Studies on the Eastern Oyster, an aquatic invertebrate, reported increased vulnerability to a protozoan pathogen when hosts were pre-exposed to pollutants (Chu and Hale 1994; Fisher et al. 1999). Similarly, negative effects of pesticide exposure on disease resistance in the invertebrate water flea *Daphnia magna* were found (Coors and De Meester 2008; Coors et al. 2008). Also for higher plant parasite systems there is evidence that pesticides can lower plant defence causing increased disease severity (Altman and Campbell 1977; Johal and Huber 2009).
However, there is a risk of oversimplification since interactions between stress and disease are likely to be much more complex (Lafferty and Holt 2003). Host susceptibility/parasite infectivity measured at the individual level represents only one aspect of host/parasite fitness. The spread of disease, however, occurs at the population level. A myriad of interactions between different fitness traits under a large variety of environmental stressors impede clear-cut predictions about the final outcome of disease in nature. For example, anthropogenic stressors may reduce host population density with negative effects on parasite transmission. Such a reduction in parasite transmission may outweigh increased host susceptibility with the net effect of a decrease in disease spread. Ecosystem stressors can also pose a direct negative constraint on parasites. Especially the exposure of free living parasite stages to pollutants can severely reduce their survival and/or infectivity (Pietrock and Marcogliese 2003). A stressed host may also provide a poor environment for parasite reproduction (Bittner et al. 2002; Seppälä et al. 2008). Therefore, elucidating the effects of anthropogenic factors on both hosts and parasites and at the population level is needed to understand the mechanisms underlying changes in disease dynamics.

Host – Parasite – Herbicide model system

The host: *Asterionella formosa*

The host *Asterionella formosa* is a freshwater diatom, easily recognized by its star shaped colonies. It is a characteristic phytoplankton species of spring blooms in temperate lakes where it is often the dominant phytoplankton species (Lund et al. 1963; Maberly et al. 1994). Described as having a cosmopolitan distribution, *A. formosa* occurs in lakes of different trophic status and with different physical and chemical characteristics. Reproduction in this species is predominantly asexual and characterized by a “shrinking division” mode, which is specific for diatoms. This process leads to a progressive reduction in cell size with every new asexual generation. In diatoms the original cell size is re-constituted by occasional sexual reproduction. So far, however, no sexual reproduction has been observed for *A. formosa*. However, rare sexual events are assumed since these may explain the high genetic diversity in a Dutch lake population (De Bruin et al. 2008) and regular size rejuvenation in the Lake Zurich population (Nipkow 1927).
The parasite: *Zygorhizidium planktonicum*

The parasite *Zygorhizidium planktonicum* is an aquatic fungus belonging to the Chytridiomycetes (James et al. 2006). As all Chytridiomycota, *Zygorhizidium* is characterized by uniflagellated motile zoospores that actively search for host cells using chemotaxis. It is an obligate, host-specific parasite of *A. formosa* (Van Donk and Ringelberg 1983b) and *Synedra acus* (Canter 1953). Hence, the parasite cannot survive and/or reproduce in absence of its host. *Z. planktonicum* is an extremely virulent parasite i.e. every infection inhibits host reproduction and leads to host cell death.

**Parasite life cycle:**

The life cycle of this chytrid parasite begins with the attachment of a motile zoospore to the surface of a host cell. After zoospore encystment a germ tube is formed which enters the host cell through the girdle zone. Via the germ tube nutrients are extracted from the host cell and used for the development of the sporangium. New zoospores are formed either asexually or sexually and are released from the sporangium by dehiscence (Doggett and Porter 1996; Ibelings et al. 2004)(Fig. 1).
Fig. 1 Life cycle of *Zygorhizidium planktonicum* (Doggett and Porter 1996). The asexual infection phase (A–D) is characterized by uninucleate haploid thalli (A) that differentiate into operculate zoosporangia (B). Upon release (C), the uniflagellate zoospores (D) give rise to either asexual thalli or incipient gametangia (E). Sexual reproduction is evidenced by heterothallic gametangia (E–G), which fuse by means of a single epibiotic conjugation tube. Plasmogamy immediately follows wall fusion, and the donor protoplast migrates into the recipient gametangium, which in turn becomes the thick-walled resting spore (G). After wall deposition, the nuclei fuse and the zygotic nucleus remains undifferentiated throughout spore dormancy (H). Meiosis occurs within the newly formed meiosporangium after resting spore germination (I). The haploid zoospores are released by an operculate meiosporangium (J).

Both parasite life stages, zoospores and sporangia, can be strongly influenced by environmental conditions. The free living transmission stages depend entirely on their internal energy storage giving them a limited time (in the order of days) to find a suitable host (Holfeld 2000b). During their dispersal they are exposed to predation (Kagami et al. 2007) and water turbulence can interfere with their swimming behaviour (Kuhn and Hofmann 1999). Severe light limitation decreases zoospore infectivity and dark conditions even completely inhibit infections. This observation has led to the hypothesis that zoospores depend on photosynthesis exudates of the host to recognize and localize host cells (Bruning 1991c; Canter and Jaworski 1981b). Once attached, the zoosporangia completely depend on the host cell for their growth until maturation. During this stage sporangia experience both direct and host mediated environmental influences. A light limited as well as phosphorous limited host environment negatively affects zoospore
production (Bruning 1991a,b,c). Increasing temperatures have also been shown to decrease sporangia maturation time, zoospore production and zoospore infective lifetime leading to shorter generation times and lower per capita potential fecundity at warmer conditions (Bruning 1991b). Moreover, at temperatures below 3 C the parasite is largely inactive and forms resting spores (Van Donk and Ringelberg 1983).

**The herbicide:** Diuron

Pesticides are ubiquitous contaminants in aquatic ecosystems. In agricultural areas, pesticides enter stream- and lake ecosystems via surface runoff or drainage flows from fields, and as a result of inappropriate handling or disposal of spray mixtures (Wittmer et al. 2010). Herbicides, in particular, account for more than 35% of the pesticide volume used in Europe and are therefore frequently found in the aquatic environment (Cedergreen and Streibig 2005). Because of their plant like biochemical and physiological properties, micro-algae represent important non-target organisms for herbicides. Studies from single species toxicity tests have reported a wide variation in algal sensitivity to different kinds of herbicides. Algal community structure may change in response to herbicide pollution (Schmitt-Jansen and Altenburger 2005). There is also evidence for genotypic variation for sensitivity to toxicants within species (Behra et al. 1999). However, until now there are no studies that evaluate the interaction between herbicides and natural stressors such as parasitism in phytoplankton.

Diuron, DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), is a herbicide that inhibits photosynthesis by binding to the D-1 protein of the Photosystem 2 reaction center, thereby blocking photosynthetic electron transport (Duke 1990). It is one of the most frequently and permanently observed agrochemicals in surface waters (Blanchoud et al. 2004; Chevre et al. 2006) and is highly persistent in the environment (Giacomazzi and Cochet 2004). Diuron is ranked as one of the most toxic phenylureas for algae (Backhaus et al. 2004).

**Genetic diversity**

Genetic diversity forms the basis for adaptation of species to a changing world, whether these changes are due to natural factors like coevolving parasites or human caused environmental pressures. There is increasing evidence that populations become more susceptible to disease as their genetic diversity decreases. At the same time parasites can be important drivers behind species and genetic diversity in natural populations (Altizer et al. 2003, Spielman et al. 2004).
Mechanisms that influence genetic variation and changes in allele frequencies within populations are:

1. Mutation: the ultimate source of novel genetic information caused by changes in the DNA sequence such as point mutations, translocations, deletions, insertions or gene duplications.
2. Recombination: re-arranges existing genes into new combinations
3. Gene flow: the exchange of genes between populations caused by the movement of individuals between separate populations. Gene flow can introduce new alleles in a population.
4. Genetic drift: the change in allele frequencies due to random sampling. Drift can lead to chance loss of genes and genotypes.
5. Inbreeding: mating between close relatives or in extreme cases between genetically identical individuals (selfing). Inbreeding promotes homozygosity and decreases genetic diversity within populations.
6. Natural selection: biotic and abiotic environmental factors that cause the differential survival and reproduction of genotypes within a population

Many diatom species and other protists commonly reproduce asexually, alternated with sexual events. The frequency of sexual reproduction remains unknown for many protists (Weisse 2008). It is, however, one of the key factors that influences the genetic structure of organisms that reproduce both asexual and sexual (De Meester et al. 2006). Due to their small sizes and large population sizes, preventing local extinction, they are believed to be ubiquitous dispersed (Finlay et al. 2002). However, see (Casteleyn et al. 2010; Evans et al. 2009; Medlin 2007; Vanormelingen et al. 2008) for opinions that contradict this view. If the population structure is largely clonal, effective population size may be low (Weisse 2008). Population genetic studies on freshwater phytoplankton species have lagged behind those of other organisms. Knowledge on the extent of their genetic diversity and structure will give insight on the evolvability of natural phytoplankton populations.

Aims of the thesis
In this thesis I used a diatom host - chytrid parasite system to explore how an anthropogenic pollutant (herbicide) affects the strength and direction of host-parasite interactions. Another objective was the characterization of genetic diversity and spatial populations structure of the planktonic diatom host Asterionella formosa.
Outline of the thesis

In nature, organisms experience simultaneous biotic and abiotic stressors. Parasites are ubiquitous and they represent an important natural stress factor that influences host populations. There is increasing evidence from a variety of host-parasite systems that parasitism acting in concert with other stressors has more detrimental effects on host organisms than either single stressor on its own. In chapter 1 I studied how parasite stress interacts with an anthropogenic stressor (herbicide). A full factorial experiment was conducted to assess the independent and combined effects of the widely used herbicide diuron and a chytrid parasite on the fitness of genetically different monoclonal diatom populations. Furthermore, I evaluated how herbicide exposure influenced infection dynamics, parasite fitness and the impact of infectious disease on host populations. Whereas herbicide exposure initially posed a constraint on disease transmission it enhanced the spread of disease over time. Consequently this shifted the nature of the parasite-toxicant stressor interaction from antagonistic (on exponential host growth) towards additive (on final host population density). Further this study shows that phenotypic traits (host cell volume) can play a significant role in response to pollution and parasite spread and may obscure clear genotypic differences. This chapter highlights that anthropogenic stress can modify infection dynamics and impact of disease on host populations through the complex interplay between the stressor, host and parasite growth dynamics and host population phenotype. The underlying mechanisms are, however, difficult to pinpoint with this experimental epidemiological setup. Changes in host density and/or physiology occur as the experiment progresses. Furthermore, parasite population growth (e.g. spread of disease) is an integrative measure of parasite fitness, including several parasite fitness traits such as infectivity (transmission efficiency), development time, per capita reproductive output and survival. Changes in the surrounding and immediate host environment may affect traits differently and affect spread of disease. Therefore in chapter 2 I experimentally disentangled the main and interactive effects of immediate host and external environmental variation on single parasite fitness traits. The results of this study suggest that successful parasite transmission is mainly affected by the external environment (herbicide exposure and host density) while parasite reproduction was mainly driven by the size (quantity of resources) of the host environment. Herbicide exposure interfered with host finding mechanisms at lower host density. This study highlights that for parasites with free living motile stages an efficient host finding mechanism is a relevant fitness trait involved in successful transmission. In many infection experiments this is not accounted for when high host densities are used and this may give unrealistic estimates of parasite transmission potential. In chapter 3 I studied the genetic
diversity and population structure of the host Asterionella formosa in different lakes and on different spatial scales with microsatellite markers. An intriguing pattern revealed higher genetic differentiation within lakes than among them and indicated the potential presence of cryptic species within A. formosa.

References


Holmes JC (1996) Parasites as threats to biodiversity in shrinking ecosystems. Biodivers Conserv 5:975-983


Jaenike J (1978) An hypothesis to account for the maintenance of sex within populations. Evol Theor 3:191-194

James TY et al. (2006) A molecular phylogeny of the flagellated fungi (Chytridiomycota) and description of a new phylum (Blastocladiomycota). Mycologia 98:860-871


Kreutz LC, Barcellos LJG, Marteninghe A, dos Santos ED, Zanatta R (2010) Exposure to sublethal concentration of glyphosate or atrazine-based herbicides alters the phagocytic function and increases the susceptibility of silver catfish fingerlings (Rhamdia quelen) to Aeromonas hydrophila challenge. Fish Shellfish Immun 29:694-697


Nipkow HF (1927) Ueber das Verhalten der Skelette planktischer Kieselalgen im geschichteten Tiefenschlamm des Zürich- und Baldeggersees (Dissertation). In. ETH Zürich
Rohr JR et al. (2008b) Agrochemicals increase trematode infections in a declining amphibian species. Nature 455:1235-U1250
Wittmer IK et al. (2010) Significance of urban and agricultural land use for biocide and pesticide dynamics in surface waters. Water Res 44:2850-2862
Chapter 1

Herbicides in the environment alter infection dynamics in a microbial host-parasite system

Silke Van den Wyngaert, Alena Gsell, Piet Spaak and Bas W. Ibelings

(accepted in Environmental Microbiology)

Summary

Parasites play an important role in the regulation of host population growth. How these ubiquitous stressors interact with anthropogenic stressors is less often studied. In a full factorial experiment we explored the independent and combined effects of the widely used herbicide diuron and a chytrid parasite on the fitness of genetically different monoclonal diatom populations. Furthermore we evaluated how herbicide exposure influenced infection dynamics, parasite fitness and the impact of infectious disease on host populations. We found no evidence of host genetic variation for diuron sensitivity and parasite resistance. Instead, host population phenotype was a decisive factor in controlling parasite growth. Although herbicide exposure initially posed a constraint on disease transmission, it enhanced the spread of disease over time. Consequently the nature of the parasite-toxicant stressor interaction shifted from antagonistic (on exponential host growth) towards additive (on final uninfected host density). We conclude that herbicide exposure can modify infection dynamics and impact of disease on host populations through the complex interplay between host and parasite growth dynamics and host population phenotype.
Introduction

In nature, organisms are exposed simultaneously to biotic and abiotic stressors. Depending on the stressor combination, joint effects can be additive, synergistic or antagonistic. Although an increasing number of studies consider the impact and interaction of multiple stressors, it remains difficult to make general predictions about the outcome of their combined effects in nature. This lack of knowledge has become a major concern in ecological risk assessment (Crain et al. 2008; Holmstrup et al. 2010).

Parasitism represents an important biotic stress factor that influences host populations in the field. Considering the high abundance and diversity of parasites we can fairly say that every species is affected by parasites (Windsor 1998). They can play a significant role in shaping population dynamics by altering the genetic structure and diversity of their host populations (Decaestecker et al. 2007; Duncan and Little 2007; Jokela et al. 2003). Effects of parasitism and anthropogenic environmental stressors such as chemical pollutants interact in intricate ways. Host organisms exposed to chemical stressors may lack the energy for mounting an efficient defence against parasite attack and therefore become more susceptible to infection (Holmes 1996). Studies on vertebrate host parasite model systems provide evidence for this line of reasoning: e.g. an amphibian-trematode system (Koprivnikar et al. 2007; Rohr et al. 2008a; Rohr et al. 2008b) or fish-pathogen systems (Kreutz et al. 2010). Studies on the Eastern Oyster, an aquatic invertebrate, also reported increased vulnerability to a protozoan pathogen when hosts were pre-exposed to pollutants (Chu and Hale 1994; Fisher et al. 1999). Similarly, negative effects of pesticide exposure on disease resistance in the invertebrate water flea Daphnia magna were found (Coors and De Meester 2008; Coors et al. 2008). Also for higher plant parasite systems there is evidence that pesticides can lower plant defence causing increased disease severity (Johal and Huber 2009). However, interactions between stress and disease are much more complex (Lafferty and Holt 2003). Ecosystem stressors may reduce host density and/or host quality and therefore have negative effects on parasite transmission and/or parasite reproduction (Bittner et al. 2002; Seppälä et al. 2008). Furthermore, host genotypes may harbour large genetic variation for traits involved in infection and pollution resistance. Whether these traits are positively or negatively correlated will affect selection processes and consequently the impact of multiple stressors on host population dynamics.
Phytoplankton, i.e. cyanobacteria and eukaryotic microalgae, as primary producers provide many important ecosystem services, form the basis of entire aquatic food webs and play a key role in the global carbon cycle (e.g. Falkowski et al. (2000)). Therefore, understanding phytoplankton responses to combined natural and anthropogenic stressors is important because of knock-on effects at higher trophic levels and whole ecosystem functioning. Although still largely understudied, many phytoplankton species have obligate, host specific parasites that have the potential to alter phytoplankton population dynamics and succession (Canter 1950; Canter and Lund 1948; Ibelings et al. 2011). In addition, phytoplankton species are relevant non-target organisms for a multitude of pesticides, especially for herbicides, which are applied in agricultural weed-control and reach stream- and lake ecosystems via run-off from the land (Cedergreen and Streibig 2005). Peak concentrations of herbicides are commonly found in spring after field application and rain events (Rabiet et al. 2010). This is also the time where the phytoplankton spring bloom develops and chytrid epidemics occur (Ibelings et al. 2004). We therefore believe that this stressor interaction is relevant in nature and may affect phytoplankton population dynamics and disease in the field. However, to our knowledge, no study so far has explored the interaction between chemical pollution and disease in phytoplankton.

In this study we address three main questions using a well studied microbial host-parasite system, consisting of the diatom Asterionella formosa Hassall host, and the chytrid fungus Zygorhizidium planctonicum Canter as parasite, exposed to the herbicide diuron: 1) Do host genotypes show variation for parasite resistance and herbicide resistance traits? If so, are these two traits correlated? 2) How does herbicide exposure interact with parasite stress: is the nature of the interaction additive, synergistic or antagonistic? 3) Does herbicide exposure alter infection dynamics and impact of parasitism on the algal host population? We demonstrate experimentally that environmental pollution has the potential to alter infection dynamics and the severity of disease in this diatom-chytrid host-parasite system.

Results

Single and combined effects of diuron and parasite on host fitness

For average host population growth rate the AIC-selected model discarded all random interaction terms but included the interaction terms between the fixed factors diuron x parasite and between both fixed factors and the covariate host population cell volume. Variation explained by the random factor host genotype compared to the residual variance was very low (almost 0%). The
interaction between mean host population cell volume and stressors was significant for the parasite treatment and marginally significant for the diuron treatment (Table 1). The estimates of the interaction terms were low, however, and the interaction was mainly driven by the host population with the smallest mean cell volume. The combined stress treatment decreased host fitness compared to the control treatment but the decrease was less (with the exception of Strain 26) than expected by the reference model of independent action (IA) (Fig. 1a). This indicates an antagonistic interaction which was substantiated by a significant two way interaction between diuron and parasite (Table 1).

Table 1: Mixed-effects model to test for the effects of the single and combined effects of diuron and parasite on maximum specific host population growth rate

<table>
<thead>
<tr>
<th></th>
<th>Estimate</th>
<th>Std. Error</th>
<th>t value</th>
<th>pMCMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>0.4204</td>
<td>0.0593</td>
<td>7.093</td>
<td>0.0001</td>
</tr>
<tr>
<td>diuron (D)</td>
<td>-0.2062</td>
<td>0.0569</td>
<td>-3.621</td>
<td>0.0014**</td>
</tr>
<tr>
<td>parasite (P)</td>
<td>0.0524</td>
<td>0.0621</td>
<td>0.843</td>
<td>0.426</td>
</tr>
<tr>
<td>cell volume (CV)</td>
<td>0.0003</td>
<td>0.0002</td>
<td>1.309</td>
<td>0.2686</td>
</tr>
<tr>
<td>D×P</td>
<td>0.0645</td>
<td>0.0173</td>
<td>3.731</td>
<td>0.0008***</td>
</tr>
<tr>
<td>D×CV</td>
<td>0.0005</td>
<td>0.0002</td>
<td>2.01</td>
<td>0.06</td>
</tr>
<tr>
<td>P×CV</td>
<td>-0.0006</td>
<td>0.0003</td>
<td>-2.414</td>
<td>0.0246*</td>
</tr>
</tbody>
</table>

For final uninfected host density, the AIC-selected model also discarded all random interaction terms but included the three way interaction term between the fixed factors diuron and parasite and the covariate mean host cell volume (Table 2). The significant three way interaction indicates that the interaction between diuron and parasite depends on host population cell size (Table 2). The interaction between diuron and parasite was therefore analyzed separately for each of the five host populations (corresponding to each level of the covariate host population cell volume). The results of this analysis show that the combined stress treatment also decreased the final uninfected host density in each population separately. However, for the host population with the smallest cell size (strain 37) this decrease was less than expected by IA (antagonistic interaction; F = 11.83, P = 0.0063, Fig 1b). The interaction became additive for populations with larger cell size. This was indicated by non-significant interactions between diuron and parasite and by the expected IA value lying within the confidence interval of the observed uninfected host density (Fig.1b).
**Fig. 1:** Average host growth rate (a) and final uninfected host density (b) of the five *A. formosa* strains exposed to no stressors (squares), single stressors; parasite (triangles up), diuron (triangles down) and combined stressors (circles). Filled symbols denote observed responses (means and 95% confidence intervals) and empty symbols represent additive effects predicted by the model of independent action. The mean cell volume of each host strain is given in parentheses below each host strain name.

**Table 2:** Mixed-effects model to test for the effects of the single and combined effects of diuron and parasite on final uninfected host density

<table>
<thead>
<tr>
<th>Final uninfected host density:</th>
<th>Estimate</th>
<th>Std. Error</th>
<th>t value</th>
<th>pMCMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>461844</td>
<td>121025.5</td>
<td>3.816</td>
<td>0.0004</td>
</tr>
<tr>
<td>diuron (D)</td>
<td>-392947.7</td>
<td>104548.2</td>
<td>-3.759</td>
<td>0.0002***</td>
</tr>
<tr>
<td>parasite (P)</td>
<td>-36263.3</td>
<td>87471.3</td>
<td>-0.415</td>
<td>0.6589</td>
</tr>
<tr>
<td>cell volume (CV)</td>
<td>-897.2</td>
<td>495.8</td>
<td>-1.81</td>
<td>0.0087**</td>
</tr>
<tr>
<td>D×P</td>
<td>295654.3</td>
<td>123703</td>
<td>2.39</td>
<td>0.0130*</td>
</tr>
<tr>
<td>D×CV</td>
<td>1225.7</td>
<td>428.3</td>
<td>2.862</td>
<td>0.0032**</td>
</tr>
<tr>
<td>P×CV</td>
<td>-118.2</td>
<td>358.3</td>
<td>-0.33</td>
<td>0.7253</td>
</tr>
<tr>
<td>D×P×CV</td>
<td>-1066.9</td>
<td>506.8</td>
<td>-2.105</td>
<td>0.0278*</td>
</tr>
</tbody>
</table>
The host fitness reduction by the two stressors followed an opposite trend with host populations of smaller mean cell volume being more sensitive to diuron, but suffering less from parasite stress, and vice versa for populations with larger mean cell volume (Figs. 2a&b). This trade off pattern, mediated by host cell volume, was expressed strongest in the response variable percentage reduction in final uninfected host density (Fig. 2b).

![Graph](image)

**Fig. 2:** Relationship between host population cell volume (mean ± SE) and host fitness inhibition; a) % growth reduction, b) % reduction in final uninfected host density. The broken line shows linear regression analysis for the stressor diuron (black circles) and the unbroken line shows linear regression analysis for the stressor parasite (white circles)

**Parasite fitness**

Parasite population growth rate increased significantly in all host populations exposed to diuron (Table 2, Fig. 3) and was positively related to mean host population cell volume, independent of herbicide treatment (no diuron; linear regression, $r^2 = 0.245$, $P = 0.012$, $n = 25$, diuron; $r^2 = 0.276$, $P = 0.007$, $n = 25$, Fig. 3). Tukey HSD posthoc analysis revealed a significant difference between parasite growth rate on the host populations with the most extreme smallest and largest mean cell volume ($P = <0.011$).
Table 3: Mixed-effects model to test for the effect of diuron exposure on parasite population growth rate.

<table>
<thead>
<tr>
<th></th>
<th>Estimate</th>
<th>Std. Error</th>
<th>t value</th>
<th>pMCMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-0.8175</td>
<td>0.7452</td>
<td>-1.097</td>
<td>0.342</td>
</tr>
<tr>
<td>diuron (D)</td>
<td>0.5656</td>
<td>0.0969</td>
<td>5.835</td>
<td>0.001**</td>
</tr>
<tr>
<td>cell volume (CV)</td>
<td>0.0124</td>
<td>0.003</td>
<td>4.086</td>
<td>0.004**</td>
</tr>
</tbody>
</table>

Fig. 3: Relationship between parasite population growth rate (mean ± SE) and host population cell volume (mean ± SE). The broken line shows linear regression analysis for the parasite exposed treatment with diuron (black circles) and the unbroken line shows linear regression analysis for the parasite exposed treatment without diuron (white circles).

Impact of diuron on parasite infection dynamics

The infection dynamics differed significantly between the two treatments (time × treatment interaction; F = 10.49, P < 0.001). Since the three way interaction between the covariate (mean host population cell volume), time and treatment was not significant (F = 0.58, P = 0.67) the data from all host populations were pooled together in order to better visualize the infection dynamics of both treatments. Infection started immediately in the non diuron treatment whereas an infection lag phase occurred in the presence of diuron. After day 4 the slope of infection increase was steeper with, than without diuron (Fig. 4a). Multiple infections were rare so that the increase in number of infections was equivalent to the increase in number of sporangia. Therefore, parasite
fitness (based upon the exponential increase of number of sporangia) was also higher in the presence of diuron (see results above). Total host density in the parasite exposed treatment increased over time but remained overall lower in the presence of diuron (Fig. 4b). This resulted in a final infection prevalence that was almost twice as high in the diuron treatment as compared to the non diuron treatment (Fig. 4c).

Fig. 4: Comparison of a) the increase of infections (mean ± SE), b) host density (mean ± SE) and c) prevalence of infection (mean ± SE) between the parasite exposed treatments in the absence of diuron (white circles) and presence of diuron (black circles). Data pooled together for all five host populations.


Discussion

In this study we investigated how abiotic and biotic stressors interact, resulting in environment by environment (E x E) interactions. In particular we studied the interaction between a herbicide that inhibits algal photosynthesis and a virulent chytrid parasite in their effects on a common planktonic diatom. In addition we included a third interaction term “host genotype” (G) in order to identify whether genotypic heterogeneity in response to single and multiple stressors does occur (i.e. host G x E and G x E x E).

Host population phenotype and response to stress

Contrary to many other studies we found no evidence for genetic variation in resistance traits and host G x E interactions (Blanford et al. 2003; Mitchell et al. 2005). It was previously shown that different A. formosa genotypes differed in their susceptibility to two different Z. planktonicum strains and that parasite strains differed in their ability to infect particular host genotypes (De Bruin et al. 2004). However, from the 17 different host genotypes tested in that paper the majority (9 genotypes) were severely infected by both parasite strains. Therefore the number of host genotypes used in this experiment - which were genetically differentiated based on AFLP marker analysis - was presumably too low to detect differences in susceptibility to parasitism. The study of De Bruin et al. (2004), however, did not consider effects of host population phenotype. Our study shows that host population phenotype, i.e. mean cell volume, plays a role in the response to single and multiple stressors and is positively correlated with parasite growth. In diatoms, asexual reproduction results in a progressive reduction in cell size. Differences in cell size between genotypes are most likely explained by genotypes being at different stages of cell-size reduction. The first question of our study (i.e. do host genotypes show variation for parasite resistance and herbicide resistance traits?) can therefore not be answered since we are unable to disentangle phenotype from genotype.

The host population phenotype x environment interactions suggest that populations with smaller mean cell size are more sensitive to the herbicide diuron but suffer less from parasite stress, while the opposite is true for populations with larger mean cell size (Table 1, Fig. 2). We are aware that slope estimates for the interaction terms were low and the linear relationship between fitness reduction and cell volume was only significant for percent inhibition of final uninfected host density with diuron. Our experiment was initially not designed to investigate phenotypic responses and only the host population with smallest mean cell volume significantly differed from
all other host populations. Therefore we lack power to properly estimate the effect of host cell volume on stress response, but a trend was visible. Several studies found a negative relationship between cell volume and toxicant sensitivity in phytoplankton (Lockert et al. 2006; Tang et al. 1998). A similar relationship for invertebrate crustaceans was found by (Vesela and Vijverberg 2007) with smaller sized daphnids being more sensitive to heavy metal toxicity. In contrast, a positive relationship between body size and infection has been demonstrated in several other host-parasite systems with free-living parasite transmission stages: e.g. *Daphnia*-yeast system (Duffy et al. 2011; Hall et al. 2007) or a snail-trematode system (Seppälä et al. 2011). The proposed mechanisms that lead to this relationship are the higher feeding rate of larger hosts which subsequently lead to higher parasite clearance rates and the increased production of parasite transmission stages on larger hosts (Duffy et al. 2011). Since *A. formosa* is not a filter feeding organism, the higher feeding rate mechanism can obviously be excluded. However, increased parasite production on larger host strains would be a possible mechanism. The correlation between host population cell size and parasite population growth which we observed, could well be explained by increased parasite reproduction - i.e. increased zoospore production - on larger host cells as more nutrients can be extracted and exploited by the parasite. Moreover, a positive correlation between parasite sporangium size and host cell size has also been observed in another diatom-chytrid association (Holfeld 2000). This ecological dependence of parasitism on host phenotype likely rivals and/or interacts with genetic mechanisms that often get more attention in the literature (Hall et al. 2009).

**Interaction between stressors**

Our second objective was to identify the direction of the interaction (synergistic versus antagonistic) between herbicide and parasite exposure. There is increasing consensus that parasitism acting concertedly with other stressors has stronger detrimental effects on host organisms than either stressor acting alone presumably due to reduced host immune defences (Marcogliese et al. 2009).

Our results show that the direction of stressor interactions depends on the focal host fitness parameter (reflecting different stages of host population growth – see paragraph on infection dynamics) and on the host population phenotype. The reduction in host growth rate in the combined stress treatment was less than the sum of both single stressors and resulted in an antagonistic interaction between parasite and diuron. However, when the final uninfected host
cell density is considered, a three way interaction between mean population cell size, diuron and parasite indicates that the interaction between the two stressors is dependent on host population phenotype, ranging from antagonistic for the host population with smallest mean cell size to additive for populations with larger mean cell size. However, this effect was mainly driven by the smallest host population where the single effect of diuron was very strong and the negative effect of parasite remained weak.

Host defence strategies against parasites range from avoidance or structural resistance to immune responses associated with excretion of defence molecules (Rigby et al. 2002) or recognition of pathogen associated molecular patterns (Chisholm et al. 2006) leading to programmed cell death (Lam et al. 2001). For some clonal strains of A. formosa a hypersensitive death reaction to infection by Rhizophydi um planktonicum has been observed resulting in growth arrestment and finally death of the parasite (Canter and Jaworski 1979). However, the exact mechanisms that lead to successful inhibition of infection in the A. formosa - chytrid interaction are not known and we did not observe any signs of hypersensitive reaction during our experiment. Our results on the combined effect of parasite and diuron may be not so much linked to host immune defence but more to the complex interplay between host population phenotype and host and parasite population growth dynamics.

**Infection dynamics**

Infection dynamics differed in the presence or absence of the environmental stressor diuron (Fig. 4a). Diuron exposure resulted in higher parasite growth (Fig. 3) and a reduction in uninfected host density (Fig 1b). However, initially, the infection started off slower when the herbicide was present, which explains the antagonistic interaction between diuron and parasite on exponential host population growth rate. The reduction of host growth rate and thereby host cell density through diuron exposure could directly constrain parasite transmission efficiency. However, the initial host density in our experiment was relatively high compared to threshold host densities allowing epidemics in the field (Ibelings et al. 2011). Alternatively, low initial infection success may be explained by the fact that many parasites with free living stages use chemical cues to track their host (Gerardo et al. 2006; Kuhn 1997). Previous experiments have shown that zoospores are unable to infect A. formosa in darkness or at very low irradiance (Bruning 1991b; Canter and Jaworski 1981). The release of extracellular organic carbon from living algal cells increases with photosynthetic production and generally correlates with increased irradiance (Espeland and
Wetzel 2001). Therefore it is supposed that zoospores are attracted by specific extracellular products from *A. formosa* that are excreted during active photosynthesis (Bruning 1991b). The concentration of diuron used in this experiment provokes ca. 50% photosynthesis inhibition (see supporting information, Fig. S1A), so that diuron probably interfered with zoospore behaviour and negatively affected host finding mechanisms. Nevertheless infection in the diuron treatment caught up with infections in the controls, and from day four onwards parasite infections even increased more rapidly in presence than in absence of diuron. How to explain this? Despite the inhibiting effect of diuron on photosynthesis host cell density continuously increased during the experiment, also in presence of the herbicide. Higher host densities may have increased chance contacts between host cells and zoospores so that the herbicide imposed constraints on chemotaxis are less relevant for infections in the late stages of the experiment, when host cell density had sufficiently increased.

Although parasite transmission was initially negatively affected by diuron, the overall parasite growth rate increased in the presence of the herbicide. As already mentioned above, the effect of increasing host density may have annulled the possible transmission constraints imposed by lower initial host density. However, this does not explain the steeper infection increase with diuron after the second parasite generation. The effect of diuron on parasite growth can be direct or indirect. For example, some studies have shown a direct growth stimulating effect of herbicides on pathogenic soil fungi (Altman and Campbell 1977; Davis et al. 1976) and the potential of several asco-, basidio-, and zygomycetes to metabolize phenylurea herbicides (Gondim-Tomaz et al. 2005; Ronhede et al. 2005). On the other hand, continuous exposure to herbicides could increase host susceptibility. Diuron affects photosynthetic electron transport and thereby causes the inhibition of sugar production in the Calvin cycle through the depletion of ATP and NADPH supplied by the light reaction. Inhibition of photosystem II electron transport also generates reactive oxygen species (ROS) that have the potential to cause membrane protein damage (Cobb and Reade 2010). Longer exposure to the herbicide may therefore disrupt physiological processes which could increase host susceptibility to parasites in the longer term. The possible alteration of host physiology through diuron exposure may also have affected parasite development time. Phosphorous limitation of *A. formosa* for example was found to reduce sporangia development time (Bruning and Ringelberg 1987). However, it also reduced the number of zoospores per sporangium. Hence the overall effect of phosphorous limitation on parasite growth was negative (Bruning and Ringelberg 1987). Faster sporangia development time could be an underlying
mechanism for higher infection rates if zoospore production is not reduced. Finally parasite growth rate integrates different life history traits, each of which may interact differently with changes in the batch culture environment as the experiment progresses (Bruning 1991a; b; Vale and Little 2009). It is therefore difficult to pinpoint the underlying mechanisms of the observed differences in infection dynamics and the outcome between the treatments (Pulkkinen and Ebert 2004).

Does stress increase the impact of disease on host populations?

Lafferty and Holt (2003) differentiate between the spread of disease ($R_0$) which describes the parasite population growth and the impact of disease on host population growth. In our system, the spread of disease was initially negatively related to the stressor diuron. This resulted in an antagonistic interaction between diuron and parasite with a weaker negative effect of the parasite on the exponential host population growth rate in the presence of diuron. The presence of this herbicide did therefore not implicate an increased impact of disease on exponential host population growth. However, this interaction between stress and spread of disease shifted from antagonistic (early infection phase) towards additive which is reflected by the increased infection rate after day four in presence of diuron. Together with this shift towards an increased $R_0$, also the impact of disease on host populations increased (lower density of uninfected host cells, Fig.4b, and higher prevalence of infection, Fig.4c). Although we do not have a mechanistic explanation yet for this shift this would indicate a risk of more severe epidemics when host populations are exposed to the photosynthesis inhibiting herbicide diuron. However, care must be taken with this general statement since our results suggest that mechanisms of density dependent efficiency of parasite transmission are in operation. Therefore the outcome of our laboratory infection experiment is probably highly dependent on initial host and parasite densities (Ben-Ami et al. 2008). Moreover, the initial host density in our experiment was relatively high compared to natural host densities in the field (Ibelings et al. 2011). Therefore, the negative effect of diuron exposure on parasite transmission could be even more pronounced under natural conditions, resulting in the reduction of epidemics. Furthermore, when our hypothesis is true that reduced photosynthesis of the algal hosts negatively affects parasite transmission (through chemotaxis interference), one could generalize that any herbicide (or any other stress factor) affecting photosynthesis will result in lower chytrid infection rates. This hypothesis requires further investigation. The strength and impact of the interaction between parasite and herbicide probably also depends on the concentration and duration of herbicide exposure. Our experimental setup
was limited to one concentration of diuron and one exposure scenario (continuous exposure) causing continuous inhibition of photosynthesis (Fig. S1). However, exposure to herbicides in the aquatic environment often occurs as repeated pulses with possible recovery of photosynthesis and algal growth between exposures (Vallotton et al. 2008). Further studies are needed to elucidate how such a pulsed exposure scenario would affect chytrid infection.

**Conclusion**

We present the first experimental study on the impact of disease in phytoplankton populations exposed to an anthropogenic chemical stressor. Simultaneous exposure of host populations to a parasite and a toxicant allowed the observation of how initial disease transmission and later disease development are affected at the population level. An important finding of this study is that environmental stress modifies the infection dynamics, thereby changing the nature of the stressor interaction from antagonistic towards additive. We therefore gained valuable insights into the dynamic nature of interactions between environmental pollution and disease. Further, our results showed that host population phenotype can be a decisive factor in controlling parasite growth. We conclude that anthropogenic stress can modify infection dynamics and the impact of disease on host populations through the complex interplay between stressor, host and parasite growth dynamics and host population phenotype.

**Experimental Procedures**

**Host-Parasite study system**

The host *Asterionella formosa* is a cosmopolitan freshwater diatom which forms stellate colonies. Reproduction in this species is predominantly asexual and characterized by a “shrinking division” mode which is specific for diatoms. This process leads to a progressive reduction in cell size. So far, no sexual reproduction has been observed for *A. formosa*. However, rare sexual events are assumed since these may explain the high genetic diversity in a Dutch lake population (De Bruin et al. 2008) and regular size rejuvenation in the Lake Zurich population (Nipkow 1927).

The parasite *Zygorhizidium planktonicum* is an aquatic fungus belonging to the Chytridiomycetes (James et al. 2006). It is an obligate, host-specific parasite of *A. formosa* (Van Donk and Ringelberg 1983). Hence, the parasite cannot survive and/or reproduce in absence of its host. *Z. planktonicum* is an extremely virulent parasite i.e. every infection inhibits host reproduction and leads to host cell death. The life cycle of this chytrid parasite begins with the attachment of a motile zoospore to
the surface of a host cell. After zoospore encystment a germ tube is formed which enters the host cell through the girdle zone. Via the germ tube nutrients are extracted from the host cell and used for the development of the sporangium. New zoospores are formed either asexually or sexually and are released from the sporangium by dehiscence (Doggett and Porter 1996; Ibelings et al. 2004).

Experimental design
All host and parasite strains were isolated during the spring bloom of 2008 from Lake Maarsseveen, (52.142828 N, 5.085711 E, The Netherlands). We conducted a full factorial experiment with five different host genotypes (based upon AFLP fingerprinting, Gsell et al. in press), one parasite genotype and four treatments: 1) stress-free (positive control), 2) diuron, 3) parasite, 4) diuron × parasite. All treatment combinations were tested in batch cultures, incubated in water baths at 18 °C and 100 µE m⁻² s⁻¹ irradiance. The total volume of a batch was 65 mL (in 100 mL Erlenmeyer flasks) and each treatment was replicated five times resulting in 100 experimental units. Each flask was manually shaken once per day. The experiment was started with an initial host density of 10 000 cells mL⁻¹. Approximately 0.5 mL of an old infected A. formosa culture (100% prevalence of infection) was added to obtain an initial parasite sporangia concentration of 500 sporangia mL⁻¹ corresponding to a starting prevalence of infection of 5%. Based on the results of a previous dose response experiment with diuron (DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea; CAS 330 54 1, PESTANAL® analytical standard, Sigma-Aldrich) and three different A. formosa strains from Lake Maarsseveen, a sublethal concentration of 8 µg L⁻¹ diuron was added. This concentration results in ca. 25% growth inhibition and 50% photosynthesis inhibition (see supporting information). Diuron, is a herbicide that inhibits photosynthesis by binding to the D-1 protein of the Photosystem 2 reaction centre, thereby blocking photosynthetic electron transport (Duke 1990). It is one of the most frequently and permanently observed agrochemicals in surface waters (Blanchoud et al. 2004; Chèvre et al. 2006) and is highly persistent in the environment (Giacomazzi and Cochet 2004). Peak concentrations of this herbicide of up to 28 µg L⁻¹ are found in the environment (Field et al. 2003), therefore a concentration of 8 µg L⁻¹ can be considered ecologically plausible and relevant. The experiment was conducted for 8 days (if experiments last longer (multiple) infections gets so widespread that infection is impossible to quantify accurately) and every two days 3 mL subsamples were fixed with a mixture of paraformaldehyde (0.01% final concentration) and glutaraldehyde (0.1% final concentration). Before the experiment was started, approximately 60 cells (from 60 different colonies) of each host population were measured and
their cell biovolume was calculated assuming the shape of a rectangular box \( V = \text{length} \times \text{width}^2 \). We counted total abundance of host cells \((N)\), abundance of infected \((N_i)\) and uninfected \((N_u)\) host cells, and abundance of fungal sporangia and attached zoospores using an inverted fluorescence microscope (Fluovert FS, Leitz) according to the Uthermoehl method (see Van Donk and Ringelberg 1983). Average host population growth rate and parasite population growth rate were calculated from the exponential increase of total host cells \(\text{mL}^{-1}\) and sporangia \(\text{mL}^{-1}\) respectively (Guillard 1973): \( K' = \ln \left( \frac{N_2}{N_1} \right) / (t_2 - t_1) \). Where: \(N_1\) and \(N_2\) is abundance at \(t_1\) and \(t_2\) respectively. A second host fitness parameter was determined; which we termed “final uninfected host density”. It was calculated as the abundance of live, uninfected host cells at the last day of the experiment minus the initial host cell abundance. To assess host genotype sensitivity for diuron and parasites the percentage of host growth rate and final host density inhibition compared to the control treatment was calculated.

**Statistical analysis**

Statistical analyses were performed using the free software R, version 2.8.1 (R Development Core Team 2008). The effects of the single and combined stress treatments on two fitness parameters of the five *A. formosa* genotypes were analyzed by a linear mixed effect model (lmmer function of the lme4 package) with average growth rate and final uninfected host density as dependent variables, diuron exposure (yes/no) and parasite exposure (yes/no) as fixed factors and host genotype as random factor. To account for cell size differences between the host genotype populations mean host cell volume was included as covariate in the model. Models were fitted using the maximum likelihood method in R and model selection was conducted by standard Akaike information criterion (AIC) methods, i.e. starting from the model including all higher order interactions and progressively deleting the least significant interaction term. In a similar analysis the effect of diuron on parasite fitness was tested with parasite population growth rate as dependent variable, diuron exposure (yes/no) as fixed factor, host mean cell biovolume as covariate and host genotype as random factor. The significance of the fixed factors in the linear mixed effects models was estimated by Markov Chain Monte Carlo (MCMC) simulations with the function pvals.fnc from language R package (Baayen et al. 2008). To analyze the infection dynamics a repeated measure analysis was performed using the lme function of the nlme package. Sampling day was the repeated factor and number of infections the dependent variable. Diuron exposure (yes/no) was fixed and the mean host population cell volume was included as covariate. The best fitting covariance structure was determined by comparing AIC values. To meet the
assumptions of normality (tested with q-q plots) and homogeneity of variance (Bartlett test) the data for number of infections was log transformed.

**Prediction of joint effects**

We assume that parasitism and the photosynthesis inhibiting herbicide diuron act independently from each other. Therefore the Bliss model of independent action (Bliss 1939) was used to calculate the reference value for additive effects of combined stressors, which is obtained by taking the sum of each single stressor minus their product (Andersen et al. 2009). Deviations from this predicted additive effect are thus an indication of antagonistic (less than predicted) or synergistic (greater than predicted) effects.

**Acknowledgements**

We thank Christoph Tellenbach for statistical advice and helpful comments on the manuscript. Further we thank Prof. Ellen van Donk for providing the lab facilities to conduct the experiment. This research was funded by the ETH Board (CCES7 GEDIHAP).

**References**


Canter HM, Jaworski GHM (1979) Occurrence of a hypersensitive reaction in the planktonic diatom Asterionella formosa Hassall parasitized by the chytrid Rhizophydiun planktonicum Canter Emend., in culture. New Phytol. 82:187-


James TY et al. (2006) A molecular phylogeny of the flagellated fungi (Chytridiomycota) and description of a new phylum (Blastocladiomycota). Mycologia 98:860-871


Kreutz LC, Barcellos LJG, Marteninghe A, dos Santos ED, Zanatta R (2010) Exposure to sublethal concentration of glyphosate or atrazine-based herbicides alters the phagocytic function
and increases the susceptibility of silver catfish fingerlings (*Rhamdia quelen*) to *Aeromonas hydrophila* challenge. Fish Shellfish Immunol. 29:694-697


Nipkow HF (1927) Ueber das Verhalten der Skelette planktischer Kieselalgen im geschichteten Tiefenschlamm des Zürich- und Baldeggersees (Dissertation). In. ETH Zürich


Rohr JR et al. (2008b) Agrochemicals increase trematode infections in a declining amphibian species. Nature 455:1235-U1250


Supporting information:

The effect of diuron on growth and photosynthetic yield of three *A. formosa* strains

**Material and Methods**

A dose response experiment was conducted with three different clones of *Asterionella formosa* (S53, S183, S190; origin Lake Maarsseveen, The Netherlands) and seven concentrations of the herbicide diuron (0 (control), 0.023, 0.23, 2.3, 23.3, 233.1 and 1165 µg L\(^{-1}\)) in three replicates, resulting in 21 experimental combinations and 63 experimental units. The algal strains were cultured in CHU-10 medium (Stein 1973) as uniclonal but non-axenic batch cultures. Prior to the experiment, cultures were kept in semi-continuous batch cultures under the experimental conditions (18 °C, 100 µE m\(^{-2}\) s\(^{-1}\) irradiance, 14:10 light: dark cycle).

At the start of the experiment 10 000 cells mL\(^{-1}\) were inoculated and the experimental diuron concentrations were added in a total volume of 150 mL CHU-10 medium. To estimate growth rate, samples were taken daily at the same moment of the light cycle until day 7. The number of *Asterionella* cells mL\(^{-1}\) was calculated from optical density measurements at 685nm (OD\(_{685}\)) in 5cm cuvettes using a Varian CaryWin UV-Vis spectrometer. The linear function of OD\(_{685}\) versus cell density was derived initially by direct microscopic counts of algal cells at 200 x magnification using an utermöhl counting chamber. Population growth rates during the exponential growth phase were calculated by linear regression of ln(OD\(_{685}\)) against time (Stein 1973). Photosynthetic yield of one replicate per strain was measured 30 minutes after the start of the experiment and subsequently on day 1, 2, 3, 4 and 7 with a PhytoPam fluorometer, using the saturating pulse method (Walz).

The percentage inhibition of growth for each treatment replicate and for photosynthetic yield was calculated from the equation:

\[
\%I = \frac{(\mu_C - \mu_T)}{\mu_C} \times 100
\]

where:

\%I: percentage inhibition in growth rate/photosynthetic yield

\(\mu_C\): mean value for growth rate/mean photosynthetic yield over the sampling period in the control

\(\mu_T\): value for growth rate/mean photosynthetic yield over the sampling period in the treatment
The average percentage of growth and photosynthesis inhibition for each strain was plotted against the logarithm of the diuron concentrations. EC50 values (concentration resulting in 50% growth and photosynthesis inhibition) were obtained by fitting a sigmoidal dose-response curve (four-parameter logistic equation) using sigmaplot version 10.0.

Results
Photosynthetic yield remained constant in all strains and treatments over the entire experimental period (Figure S1). Figures S2 A and B show the photosynthetic yield and growth inhibition dose-response curves for the three different A. formosa strains. The EC50 for photosynthetic yield inhibition was lower and showed less variation among strains (mean = 7.5, SD = 4.8) than the EC50 for growth inhibition (mean = 15.9, SD = 0.09).

Figure S2: Daily measurements of photosynthetic yield for all treatments in the three A. formosa strains over the experimental period (7 days).
Figure S2: Fitted dose-response curves show the variation in A) photosynthesis inhibition and B) growth inhibition (mean ± SD) among three different A. formosa strains after seven days of diuron exposure. The EC$_{50}$ values for each strain are back transformed.
Chapter 2

Parasite fitness traits under environmental variation: the case of a diatom infecting chytrid fungus

Silke Van den Wyngaert, Olivier Vanholsbeeck, Piet Spaak, Bas W. Ibelings
(to be submitted to International Journal of Parasitology)

Abstract

Parasite environments are heterogeneous at different levels. A first level of variability is the host itself. A second level of variation represents the external environment that parasite share with their hosts. Both levels are expected to affect parasite fitness traits and consequently how parasites spread in the host population. We used a full factorial design to disentangle the main and interaction effects of variation in the immediate host environment, here the diatom Asterionella formosa (variables host cell volume and host condition through herbicide pre-exposure) and variation in the external environment (variables host density and acute herbicide exposure) on three fitness traits (transmission efficiency, development time and reproductive output) of a chytrid parasite. The chytrid fungus uses chemical cues to locate its host, i.e. free swimming zoospores find the host through chemotaxis. We found that herbicide exposure had a negative effect on parasite transmission efficiency under low, but not high, host density, possibly because the photosystem 2 blocking herbicide interfered with photosynthesis dependent chemotaxis in a density dependent way. Theoretical support for this explanation is provided by an agent based simulation model (ABM). Host size played only a minor role for direct transmission efficiency but substantially affected parasite reproductive output and to a lesser extent sporangia development time. Changes in host condition through herbicide pre-exposure did not increase transmission efficiency and neither did it greatly affect sporangia development time and reproductive output. This suggests that successful parasite transmission is mainly affected by the external environment (host density and acute herbicide exposure), and that parasite reproduction is mainly driven by host cell size. We conclude that the characterization of individual parasite fitness traits in multiple levels of heterogeneous environments allows more accurate predictions of parasite spread and disease dynamics.
Introduction

How an infectious disease spreads through a host population is driven by the complex interplay between the host, parasite and environment (disease triangle; Stevens 1960). From a parasite’s perspective, the environment can be viewed in two dimensions: (i) the host itself (immediate environment), nested in (ii) the surrounding environment (habitat of the host, host population density), with both dimensions being highly inter-dependent (Loot et al. 2011; Thomas et al. 2002). The immediate (i.e. the host) environment consists of both genetic and ecological components. A substantial part of research on host parasite interactions has focused on the genetic mechanisms underlying parasite infectivity/host susceptibility (reviewed in Wolinska and King 2009). However ecological mechanisms, either directly through phenotypic traits of individual hosts or indirectly through environmental induced changes in host condition may also affect parasite fitness and consequently play a large role in parasite population dynamics (Hall et al. 2007a; Hall et al. 2009; Krist et al. 2004; Seppälä et al. 2008).

Parasite population growth (e.g. spread of disease) is an integrative measure of parasite fitness including several parasite fitness traits such as infectivity (transmission efficiency), development time, per capita reproductive output and survival. Each of these traits can be differentially affected by the immediate host and/or external environment. Vale and Little (2009) for example disentangled parasite fitness traits in a Daphnia-bacteria host parasite system and demonstrated that infectivity depended mainly on host genetics (immediate host environment) whereas parasite spore production was determined by temperature (external environment). Another study (Gendron et al. 2003) found that pre-exposure of leopard frogs to a pesticide mixture did not influence host recognition or penetration components of the transmission process of the parasitic lungworm. However the parasite matured and reproduced earlier in hosts that were exposed to pesticides in their environment. These results highlight that in most cases accurate measures of parasite fitness in heterogeneous environments requires knowledge of more than one parasite fitness trait. Moreover, both the host and external environment rarely stay constant, making it extremely challenging to predict disease dynamics in nature (Duncan et al. 2011).

In this study we aim at experimentally disentangling the main and interactive effects of immediate host and external environmental variation on single parasite fitness traits such as transmission efficiency, development time and reproduction. We designed our experiments based on the results of a previous epidemiological experiment that we conducted with the host parasite model
system *Asterionella formosa* (as diatom host) and *Zygorhizidium planktonicum* (as chytrid parasite) exposed to an environmental pollutant, the herbicide diuron. Here we observed that exposure to diuron modified the parasite population dynamics and that the spread of infection positively correlated to mean host population cell volume (Van den Wyngaert et al. submitted). Compared to the control treatment, herbicide exposure prompted an initial inhibition of transmission, though over longer time – with an increase in host density - an enhanced spread of disease was observed upon diuron exposure. In the present study we used a full factorial design to disentangle the main and interaction effects of variation in the immediate environment, which the host *A. formosa* constitutes for the parasite (variables host cell volume and host condition through herbicide pre-exposure) and variation in the external environment which host and parasite share (variables host density and acute herbicide exposure) on parasite fitness.

Hypothesis #1 revolves around density dependence of zoospore transmission. We hypothesize that acute herbicide exposure initially constrains parasite transmission either through a direct host density effect (diuron reduces host population growth through inhibition of photosynthesis) or by interfering with host finding mechanisms based on chemotactic cues. Specific to our algal - chytrid model system it was found that chytrid zoospores are unable to infect host cells in the dark (Bruning 1991e; Canter and Jaworski 1981a) and it is thought that zoospores depend on photosynthesis exudates of the host to recognize and localize host cells. The herbicide diuron inhibits photosynthesis (Duke 1990) and could thereby interfere with chemotaxis. Hence we hypothesize that a higher host density increases contact rates between host and parasite, thereby following the mass-action principle (McCallum et al. 2001), rendering chemotaxis less relevant for transmission success (Kuhn 1997). Hypothesis #2 revolves around the damaging effects of environmental (herbicide) stress on host condition and disease resistance. We hypothesize that increased host exposure time to the herbicide stressor reduces the energy budget of the host and this ultimately makes them more susceptible to infection (Rohr et al. 2008a; Saarinen and Taskinen 2005). Hypothesis #3 revolves around the effects of host quality on parasite fitness. Here, we hypothesize that the increased spread of infection in host populations with larger cell volume (Van den Wyngaert et al., submitted) is mainly driven by a higher parasite reproductive output on larger host cells due to a richer (larger quantity of nutrients) host environment (Smith 2007). However, we also expect that diuron pre-exposure negatively affects host nutrient quality and thereby reduces zoospore production and/or development time (Frost et al. 2008; Seppälä et al. 2008). Through our infection experiments, and supported by an agent based model, we
demonstrate that in order to better understand and predict disease dynamics it is useful to implicitly distinguish those factors that determine the immediate (i.e. host) and general external parasite environment.

**Material and Methods**

**Host-Parasite study system**

The host *Asterionella formosa* is a cosmopolitan freshwater diatom which forms stellate colonies. Reproduction in this species is predominantly asexual and characterized by a “shrinking division” mode, which is specific for diatoms. This process leads to a progressive reduction in cell size. The parasite *Zygorhizidium planktonicum* is an aquatic fungus belonging to the Chytridiomycetes (James et al. 2006). It is an obligate, host-specific parasite of *A. formosa* (Van Donk and Ringelberg 1983b). Hence, the parasite cannot survive and/or reproduce in absence of its host. *Z. planktonicum* is an extremely virulent parasite i.e. every infection inhibits host reproduction and leads to host cell death. The life cycle of this chytrid parasite begins with free swimming motile stages called zoospores that actively find their host and attach to the surface of the host cell. After zoospore encystment a germ tube is formed, which enters the host cell through the girdle zone. Via the germ tube nutrients are extracted from the host cell and used for the development of the sporangium. New zoospores are formed either asexually or sexually and are released from the sporangium by dehiscence (Doggett and Porter 1996; Ibelings et al. 2004).

**General experimental set up**

To test our three hypotheses we conducted two experiments. With a short-term infection experiment we disentangle the effects of the external environment and the immediate host environment on the early stages of infection, i.e. parasite transmission efficiency. With the second experiment we continue beyond transmission and study the production of new parasite zoospores, which we hypothesize to be dependent on host defence and host nutritional quality (hypotheses # 2 and 3).

All host and parasite strains were isolated during the spring bloom of 2008 from Lake Maarsseveen, (52.142828 N, 5.085711 E, The Netherlands). They were cultured in CHU-10 medium (Stein 1973) as uniclonal but non-axenic batch cultures.

Prior to the experiments, we kept the host strains and the parasite strain in exponential growth in 60 mL and 120 mL, respectively, batch cultures under the experimental conditions (18 °C, 100 µE m⁻² s⁻¹ irradiance). Six days before the start of the experiments we transferred one subpart of the
host strains to control conditions and another subpart we incubated with a sublethal concentration of 8 µg L⁻¹ diuron (CAS 330 54 1, PESTANAL® analytical standard, Sigma-Aldrich). This concentration provokes ca. 50% inhibition of the maximum photosynthetic yield and was determined from a preliminary toxicity test performed with three different A. formosa strains from Lake Maarsseveen (Van den Wyngaert et. al, submitted). Before the experiment was started, we measured photosynthetic yield of both control and diuron incubated host strains (Walz PhytoPam) to ensure that the diuron exposure had effectively reduced photosynthesis. We obtained the parasite zoospore suspension by filtering the parasite culture through a 7 µm mesh sized plankton net. We verified whether filtration and exposure to the experimental diuron concentration had any negative effect on zoospore survival by microscopic inspection of the swimming activity of the zoospores.

Experiment 1: parasite transmission efficiency

In a full factorial experiment we examined the effect of the external environment, (i) host population density (low; 10’000 cells mL⁻¹ versus high: 80’000 cells mL⁻¹) and (ii) acute diuron exposure and the immediate host environment, (iii) mean host population cell volume and (iv) diuron pre-exposure on parasite transmission efficiency. We used one parasite strain and five monoclonal host populations which we grouped in two different size classes (small; mean cell volume = 253 µm⁻³ (±6.04) and large; mean cell volume = 301 µm⁻³ (±3.10), see Fig. S1). We divided the experiment in two replicated temporal blocks (one week apart) which each contained three replicates of all treatments. The experiment involved 8 treatment combinations: 5 host populations x 2 diuron (yes/no) x 2 host density (low, high) x 2 diuron pre-exposure (yes/no) x 2 blocks x 3 replicates = 240 experimental units. The infection experiment was conducted in 24 well plates with each well having a total volume of 2.5 mL. First we added the host and subsequently the amount of diuron to the respective treatments to reach a final concentration of 8 µg L⁻¹. Thereby we considered the amount of diuron already contained in the pre-exposed host strains. Since we did not want to manipulate the host differently compared to the other treatments (by filtering and washing), we have to notify that in case of the high host density conditions the amount of diuron was still relatively high in the pre-exposed, non diuron added treatment (approximately 6 µg L⁻¹). In the low host density treatment, however, diuron that was transferred from the pre-exposed host strain batches was diluted to a concentration in the range of nanograms L⁻¹. This concentration did not have an effect on photosynthetic ability (as measured with the PhytoPam (Waltz, Germany)). After a 30 minute incubation time we added 0.5 mL of
zoospore suspension to each treatment. To determine the zoospore concentration we added lugol’s solution to a 1 mL subsample of the filtered zoospore suspension in order to let the zoospores settle. We counted a minimum of 30 fields of view using an inverted microscope (Fluovert FS, Leitz). For block 1 and block 2 zoospore concentrations were 6728 mL$^{-1}$ and 4407 mL$^{-1}$, respectively. After 3 hours of parasite exposure we fixed the samples in the well plates with a mixture of paraformaldehyde (0.01% final concentration) and glutaraldehyde (0.1% final concentration). Samples were subsequently transferred into sampling tubes and stored cool and dark until further processing. We counted for each treatment ca. 350 host cells using an inverted microscope (Fluovert FS, Leitz) according to the Utermöhl method (Van Donk and Ringelberg 1983) and determined the number of infected cells and number of attached zoospores. We calculated parasite transmission efficiency as the proportion of attached zoospores from the initially added zoospore numbers.

**Experiment 2: sporangia development time and zoospore production**

In a full factorial experiment we tested for the effects of the external environment, (i) acute diuron exposure and the immediate host environment, (ii) host cell volume and (iii) diuron pre-exposure of host cells on sporangia development time and zoospore production. The experiment involved 4 treatment combinations: 2 host populations x 2 diuron (yes/no) x 2 diuron pre-exposure (yes/no) x 3 replicates = 24 experimental units. We conducted the infection experiment in 6 well plates with each well having a total volume of 10 mL, containing 5 mL of filtered zoospore solution. Based on an earlier experiment with the chytrid *Rhizophydium planktonicum* the sporangia development time was determined to be approximately two days under the experimental temperature conditions (18 °C) used in our experiment (Bruning 1991c). For each treatment we had a control where we took subsamples at 4 or 5 hours time intervals from day 2 onwards to verify the start of sporulation. After 62 hours we saw the first empty sporangia and from this time onwards we took 1 mL subsamples in 2.5 or 3 hour time intervals. We counted for each treatment and time interval at least 45 sporangia and calculated the percentage of empty sporangia. We determined the sporangia development rate as the time where 25% of sporangia had sporulated. From the same samples we measured the shortest diameter of 35 empty sporangia per treatment and calculated the sporangia volume assuming a spherical shape (Bruning 1991e). Sporangium volume can be used as an approximation for zoospore production (Bruning 1991e). In addition we measured the host cell volume associated with the empty sporangium assuming the shape of a rectangular box ($V = \text{length} \times \text{(width)}^2$). We recorded phase contrast images ($\times$ 800 magnification) with a digital
camera (Leica DFC290 HD), using an inverted microscope (Leica DMI4000 B) and used Leica image analysis software (LAS) for the measurements.

**Statistical analysis**
We performed a factorial ANOVA to analyze the effect of host population cell volume, acute diuron exposure, diuron pre-exposure and host density on parasite transmission efficiency. We included block as a fixed factor in the model since zoospore abundance differentiated between the two blocks. We used a logit transformation of the response variables as this is preferred over arcsine square root transformation of proportion data (Warton and Hui 2011). Afterwards we used planned contrasts to test whether transmission efficiency was host size dependent. To analyze the effect of host population cell volume, diuron and diuron pre-exposure on sporangia development time we used the 25% sporulation time determined from the three replicates as the dependent variable and host cell volume, diuron pre-exposure and acute diuron exposure as fixed independent variables. We further performed post hoc pairwise comparisons between treatments. A similar analysis was performed for sporangia volume, including the associated host cell volume as a covariate to control for cell size effects between treatments. In both analyses, sporangia development time and sporangia volume complied with the assumptions of normality and homogenous variance, judged by visual inspection of residuals. We also conducted linear regressions to investigate the relationship between sporangia volume and host cell volume. All statistical analyses were performed using SPSS (Version 17, SPSS Inc., Chicago, Ill).

**Agent based model (ABM)**
The model is implemented using Uglylab, an agent based modelling tool we developed in Java and which is freely downloadable from [http://www.computational-biology.org](http://www.computational-biology.org). The complete model description can be found in the supplementary material (S2). The purpose of the model is to test the hypothesis of density dependence of chemotaxis (active host finding mechanism) for parasite transmission success and to compare the predicted patterns with our experimental results. We therefore defined four scenarios that simulate the experimental treatments: (1-2) low and high host density with chemotaxis (control) (3-4) low and high host density without chemotaxis (diuron exposure). The initial model contains two agent classes: hosts and zoospores (free living life stage of the parasite) which are normally distributed in a 3 dimensional space representing a volume of 0.125 ml. We considered a host being a colony of 8 cells because these are the most commonly observed
ones in nature. We determined the initial number of host colonies and zoospores according to the experimental host and zoospore densities. We introduced 5 host pools normally distributed around the mean host cell size of 40 µm (represents the larger host size class) to introduce host cell size variation.

The model contains 3 rules:

1) Zoospore movement: When chemotaxis is not active, zoospores show a random walking behaviour. When chemotaxis is activated, zoospores show a random walking behaviour until hosts are encountered in the defined sphere of chemotaxis influence. Once inside the sphere of chemotaxis, the algorithm computes a vector representing the direction of the chemotaxis strength. The chemotaxis strength is the sum of the individual strength of each host present in the sphere of chemotaxis influence. A host exerts a force inversely proportional to the distance host-zoospore and tends to zero as the distance tends to the radius of the sphere of influence.

2) Host movement: The host movement rule simulates Brownian motion (small-scale random motions) just to avoid that zoospores are caught into a basin of attraction when chemotaxis is active.

3) Infection: The infection rule has two parameters, the infection probability (PI) and the infection zone which is given by the individual sizes of the host. If a zoospore enters the infection zone it will infect the host with a probability PI.

Since we are interested in rapid infection processes we used very short discrete time steps of 1 second. Simulations were run for 10800 seconds (3 hours) which corresponds to the duration of the infection experiment.

Results

Experiment 1: parasite transmission efficiency

Although there were significant block × host density and block × diuron pre-exposure interactions (Table 1), the direction of the slopes did not change and reaction norms did not cross between blocks (host density; block 1, slope = 1.05, p = <0.001, block 2, slope = 2.11, p = <0.001, pre-exposure; block 1, slope = -0.04, p = 0.85, block 2, slope = -0.45, p = 0.12). The block with higher initial zoospore concentration had a higher overall transmission efficiency. Diuron exposure had a negative effect on parasite transmission efficiency, however only in the low host density treatment (Fig. 1a).
Table 1: ANOVA results of the effect of host strain (HS), block (B), host density (HD), diuron pre-exposure (EXP) and diuron (D) on the proportion of *Z. planktonicum* zoospores successfully infecting *A. formosa* cells. All 3-way and higher-order interactions were not significant and therefore are not included in this table. Bold: significant

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Host strain (HS)</td>
<td>4</td>
<td>3.42</td>
<td>3.63</td>
<td>0.007</td>
</tr>
<tr>
<td>Block (B)</td>
<td>1</td>
<td>17.94</td>
<td>19.03</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Host density (HD)</td>
<td>1</td>
<td>135.51</td>
<td>143.74</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diuron pre-exposure (EXP)</td>
<td>1</td>
<td>4.58</td>
<td>4.86</td>
<td>0.029</td>
</tr>
<tr>
<td>Diuron (D)</td>
<td>1</td>
<td>0.07</td>
<td>0.07</td>
<td>0.785</td>
</tr>
<tr>
<td>B × HD</td>
<td>1</td>
<td>13.3</td>
<td>14.11</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>B × EXP</td>
<td>1</td>
<td>4.12</td>
<td>4.37</td>
<td>0.038</td>
</tr>
<tr>
<td>B × D</td>
<td>1</td>
<td>0.19</td>
<td>0.2</td>
<td>0.655</td>
</tr>
<tr>
<td>HD × D</td>
<td>1</td>
<td>19.51</td>
<td>20.69</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HD × EXP</td>
<td>1</td>
<td>0.005</td>
<td>0.005</td>
<td>0.942</td>
</tr>
<tr>
<td>EXP × D</td>
<td>1</td>
<td>7.76</td>
<td>8.23</td>
<td>0.005</td>
</tr>
<tr>
<td>HS × B</td>
<td>4</td>
<td>0.99</td>
<td>1.05</td>
<td>0.382</td>
</tr>
<tr>
<td>HS × HD</td>
<td>4</td>
<td>1.79</td>
<td>1.9</td>
<td>0.114</td>
</tr>
<tr>
<td>HS × EXP</td>
<td>4</td>
<td>0.21</td>
<td>0.22</td>
<td>0.926</td>
</tr>
<tr>
<td>HS × D</td>
<td>4</td>
<td>1.27</td>
<td>1.35</td>
<td>0.254</td>
</tr>
<tr>
<td>Error</td>
<td>154</td>
<td>0.943</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Parasite transmission efficiency increased with high host density in both the control and diuron treatment. This increase was higher in the presence of diuron which resulted in a significant interaction between host density × diuron (Table 1, Fig. 1a). Host pre-exposure to diuron decreased parasite transmission efficiency in the non diuron added treatment whereas additional diuron exposure did not lower parasite transmission. This resulted in a pre-exposure × diuron interaction (Table 1, Fig. 1b). Host strain had a significant effect on parasite transmission efficiency (Table 1). Lower parasite transmission efficiency in host populations with smaller mean cell volume compared to populations with larger mean cell volume was shown by a significant contrast effect ($T_{228} = 2.51$, $p = 0.013$), though it accounted only for 3% of the total variance.
**Fig. 1:** Interaction effects of diuron exposure and a) host density, b) diuron pre-exposure on parasite transmission efficiency (± SE).

**Experiment 2: sporangia development rate and zoospore production**

Sporangia development time was slightly shorter on the largest host strain (Table 2a, Fig 2). Tukey HSD post hoc analysis indicated that only the treatment with diuron addition was significantly different from all other treatments, i.e. sporangia development time increased (Fig. 2).

**Fig. 2:** Effects of the four treatments; control (c), control+diuron (c+d), diuron pre-exposure (p-exp) and diuron pre-exposure+diuron (p-exp+d) on sporangia development time (± SE). Black and grey bars represent the smaller and larger host strain respectively. Letters indicate statistical differences between treatments in pairwise post-hoc tests (Tukey HSD; p < 0.001).
For the dependent variable sporangia size there was a significant host strain effect, with the smallest host strain (S37) producing smaller sporangia than the largest host strain (S43) (Table 2b, Fig 3). When we apply the conversion factor for estimating number of zoospores (as determined by Bruning 1991e; sporangia volume x 0.166), then the largest host strain produced on average about 1.5 times more zoospores than the smallest (25 and 16 zoospores per sporangium respectively). Both the covariate host cell volume and diuron pre-exposure significantly influenced zoospore production and there was a significant interaction between diuron pre-exposure and diuron (Table 2b). However, the biological importance of diuron pre-exposure and the interaction effect can be considered marginal since it translated only to a small absolute difference in zoospore production between pre-exposure yes or no (20 and 21 zoospores, respectively).

Table 2: Three-way ANOVA for (a) sporangia development and (b) sporangia volume (zoospore production) by host strain (HS), diuron pre-exposure (EXP) and diuron (D) In case of sporangia size we included the covariate host cell volume (CV) Bold: significant.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Sporangia development time</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Host strain (HS)</td>
<td>1</td>
<td>69.87</td>
<td>54.76</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diuron pre-exposure (EXP)</td>
<td>1</td>
<td>25.94</td>
<td>20.33</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diuron (D)</td>
<td>1</td>
<td>33.73</td>
<td>26.43</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HS x EXP</td>
<td>1</td>
<td>0.18</td>
<td>0.14</td>
<td>0.716</td>
</tr>
<tr>
<td>HS x D</td>
<td>1</td>
<td>2.01</td>
<td>1.58</td>
<td>0.227</td>
</tr>
<tr>
<td>EXP x D</td>
<td>1</td>
<td>4.38</td>
<td>3.43</td>
<td>0.083</td>
</tr>
<tr>
<td>HS x EXP x D</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0.993</td>
</tr>
<tr>
<td>Error</td>
<td>16</td>
<td>1.28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(b) Sporangia volume</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Host strain (HS)</td>
<td>1</td>
<td>42719</td>
<td>39.17</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Host cell volume (CV)</td>
<td>1</td>
<td>74664</td>
<td>68.46</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diuron pre-exposure (EXP)</td>
<td>1</td>
<td>7734</td>
<td>7.09</td>
<td>&lt;0.008</td>
</tr>
<tr>
<td>Diuron (D)</td>
<td>1</td>
<td>576</td>
<td>0.53</td>
<td>0.468</td>
</tr>
<tr>
<td>HS x EXP</td>
<td>1</td>
<td>2.12</td>
<td>0.002</td>
<td>0.965</td>
</tr>
<tr>
<td>HS x D</td>
<td>1</td>
<td>1738</td>
<td>1.59</td>
<td>0.208</td>
</tr>
<tr>
<td>EXP x D</td>
<td>1</td>
<td>5713</td>
<td>5.24</td>
<td>0.023</td>
</tr>
<tr>
<td>HS x EXP x D</td>
<td>1</td>
<td>1721</td>
<td>1.58</td>
<td>0.21</td>
</tr>
<tr>
<td>Error</td>
<td>251</td>
<td>1091</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The covariate host cell volume was positively related to sporangia volume, independent of host strain and treatment (linear regression; S37, $r^2 = 0.21$, $p < 0.001$, $n = 137$; S43, $r^2 = 0.20$, $p < 0.001$, $n = 147$, Fig 3)

![Graph showing the relationship between host cell volume and sporangia volume for both host strains (S37; black circles, S43; white circles).](image)

**Fig. 3:** Relationship between host cell volume and sporangia volume for both host strains (S37; black circles, S43; white circles).

**Simulation results**

The simulations are presented as dynamic infection processes over a 3h time span (equal to the experimental time). Since the objective of the model is to compare the simulation results with the outcome of the experimental results we only report about the endpoint. Just by ex- or including chemotaxis and keeping all other parameter values constant, the model reproduced patterns similar to the outcome of the experiment. For the low host density scenarios there was a clear difference between number of attached zoospores in the presence or absence of chemotaxis, although this difference was stronger in the model than observed in the experiment (57% and 36% respectively; Fig. 4). Under high host density conditions the absence of chemotaxis did not have a negative effect on zoospore attachment, even slightly increasing it (Fig 4). Here, however, the difference was weaker in the model than observed in the experiment (4% and 18% respectively).
**Discussion**

In this study we used a diatom-chytrid model system to disentangle the main and interactive effects of the immediate host environment (host cell volume, host condition through herbicide pre-exposure) and external environmental variation (host density, acute herbicide exposure) on three important parasite fitness traits; transmission efficiency, development time and reproduction.

The main results were: (1) acute herbicide exposure negatively affected parasite transmission efficiency, however, only in the low host density treatment; (2) changes in host condition through herbicide pre-exposure did not increase transmission efficiency and neither did it greatly affect sporangia development time and reproductive output; (3) host size positively correlated with parasite reproductive output and sporangia development time was faster on the largest host strain. These results suggest that successful parasite transmission was mainly affected by the external environment while parasite reproduction was mainly driven by the size (quantity of resources) of the immediate host environment. This study also shows how different external environmental factors, in this case exposure to a chemical pollutant and host density, can interact to modify parasites transmission efficiency.

In a previous experiment we already showed that diuron initially - i.e. at low host density - inhibited parasite transmission (Van den Wyngaert et al submitted). However, the experimental
design did not allow disentangling the effects of diuron on host density (reduced host population growth through inhibition of photosynthesis) and host susceptibility and through this on parasite transmission. The experimental design utilized here, where parasite transmission was assessed after three hours only, enabled us to control for host density (keep host density constant). Thereby we show that diuron itself has a negative effect on parasite transmission. However, as hypothesized, in a high host density environment this negative effect of diuron disappeared and transmission efficiency was even enhanced (Fig. 1a). Although we cannot completely exclude the fact that diuron has a direct negative effect on zoospores we did not observe any differences in zoospore swimming behaviour in the presence of diuron. Further, if diuron would act immediately on zoospores we would also expect lower transmission efficiency under high host density. Therefore, we argue that the underlying mechanism explaining these results may be the interference of the herbicide with host finding mechanisms (chemotaxis) below a threshold host density. We do not deliver direct proof of this principle but the simulation scenarios of our ABM model were able to produce patterns similar to those observed in our experiment. Hence our simulations support our first hypothesis that the importance of chemotaxis in locating hosts is host density dependent.

Several studies indicate that motile organisms display chemotactic behaviour towards suitable hosts or nutrient sources. For example the malaria mosquito uses human sweat components as host finding cues (Kelly 2001; Verhulst et al. 2011), cercariae larvae show chemo-orientation toward micromolecules excreted by snails (Haas et al. 1995; Korner and Haas 1998) or nematodes and pathogenic bacteria are attracted to substances released by their hosts (Wadhams and Armitage 2004; Zuckerman and Jansson 1984). There are also a few studies on chemotaxis of chytrid zoospores. The infamous chytrid pathogen of amphibians, *Batrachochytrium dendrobatidis*, for example showed positive movement towards nutritional cues of host origin (Moss et al. 2008), and Muehlstein et al. (1988) found evidence of chemotactic activity of a marine chytrid towards concentrated food sources. Although chemotaxis seems to be a wide spread mechanism for parasites with free living stages to increase their transmission potential, not many studies have incorporated host finding success (chemotaxis) as a parasite fitness trait explicitly in an epidemiological context.

One thing to keep in mind is that our host-parasite system naturally occurs in the pelagic zone of aquatic ecosystems. Where our experimental setting offered a physically stable environment,
under natural conditions varying degrees of water turbulence may interact with zoospore movement and host finding efficiency. An experimental study on a marine phytoplankton-parasite system showed that turbulence (by shaking flasks) reduced infection compared to unshaken flasks (Llaveria et al. 2010). A field study on chytrid parasitism of *A. formosa* in nine reservoirs found the highest rate of infections at intermediate hydraulic disturbance levels (Bertrand et al. 2004). Apparently motile parasites can benefit from a certain amount of turbulence (e.g. by increasing chance contacts), but when mixing becomes too intense this may override swimming behavioural effects (Fingerut et al. 2003). ABM’s provides a good tool to explore these physical and spatial aspects. In future work we want to include both turbulence and host patchiness in the model.

Contrary to our expectations, host pre-exposure to diuron did not increase parasite transmission and even showed a tendency to decrease it (Figure 1b). This suggests that hosts did not become more prone to infection upon longer stress exposure. Hence we reject hypothesis nr. 2. In plants, it has been shown that various biotic and abiotic stress response pathways share common nodes (Mazarei et al. 2007; Sohn et al. 2006) so that pre-exposure to one stressor may positively influence subsequent resistance to another stressor (Mitra et al. 2004; Sandermann 2000). However, diuron pre-exposed hosts transferred to clean medium had only a limited recovery time (ca. 30 min before parasite addition) thereby still experiencing inhibited photosynthesis activity upon parasite exposure. The weak interaction effect between pre-exposure and additional diuron exposure could then simply be explained by the negative effect of photosynthesis reduction by diuron on zoospore host finding which is not further enhanced by additional diuron exposure.

Our third hypothesis was that increased spread of infection observed in host populations with larger mean cell volume is mainly driven by a higher parasite reproductive output. Although we found that transmission efficiency was lower on host populations with smaller mean cell volume, the effect of host size was rather weak accounting for only 3% of the total variance. Sporulation also occurred earlier on the host population with the largest mean cell volume, but again the difference between sporulation time on small vs. large hosts was only limited (on average 3 hours which represent a difference of only 4% of the total development time). We expected to see a negative effect of prolonged herbicide exposure on sporangia volume i.e. reproductive output or development time. Since diuron affects photosynthetical electron transport it causes inhibition of sugar production in the Calvin cycle through the depletion of ATP and NADPH supplied from the light reaction. Longer pre-exposure to the herbicide is therefore expected to alter physiological processes and possibly host nutrient quality. Studies on two different *Daphnia*-parasite systems

61
showed that low quality nutrition in *Daphnia* reduced the reproductive output of its parasite (Frost et al. 2008; Hall et al. 2009). Though, in both cases poor nutrition in *Daphnia* also led too smaller sized animals. Diuron exposure does not directly affect diatom cell size but could reduce the mean cell size in the population on the long term through reduction in cell division (growth inhibition). The specific mode of reproduction in diatoms implies that diatoms progressively get smaller with every cell division. Sporangia size, however, was not directly affected by herbicide exposure or pre-exposure and this suggests that changes in host condition, with a potential decrease of nutrient quality for the parasite, did not affect zoospore reproduction. It is possible though that even when the number of parasite offspring produced was similar zoospores were of inferior quality, i.e. having a shorter infective lifetime (Seppälä et al. 2008). This remains a hypothesis to test. As expected we did find that larger hosts sustained significantly larger sporangia (e.g. produced more zoospores). When we assume that the same conversion factor can be applied as determined by Bruning (1991e), zoospore production was around 1.5 times higher on the host population with larger mean cell volume. This suggest that the increased spread of disease on host populations with larger mean cell volume indeed seems to be mainly driven through increased parasite reproduction. In vertebrates or higher plants, size differences between hosts and parasites are generally large and parasites only consume a relatively small part of their host resources. In contrast, the biovolume of mature sporangia of phytoplankton parasites may constitute around half of the host’s biovolume and parasites consume practically all of their host resources. This may explain why quantitative nutrient availability, as determined by host cell size, is such an important aspect of parasite fitness in this system.

**Concluding remarks regarding implications for disease dynamics**

Our results are somewhat contradictory to what is known from plant literature, i.e. plants exposed to external stress factor are often disposed to an enhanced likelihood of plant disease (Altman and Campbell 1977; DiLeo et al. 2010; Johal and Huber 2009; Macdonald 1982). It also differs from many animal studies where hosts in poor shape seem to be more susceptible to infection. However, some studies on invertebrate host-parasite systems also report opposite results to the mainstream of the literature (Bedhomme et al. 2004; Pulkkinen and Ebert 2004; Seppälä et al. 2008). In microalgae, many abiotic and biotic stress factors are likely to affect photosynthesis, so if decreased photosynthesis negatively affects parasite transmission, one could generalize that increased stress conditions in the environment will probably lead to a decreases spread of disease in phytoplankton populations. The fact that the negative effect of inhibition of photosynthesis on
transmission of the chytrid was clearly host density dependent (Fig 1a) does indicate that transmission may only be impaired in those host-parasite systems where chemotaxis plays a role. Furthermore we found that key phenotypic traits of the host (host size) can play an important role for parasite fitness. This result indicates that parasite genotypes that infect larger hosts (certain chytrid genotypes infect certain diatom genotypes only) will contribute proportionally more to the entire parasite population (faster development time and more parasite spores produced). Larger hosts may not be necessary the more common genotype. This could result in a reduced ability of the parasite to co-evolve with their local host population and consequently affect disease dynamics.

Finally, we highlight the importance of dissecting the response of individual parasite fitness traits in heterogeneous environments in order to better predict parasite population dynamics and the spread of disease in dynamic environments. We also want to point out that for parasites with free living motile stages efficient host finding mechanisms is a relevant fitness trait involved in successful transmission. In many infection experiments this is not accounted for when high host densities are used and this may give unrealistic estimates of parasite transmission potential.

**Acknowledgements**

We thank Christoph Tellenbach for statistical advice and Alena Gsell for helpful comments on the manuscript. This research was funded by the ETH Board (CCES7 GEDIHAP).

**References**


DiLeo MV et al. (2010) Abscisic acid in salt stress predisposition to Phytophthora root and crown rot in Tomato and Chrysanthemum. Phytopathology 100:871-879


James TY et al. (2006) A molecular phylogeny of the flagellated fungi (Chytridiomycota) and description of a new phylum (Blastocladiomycota). Mycologia 98:860-871


Kelly DW (2001) Why are some people bitten more than others? Trends Parasitol. 17:578-581


Stevens RB (1960) Plant Pathology, an Advanced Treatise


**Appendix**

**Figure S1**

![Box plots of the cell volume for the five hosts populations](image)

**Fig. S1:** Box plots of the cell volume for the five hosts populations (for each population \( n = 50 \)). The horizontal line represents the median and whiskers indicate the 5th and 95th percentiles. Letters indicate statistically different groups in pairwise post-hoc tests (Tukey HSD; \( p < 0.001 \)). Grey boxes depict the host size classes (small and large) with their mean cell volume (± SE) that we defined for experiment 1.

**S2: Agent based model (ABM)**

The model description follows the ODD (Overview, Design concepts, Details) protocol for describing simple individual -and agent-based models (Grimm et al. 2006; Jovani and Grimm 2008). This model is implemented using Uglylab, an agent based modelling tool we developed in Java. It is freely downloadable from [http://www.computational-biology.org](http://www.computational-biology.org).

**-Purpose:** The purpose of the model is to test the hypothesis of the density dependence of chemotaxis (active host finding mechanism) for parasite transmission success and to compare the predicted patterns with our experimental results.

**-State variables and scales:** The model is composed of three agents: host, zoospore (free living life stage of the parasite) and sporangium (attached life stage of the parasite). The hosts are...
characterized by the state variables: location (spatial position x,y,z), size and chemotaxis strength. Zoospores are characterized by the state variables: location (spatial position x,y,z) and swimming speed (based on values from the literature (Bruning 1991e)). The sporangium has only one state variable, its spatial position x,y,z.

Since we are interested in rapid infection processes we use very short discrete time steps of 1 second. Simulations are run for 10800 seconds (3 hours) which corresponds to the duration of the infection experiment. The host-parasite system we model represents two eukaryotic microorganisms in an aquatic environment. Therefore our simulations are performed on a micrometer scale in a continuous cubic 3D environment that represents a volume of 0.125 ml.

-Process overview and scheduling: At time t we start with a list of agents composed by hosts, zoospores and sporangia. After shuffling the list we compute each agent after another. For the agents host two rules are applied: infection rule and Brownian motion (see below section submodels). For the zoospores one rule is applied (zoospore movement rule): chemotaxis or random walk. The agent sporangium is the result of the infection rule but there is no rule applied on this agent.

-Design concepts: The model is driven by the interaction between a non-motile host and a highly motile parasite and uses the concept of chemotaxis to explore parasite transmission efficiency. In many host parasite interactions where the host is immobile and the parasite has a motile life stage, active search mechanisms are involved that allow the parasite to locate a suitable host (Gerardo et al. 2006). Chemotaxis is defined as biased or directed migration in response to diffusible chemical cues, the motion being directed to regions of highest chemical concentration. Diatoms (host in our model) are known to exude a variety of organic substances (Bjornsen 1988). These extracellular releases can build up gradients around algal cells which can be tracked by the parasite. In this model chemotaxis is not given by released chemical cues but given by the entity host.

-Stochasticity: Stochasticity is assumed in the initial spatial distribution of hosts and zoospores as well as in the random walk of host and random walk of zoospores when the chemotaxis rule is excluded or when zoospores are outside the chemotaxis zone. In addition, we introduce stochasticity in the infection process through the parameter infection probability PI.
Initialization: We compare 4 scenarios:

Scenario 1: low host density without chemotaxis.
Scenario 2: low host density with chemotaxis.
Scenario 3: high host density without chemotaxis.
Scenario 4: high host density with chemotaxis.

The initial state of the model starts with a number of hosts and zoospores which are randomly distributed in space (normal distribution following x,y,z). The low host density simulation is initialized with 155 host colonies and the high host density simulation with 1240 host colonies. All simulations are performed with the same number of zoospores (500) in a volume of 0.125ml and with similar infection probability (1%). We consider host colonies of 8 cells because these are the most commonly observed ones in nature. This means that 155 host colonies correspond to 1240 cells in a volume of 0.125ml and this number corresponds to the experimental low host density of ca. 10'000 cells ml\(^{-1}\). In a similar way, 500 zoospores correspond to 4000 zoospores ml\(^{-1}\) and 1240 colonies correspond to the experimental high host density of ca. 80'000 host cells ml\(^{-1}\). We introduce 5 host pools normally distributed around the mean host cell size of 40 µm which we adjust through size correction coefficients.

-Input data: The model does not have any external input of driving environmental variables.

Submodels: We define 3 submodels;

1) Zoospore movement: The zoospore movement rule describes the movement of zoospores in presence and absence of chemotaxis. In absence of chemotaxis zoospores show a random walking behaviour. The algorithm picks two random numbers representing respectively the inclination angle and the azimuth angle and moves the zoospores along the length of the radial distance defined in the rule parameter corresponding to the average swimming speed (distance per time step = 100 µm s\(^{-1}\), based on values from the literature (Bruning 1991e)). When chemotaxis is activated, zoospores show a random walking behaviour until hosts are encountered in the defined sphere of chemotaxis influence. This chemotaxis sphere is set as 2 x the sphere of infection, which equals the defined host size. Once inside the sphere of chemotaxis, the algorithm computes a vector representing the direction of the chemotaxis strength. The chemotaxis strength is the sum of the individual strength of each host present in the sphere of chemotaxis influence. A host exerts a force inversely proportional to the distance host-zoospore and tends to zero as the distance tends to the radius of the sphere of influence.
2) **Host movement**: The host movement rule simulates Brownian motion (small-scale random motions) just to avoid that zoospores are caught into a basin of attraction when chemotaxis is active.

3) **Infection**: The infection rule has two parameters, the infection probability (PI) and the infection zone which is given by the individual sizes of the host. If a zoospore enters the infection zone it will infect the host with a probability PI.
Chapter 3

Hidden diversity in the freshwater planktonic diatom *Asterionella formosa*.

Silke Van den Wyngaert, Markus Moest, Alena Gsell, Bas Ibelings, Piet Spaak

(In preparation, target journal Molecular Ecology)

Abstract

Many freshwater and marine algal species are described as having cosmopolitan distributions. Whether these widely distributed morphologically similar algal species also share a similar gene pool remains often unclear. In the context of island biogeography theory, stronger spatial isolation in freshwater lakes should restrict gene flow and lead to higher genetic differentiation among lakes. Using nine microsatellite loci, we investigate the genetic diversity of a widely distributed freshwater planktonic diatom, *Asterionella formosa* across different lakes in Switzerland and the Netherlands. We applied a hierarchical spatial sampling design to determine the geographical scale at which populations are structured. A subset of the isolates was additionally analysed using AFLP (Amplified Fragment Length Polymorphism) markers.

We detected three principal genetically distinct clusters with different clustering approaches and with both markers (microsatellites and AFLP). Unexpectedly we found much higher genetic differentiation within lakes than among them and strong subpopulation division within the Swiss lakes. These results indicate the potential presence of cryptic species within *A. formosa*. 
Introduction

Many freshwater and marine algal species are described as having cosmopolitan distributions which seems to be in accordance with Beijerinck’s (1919) and Baas Becking (de Wit and Bouvier 2006) famous metaphor “in micro-organisms everything is everywhere, the environment selects”, later emphasized by Fenchel and Finlay (2004). However, several studies contradict this hypothesis (Casteleyn et al. 2010; Evans et al. 2009; Medlin 2007; Vanormelingen et al. 2008). Whether these widely distributed morphologically similar algal species also share a common gene pool remains often unclear (Medlin 2007). But, this is an important question since intraspecific diversity (genetic polymorphism) represents the evolutionary and adaptive potential of a species. Restricted gene flow may lead to the fragmentation of the common gene pool and local adaptation of populations which may eventually lead to the evolution of new (cryptic) species. Therefore gene pool size (genetic diversity) and the gene flow between pools (population structure) are critical for speculating that everything is everywhere.

Molecular analyses of the genetic diversity and structure of microalgae are still scarce relative to other organisms and most of the studies done so far have dealt with marine species (Alpermann et al. 2009; Erdner et al. 2011; Härnström et al. 2011; Iglesias-Rodriguez et al. 2006; Nagai et al. 2009; Rynearson and Armbrust 2000; 2004; 2005; Rynearson et al. 2009; Rynearson et al. 2006; Casteleyn et al. 2009, 2010). A general assertion that marine environments offer few physical barriers to dispersal has often led to the assumption that protist populations are genetically homogeneous. Two studies on the marine diatom *Pseudo-nitzschia pungens* are in agreement with the assumption of panmixia and found evidence for a single largely unstructured population within two large but heterogeneous areas of the North Sea (Casteleyn et al. 2009; Evans et al. 2005). However, the majority of studies have shown opposite results and report both large and fine scale spatial and temporal population genetic structure within a variety of marine protist species (Alpermann et al. 2009; Lowe et al. 2010; Nagai et al. 2009; Rynearson et al. 2006).

Freshwater lakes are considered to represent more discrete, patchy and isolated habitats compared to the open ocean (however see e.g. Leibold and Norberg (2004) for lake plankton biodiversity in a metacommunity context). Consequently, it is hypothesized that this stronger spatial isolation restricts gene flow even more, leading to more genetically distinct protist populations in lakes compared to marine habitats. The handful of studies about genetic differentiation among and within freshwater protist populations seems to agree with this
hypothesis. Microsatellite analyses on the benthic freshwater diatom *Sellaphora capitata* found highly differentiated populations between lakes of the UK, Belgium and Australia (Evans et al. 2009). The same picture emerged also in an amplified fragment length polymorphism (AFLP) analysis of *Asterionella formosa* populations of geographically close Dutch lakes that revealed high genetic diversity among populations with clear clustering of genotypes according to the lake of origin (De Bruin et al. 2004). Similar results were obtained for the planktonic diatom *Fragilaria capucina*, analysed by random amplified polymorphic DNA (RAPD), showing highly genetically differentiated populations among seven lakes along a latitudinal gradient across North America (Lewis et al. 1997). Genetically distinct populations of the diatom *Cyclotella meneghiniana* were also found on smaller spatial scales (within 40 km, Beszteri et al. 2007). However, a contrasting example is the low differentiation of a ciliate population in four Chinese lakes which were relatively closely located to each other (Zhang et al. 2006) or the high genetic variability but absence of phylogeographic structure in *Microcystis aeruginosa* shown by van Gremberghe et al. (2011).

Eukaryotic microalgae commonly reproduce asexually alternate with rare sexual events (Weisse 2008). Despite the dominance of clonal reproduction, the majority of studies find surprisingly high levels of genetic diversity using polymorphic markers such as AFLP and microsatellites (Logares et al. 2009; Lowe et al. 2010; Rynearson and Armbrust 2000). Even during bloom conditions, clonal diversity of the marine centric diatom *Ditylum brightwellii* remained high, 87% to 95% distinct genotypes (Rynearson and Armbrust 2005).

In this study, we examine the genetic diversity and structure of populations of a freshwater planktonic diatom, *Asterionella formosa*. *A. formosa* is one of the dominant algal species in many freshwater lakes during the phytoplankton spring bloom. It is described as having a cosmopolitan distribution, occurring in lakes and ponds of different trophic status and with different physical and chemical characteristics. Two molecular studies have been published with contrasting results regarding genetic diversity within this diatom. Based on allozyme electrophoresis of three polymorphic enzyme loci, Soudek and Robinson (1983) did not detect a single genetically different isolate within a variety of North American and European lakes, suggesting that *A. formosa* is a pure clonal species or at least genetically highly homogeneous. This contradicts to the De Bruin et al. (2004) who applied AFLP and RAPD methods found every isolate to be genetically unique within a single *A. formosa* population of a Dutch lake (Lake Maarsseveen) suggesting that sexual
recombination (if only occasionally) in this species is likely to occur. These contrasting results most likely reflect the resolution power of the different markers but may also reflect different levels of genetic variation in different lake populations. Although the allozyme study did not find genetic variation within populations, it found considerable genetic differentiation among populations of different lakes but no clear geographical pattern was observed. Neighbouring lakes, even connected by streams, were genetically not more similar to each other than distant lakes (Soudek and Robinson 1983). However, a drawback of this study was that isolates were collected in different years. Ideally, individuals sampled for the estimation of population genetic structure should belong to the same generation, because allele frequencies vary not only over space, but also over time (Balloux and Lugon-Moulin 2002).

The objective of this study is to investigate the genetic diversity in A. formosa in high resolution (with nine polymorphic microsatellite markers) and to determine the geographical scale at which populations are structured during a single phytoplankton spring bloom.

**Methods**

**Sampling design and clone isolation**

All isolates of A. formosa were collected during the spring bloom 2010 (February, March, April) with exception of Lake Zwemlust (February, April 2009). From Lake Greifen and Maarsseveen we collected isolates on two different sampling dates (December 2009, March 2010) which we pooled together. In total we sampled 11 lakes following a hierarchical spatial sampling design to assess genetic diversity and to reveal population structure on a (1) inter-regional scale (Swiss lakes versus Dutch lakes), (2) regional scale (7 lakes within Switzerland (CH) and 4 lakes within the Netherlands (NL)) and (3) local scale (within lakes). In Switzerland we included lakes that were connected with each other each other, i.e. Lake Baldegg connected to Lake Hallwil by the river Aabach and the highly connected Upper and Lower Lake Zurich. Within Lake Lucerne we sampled seven different basins of this complex lake system. We divided the isolates of the seven different locations into three populations (VWS A, B and C) that represent the most separated basins; Lake Alpnach (VWS A), Horw Bay, Lake Küssnacht, Kreuztrichter, Weggis/Vitznau basin (VWS B), Gersau basin, Lake Uri (VWS C). Table 1 summarizes the sampled lakes and basins and their main characteristics.
<table>
<thead>
<tr>
<th>Lake (abbreviation)</th>
<th>country</th>
<th>Latitude longitude</th>
<th>Origin</th>
<th>Surface area (km²)</th>
<th>max depth (m)</th>
<th>trophic status</th>
<th>sampling date</th>
<th>abundance A. formosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lake Baldegg (BS)</td>
<td>Switzerland</td>
<td>47°11'47.69&quot;N 8°15'45.17&quot;E</td>
<td>natural</td>
<td>5.2</td>
<td>66</td>
<td>mesotrophic</td>
<td>15.03.2010</td>
<td>high</td>
</tr>
<tr>
<td>Lake Halwill (HW)</td>
<td>Switzerland</td>
<td>47°16'50.59&quot;N 8°13'01.79&quot;E</td>
<td>natural</td>
<td>10.2</td>
<td>47</td>
<td>mesotrophic</td>
<td>23.03.2010</td>
<td>low</td>
</tr>
<tr>
<td>Lake Greifen (GS)</td>
<td>Switzerland</td>
<td>47°20'58&quot;N 8°40'49&quot;E</td>
<td>natural</td>
<td>8.45</td>
<td>32</td>
<td>eutrophic</td>
<td>18.12.2009/24.03.2010</td>
<td>moderate</td>
</tr>
<tr>
<td>Lake Rot (RS)</td>
<td>Switzerland</td>
<td>47°04'10.56&quot;N 8°18'49.86&quot;E</td>
<td>natural</td>
<td>0.46</td>
<td>16</td>
<td>meso-eutrophic</td>
<td>18.03.2010</td>
<td>high</td>
</tr>
<tr>
<td>lower Lake Zurich (ZSU)</td>
<td>Switzerland</td>
<td>47°18'25.24&quot;N 8°34'37.8&quot;E</td>
<td>natural</td>
<td>65</td>
<td>145</td>
<td>mesotrophic</td>
<td>18.03.2010</td>
<td>high</td>
</tr>
<tr>
<td>upper Lake Zurich (ZSO)</td>
<td>Switzerland</td>
<td>47°12'33.29&quot;N 8°49'55.62&quot;E</td>
<td>natural</td>
<td>20</td>
<td>48</td>
<td>oligo-mesotrophic</td>
<td>25.03.2010</td>
<td>low</td>
</tr>
<tr>
<td>Lake Lucern:</td>
<td>Switzerland</td>
<td>47°07'47.70&quot;N 8°19'25.04&quot;E</td>
<td>natural</td>
<td>4.76</td>
<td>35</td>
<td>oligotrophic</td>
<td>24.02.2010</td>
<td>low</td>
</tr>
<tr>
<td>Lake Alpnach (VWSA)</td>
<td>Switzerland</td>
<td>47°01'25.80&quot;N 8°18'58.84&quot;E</td>
<td>natural</td>
<td>57</td>
<td>151</td>
<td>oligotrophic</td>
<td>24.02.2010</td>
<td>moderate</td>
</tr>
<tr>
<td>Horw Bay, Lake Küsnacht, Kreuztrichter, Weggis/Vitznau basin (VWSB)</td>
<td>Switzerland</td>
<td>47°11'47.69&quot;N 8°15'45.17&quot;E</td>
<td>natural</td>
<td>52</td>
<td>214</td>
<td>oligotrophic</td>
<td>24.02.2010</td>
<td>moderate</td>
</tr>
<tr>
<td>Lake Uri &amp; Gersau basin (VWSC)</td>
<td>Switzerland</td>
<td>47°01'25.80&quot;N 8°18'58.84&quot;E</td>
<td>natural</td>
<td>52</td>
<td>214</td>
<td>oligotrophic</td>
<td>24.02.2010</td>
<td>moderate</td>
</tr>
<tr>
<td>Ford Vechten (FV)</td>
<td>Netherlands</td>
<td>52°05'81.31&quot;N 5°16'42.48&quot;E</td>
<td>artificial 18th century</td>
<td>0.08</td>
<td>4</td>
<td>eutrophic</td>
<td>02.03.2010</td>
<td>?</td>
</tr>
<tr>
<td>Lake Maarsseveen (MSV)</td>
<td>Netherlands</td>
<td>52°14'28.28&quot;N 5°08'57.11&quot;E</td>
<td>artificial 1965</td>
<td>0.7</td>
<td>32</td>
<td>oligo-mesotrophic</td>
<td>26.01/25.03.2010</td>
<td>high</td>
</tr>
<tr>
<td>Lake Vinkeveen (VKV)</td>
<td>Netherlands</td>
<td>52°23'52.12&quot;N 4°96'15.98&quot;E</td>
<td>artificial 19th century</td>
<td>0.6</td>
<td>shallow</td>
<td>eutrophic</td>
<td>02.03.2010</td>
<td>?</td>
</tr>
<tr>
<td>Lake Zwemlust (Z)</td>
<td>Netherlands</td>
<td>52°19'30.40&quot;N 5°00'73.13&quot;E</td>
<td>artificial 1921</td>
<td>0.015</td>
<td>2.5</td>
<td>hypertrophic</td>
<td>25.02/21.04.2009</td>
<td>?</td>
</tr>
</tbody>
</table>
A 10 meter integrated plankton sample was collected with a plankton net (30 µm mesh size). Within 24 hours of collection, single colonies were isolated from a diluted plankton sample with an elongated glass pipette in order to obtain clonal cultures. Single colonies were transferred into individual wells of a 48-well plate containing 2 mL CHU 10 medium (Stein 1973) and maintained in a climate chamber at 14° C on a 14:10-h light:dark cycle of 35 µE m⁻² s⁻¹ for further growth. Cultures were checked for possible contaminations and/or accidental multiple colony isolation (visible when multiple algal patches were present). After 3 weeks of growth we transferred each unialgal *A. formosa* isolate into Erlenmeyer flask in order to obtain sufficient cell material for DNA extraction.

**DNA extraction**

Algal material (1.5 mL) was collected during exponential growth in 2.5 mL eppendorf tubes and was centrifuged in order to obtain an algal pellet. The liquid supernatant was removed. Afterwards the HotSHOT DNA extraction protocol was followed as described by Montero-Pau et al. (2008) using 20 µl of each buffer. Due to the fact that this was the first time that HotSHOT DNA extraction was performed on diatoms and different DNA extractions methods may affect genotyping reproducibility we also performed a preliminary quality test. We compared the HotSHOT DNA extraction method and the Qiagen Plant Tissue Mini Kit with respect to the reproducibility of the genotype profile of 6 culture strains of *A. formosa* and obtained the same genotyping profiles of all the 6 strains with peaks of comparable quality.

**Microsatellites development and genotyping**

An enriched library was made by Ecogenics GmbH (Zurich, Switzerland) from size selected genomic DNA ligated into SAULA/SAULB-linker (Armour et al. 1994) and enriched by magnetic bead selection with biotin-labelled (CT)₁₃ and (GT)₁₃ oligonucleotide repeats (Gautschi et al. 2000a; Gautschi et al. 2000b). Of 528 recombinant colonies screened, 213 gave a positive signal after hybridization. Plasmids from 155 positive clones were sequenced and primers were designed for 16 microsatellite inserts. Of these 16 primer pairs, nine yielded scoreable and polymorphic microsatellites. Details on the nine markers are summarized in Table 2. These nine markers were used in two sets of multiplex PCR reactions (MP1: Ast01, Ast02, Ast04 and MP2: Ast03, Ast05, Ast10, Ast13, Ast14). Multiplex PCR amplification was performed in a 10µl reaction volume containing 10ng of DNA, 5µl Multiplex PCR Master Mix (Qiagen), 0.3µM of forward and reverse primers each and double distilled water. PCR fragments were fluorescently labelled with FAM,
We used the following amplification protocol on a Biometra thermal cycler (Whatman Biometra): 30 cycles of 30 s at 94 °C, 1 min 30 s at 60 °C (MP1) or 56 °C (MP2), respectively, 1 min at 72 °C. Before the first cycle, a prolonged denaturation step (95 °C for 15 min) was included and the last cycle was followed by a 30 min extension at 72 °C. PCR products were 1:20 diluted with double distilled water and 10 µl HiDi + 0.25 µl size standard (GeneScan - 500 LIZ, Applied Biosystems) was added to 1 µl of diluted PCR product. PCR products were separated on an ABI PRISM® 3130 XL Genetic Analyzer 16 Capillary system and analyzed using Genemapper version 3.7 (Applied Biosystems). Genotyping (i.e. PCR, electrophoresis and allele scoring) was repeated 3 times for 25 percent of the samples to assure genotype accuracy and reproducibility. The occurrence of null alleles and PCR amplification bias against large alleles was assessed using MICRO-CHECKER (Van Oosterhout et al. 2004).

In addition we analysed a subset of the isolates (91 out of 224 isolates) with AFLP markers. AFLP analysis was performed by Keygene® (Wageningen, The Netherlands) using the same four EcoRI/MseI AFLP primer combinations as reported in De Bruin et al. (2004), a) Eco+GA & Mse+AT, b) Eco+GA & Mse+CC, c) Eco+GA & Mse+CG, d) Eco+GC & Mse+AC.

Table 2: Summary of locus characteristics, PCR details and levels of variability of nine polymorphic microsatellite loci in the freshwater diatom Asterionella formosa from 14 locations in Switzerland and the Netherlands (n = 224)

<table>
<thead>
<tr>
<th>Locus</th>
<th>Dye</th>
<th>Primer sequence (5' -&gt;3')</th>
<th>Repeat motif</th>
<th>Ts</th>
<th>Size</th>
<th>Ns</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ast01</td>
<td>NED</td>
<td>F CTACCGATAGCAGCCCAAGR ACAGAATCAAGAAGCCGAGAC</td>
<td>(AG)_14</td>
<td>60</td>
<td>108-128</td>
<td>10</td>
</tr>
<tr>
<td>Ast02</td>
<td>FAM</td>
<td>F CTGTCTCGCTCAACGGATG R GGAGCATGGTACACCCAAAG</td>
<td>(AC)_23</td>
<td>60</td>
<td>112-156</td>
<td>18</td>
</tr>
<tr>
<td>Ast04</td>
<td>PET</td>
<td>F TGCTACAACCTTGCCCTTAC R TGTCCGAAGTTTGTTGTCCC</td>
<td>(TC)_12</td>
<td>60</td>
<td>85-101</td>
<td>13</td>
</tr>
<tr>
<td>Ast08</td>
<td>VIC</td>
<td>F TTCAATTGAGGTTCCTCAC R AACGGAACACACACAACTGG</td>
<td>(AC)_13</td>
<td>60</td>
<td>102-114</td>
<td>12</td>
</tr>
<tr>
<td>Ast03</td>
<td>FAM</td>
<td>F CCGTTACAACCCATGATAG R TCCCCCTTGTGGATTTCGAC</td>
<td>(GA)<em>{13}-(GA)</em>{6}</td>
<td>56</td>
<td>60-116</td>
<td>18</td>
</tr>
<tr>
<td>Ast05</td>
<td>FAM</td>
<td>F CGGTCCAATGGTAAGACTCC R ATGGAAAGCGCGAGTGC</td>
<td>(CA)<em>{5}(TA)</em>{5}-(CA)_{17}</td>
<td>56</td>
<td>196-248</td>
<td>13</td>
</tr>
<tr>
<td>Ast10</td>
<td>NED</td>
<td>F TGTAGTGCTAGAAGCTTAGG R CAGAGGCCAGTGAGAATG</td>
<td>(GT)<em>{2}(GA)</em>{12}</td>
<td>56</td>
<td>235-245</td>
<td>5</td>
</tr>
<tr>
<td>Ast13</td>
<td>PET</td>
<td>F TTCTTGCGTGTCAAGAATGC R CAAATGGAAATGGTTGGGTC</td>
<td>(TG)_{11}</td>
<td>56</td>
<td>154-166</td>
<td>8</td>
</tr>
<tr>
<td>Ast14</td>
<td>NED</td>
<td>F CTGCGTTCGGGCTGTAGTAG R TGATGACGCTTGGTCTCAAC</td>
<td>(TG)_{10}</td>
<td>56</td>
<td>150-160</td>
<td>5</td>
</tr>
</tbody>
</table>
Genotypic richness and genetic diversity analysis

Individuals with identical multi-locus genotypes (MLGs) were detected with the program GENCLONE version 2.1. (Arnaud-Haond and Belkhir 2007). To assess if identical MLGs could be considered as a part of the same asexual lineage, the probability that two individuals sharing the same MLG originate from different sexual reproductive events ($P_{sex}$) was estimated. This probability depends on the allele frequencies in the population and the observed heterozygosities and was calculated with the program MLGsim (Stenberg et al. 2003) which uses a simulation approach and calculates significance values for the likelihood that a MLG observed more than once in a population is the result of random sexual reproduction. The number of simulations was adapted for each MLG and increased until a stable critical value was reached ($P < 0.05$). For each single population, genotypic richness ($R$) was estimated by ($R = G - 1 / N - 1$) where $G$ is the number of distinct multilocus genotypes and $N$ is the number of sample units (Dorken and Eckert 2001). Hence $R$ ranges between zero (monoclonal population) and 1 (each individual represents a genetically unique MLG). Since sample size varied between populations we performed a rarefaction analysis to calculate genotypic richness using the aRarefactWin software (v. 1.3; S. Holland, Stratigraphy Lab, University of Georgia, Athens; [www.uga.edu/~strata/software/](http://www.uga.edu/~strata/software/)). The smallest sample size (ZSO; $n = 10$) of the 13 lakes assayed was used to compare genotypic richness estimates between lakes. We further measured genetic diversity as allelic richness and expected heterozygosity. We also estimated allelic richness with a rarefaction approach using the program HP-Rare (Kalinowski 2004) which fixes $n$ as the smallest number of individuals typed for a locus in a sample. Since two markers showed an elevated number of amplification failure within some populations, we performed the analysis with 7 markers to avoid the fact that null amplification results in an underestimation of allelic richness. Weir & Cockerham (1984) $F_{IS}$ values, Nei’s unbiased expected heterozygosity ($H_e$) and observed heterozygosity $H_0$) were calculated with GENETIX version 4.05 (Belkhir et al. 1996-2004) for each locus. Hardy-Weinberg exact tests were performed using Genepop version 4.0 (Rousset 2008) assuming that the alternative hypothesis $H_1$ is a heterozygote deficiency. Linkage disequilibrium was calculated between all pairs of loci, with 10 000 dememorizations, 100 batches and 5000 iterations per batch. Critical significance levels were adjusted for multiple comparisons by using the sequential Bonferroni correction (Rice 1989).

Population genetic structure analysis

Population structure was analyzed by two different approaches: (1) using predefined populations corresponding to the 13 sampling locations (individual lakes and lake basins) and (2) using no
priori assumptions about population subdivision. We observed identical multilocus genotypes (MLGs) in our dataset, indicative of clonal structure. To explore the contribution of clonal structure on the spatial population genetic structure we created two datasets, one including all MLGs (All MLG) and another clone corrected data set, keeping only one copy of each MLG (CC).

The spatial distribution of genetic variance was analysed by hierarchical analyses of molecular variance (AMOVA) using the program Arlequin version 3.01 (Excoffier et al. 2005) with significance estimated based on 1000 permutations. Total genetic variance was partitioned into three hierarchical levels: (i) among region (CH, NL, ca. 600 km), (ii) among populations within the same region (5 – 50 km), and (iii) among individuals within populations. Pairwise $F_{ST}$ values for each possible pairwise combination of populations were estimated based on 1000 permutations in Arlequin. $F_{ST}$ and related statistics have the drawback that they may underestimate population differentiation when polymorphism is high, which is usually the case for microsatellite markers (Heller and Siegismund 2009). Therefore we also estimated Jost’s D, an alternative measure of genetic differentiation which is not dependent on marker variability (Jost 2008). Locus specific calculations of $D_{EST}$ were performed using the software program SMOGD (Crawford 2010). The overall $D_{EST}$ for each pairwise population comparison was calculated as the arithmetic mean across loci. Genetic relationships between populations were estimated as the proportion of shared alleles at each loci, i.e. allele sharing distances ($D_{AS}$) and were computed in POPULATIONS version 1.2.32 (Langella 1999). We used the resulting distance matrices to build UPGMA dendrograms with confidence determined by bootstrapping over loci (1000 replicates).

To infer population structure without a predefined population subdivision, first a factorial correspondence analysis (FCA) was performed on all individual isolates, as implemented in GENETIX version 4.05 (Belkhir et al. 1996-2004). To further identify clusters of genetically related individuals and to obtain probabilities of assignment for individuals to a cluster (or clusters when they are admixed) two different approaches were used. First the model based Bayesian clustering algorithm implemented in the software program InStruct (Gao et al. 2007) was run, which is an extension of the approach used in the program Structure. Runs with the clone corrected dataset were performed with a number of genetic clusters (K) ranging from 1 to 18. Each of the two MCMC chains used 1,000,000 iterations after a burning period of 500,000 steps. Optimal number of K (clusters) was determined by means of the Deviation Index Criterion (DIC), i.e. a model-complexity penalized measure of how well the model fits the data (Spiegelhalter et al. 2002). InStruct relaxes the assumptions of HWE although still assumes linkage equilibrium. Since clonal
organisms rarely meet this assumption the multivariate method Discriminant Analysis of Principal Components (DAPC), available in the ADEGENET package (Jombart 2008) for the R software (R development Core Team) was used. This method does not rely on a particular population genetics model and is therefore free of assumptions on HWE or linkage disequilibrium (Jombart et al. 2010). The number of clusters was assessed using the function “find.clusters”, which runs successive K-means clustering with increasing numbers of clusters (k). Selection of the ‘optimal number’ of clusters was based on visual inspection of the plots showing associated BIC (Bayesian information criterion) values for each number of clusters. In addition the criterion ‘goodfit’ was used to infer the optimal number of clusters. Separate analyses for linkage disequilibrium, $F_{IS}$ and population structure were repeated, considering the major model clusters detected by FCA and DAPC.

**Results**

**Genotypic and genetic diversity**

We found 94 distinct MLGs among the 182 individuals that could be genotyped for all 9 markers. When we excluded the 2 markers (Ast 04 and Ast 03) which contained most missing data points, we found 96 distinct MLGs among 215 individuals. Most of the MLGs were specific for their lake of origin. One MLG was shared by the two unconnected lakes CH-upper Lake Zürich and CH-Lake Hallwil. CH-upper Lake Zürich and CH-lower Lake Zürich also shared one MLG and two MLGs each were shared by the CH-Lake Lucerne basins A and B and basins B and C, respectively. One MLG was found in all three basins of Lake Lucerne. $P_{sex}$ values were very low and ascertain clonal identity in most cases (83%). For 17% of the detected identical multilocus genotypes, however, the $P_{sex}$ value was not significant, meaning that they could have originated from different sexual events.

Based on the rarefaction curves (Fig. 1) the CH lakes ZSO, ZSU, GS, HW, VWSB and VWSC had a similar degree of genotypic richness (lying within the 95 % confidence intervals for the lake with the smallest sample size). The CH lakes RS, BS, VWSA and NL lakes MSV and VKV had lower genotypic richness. Lowest genotypic richness was found in the two Dutch lakes NL-Ford Vechten and NL-Lake Zwemlust which consisted each of one single genotype (Table 3, Fig. 1).
Table 3: Genotypic and genetic diversity of *Asterionella formosa* populations

<table>
<thead>
<tr>
<th>Population</th>
<th>N</th>
<th>G</th>
<th>R</th>
<th>AR</th>
<th>SE&lt;sub&gt;AR&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS</td>
<td>22</td>
<td>10</td>
<td>0.43</td>
<td>3.64</td>
<td>0.45</td>
</tr>
<tr>
<td>HW</td>
<td>10</td>
<td>7</td>
<td>0.67</td>
<td>3.70</td>
<td>0.18</td>
</tr>
<tr>
<td>GS</td>
<td>35</td>
<td>28</td>
<td>0.76</td>
<td>4.19</td>
<td>0.52</td>
</tr>
<tr>
<td>RS</td>
<td>20</td>
<td>9</td>
<td>0.42</td>
<td>2.97</td>
<td>0.46</td>
</tr>
<tr>
<td>ZSU</td>
<td>22</td>
<td>12</td>
<td>0.52</td>
<td>3.15</td>
<td>0.29</td>
</tr>
<tr>
<td>ZSO</td>
<td>10</td>
<td>8</td>
<td>0.78</td>
<td>3.84</td>
<td>0.59</td>
</tr>
<tr>
<td>VWSA</td>
<td>13</td>
<td>5</td>
<td>0.33</td>
<td>3.28</td>
<td>0.39</td>
</tr>
<tr>
<td>VWSB</td>
<td>15</td>
<td>12</td>
<td>0.79</td>
<td>4.46</td>
<td>0.58</td>
</tr>
<tr>
<td>VWSC</td>
<td>13</td>
<td>9</td>
<td>0.67</td>
<td>4.41</td>
<td>0.46</td>
</tr>
<tr>
<td>FV</td>
<td>9</td>
<td>1</td>
<td>0.00</td>
<td>1.71</td>
<td>0.18</td>
</tr>
<tr>
<td>MSV</td>
<td>14</td>
<td>9</td>
<td>0.54</td>
<td>4.14</td>
<td>0.62</td>
</tr>
<tr>
<td>VKV</td>
<td>13</td>
<td>8</td>
<td>0.58</td>
<td>4.23</td>
<td>0.53</td>
</tr>
<tr>
<td>Z</td>
<td>20</td>
<td>1</td>
<td>0.00</td>
<td>1.71</td>
<td>0.18</td>
</tr>
<tr>
<td>Σ pop*</td>
<td>215</td>
<td>96</td>
<td>0.44</td>
<td>10.12</td>
<td>1.52</td>
</tr>
<tr>
<td>Σ pop**</td>
<td>182</td>
<td>94</td>
<td>0.51</td>
<td>11.22</td>
<td>1.60</td>
</tr>
</tbody>
</table>

* 7 marker, ** 9 marker

Sample size (N), number of genotypes (G), genotypic richness (R), mean allelic richness (AR), standard error of AR (SE<sub>AR</sub>)

![Rarefaction curves](image)

**Fig. 5**: Rarefaction curves (solid lines) show the genotypic richness of *A. formosa* in each lake and lake basin. Dotted grey lines are the 95% confidence intervals for the lake with the smallest sample size (ZSO; n = 10) which was used to compare genotypic richness estimates between lakes.
Genetic diversity, measured as allelic richness, also varied between lakes and was positively correlated with genotypic richness ($R^2 = 0.84$, $P < 0.001$, Fig. 2). Even when we excluded the two Dutch populations with extreme low genotypic richness, there was still a significant positive correlation ($R^2 = 0.51$, $P = 0.009$).

**Fig.2:** Relationship between genotypic and allelic richness of the thirteen sampled *A. formosa* populations.

*Linkage disequilibrium and heterozygosity deficiency:*  
Significant linkage disequilibrium was found in five populations with highest proportions observed in CH-Lake Greifen (67 %), CH-Lake Rot (25 %) and CH-upper Lake Zurich (28 %) (Table 4). All populations had a positive $F_{IS}$ across all loci and thus a significant heterozygosity deficiency, except for the populations of CH-Lake Lucerne basin A and C and NL-Lake Maarsseveen from which the genotypic distributions did not deviate from HWE expectations (Table 4, Table S1). For all the populations $F_{IS}$ values showed large variation across loci (Table S1).

*Population genetic structure analysis*  
**A priori grouping of individuals into individual lakes (or lake basins)**  
The results of the hierarchical analysis of molecular variance (AMOVA) and UPGMA clustering indicate that clonal structure affects genetic differentiation between populations. Overall AMOVA analysis revealed that most genetic variation was found within populations and least among countries. When all MLGs were included we found a significant genetic structuring at the three geographical levels (8.36% among countries, 21.99% among populations within countries and 69.66% within populations, Table 5). When the clone corrected dataset was used no significant structuring among countries was found (Table 5). Variation within populations increased and differentiation between populations was reduced but remained significant.
Table 4: Wright’s inbreeding coefficient (F<sub>IS</sub>) and linkage disequilibrium

<table>
<thead>
<tr>
<th>Region</th>
<th>Population</th>
<th>N</th>
<th>F&lt;sub&gt;IS&lt;/sub&gt;</th>
<th>LD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH</td>
<td>BS</td>
<td>10</td>
<td>0.28*</td>
<td>0 (36)</td>
</tr>
<tr>
<td>CH</td>
<td>HW</td>
<td>7</td>
<td>0.31*</td>
<td>0 (36)</td>
</tr>
<tr>
<td>CH</td>
<td>GS</td>
<td>28</td>
<td>0.35***</td>
<td>24 (36)</td>
</tr>
<tr>
<td>CH</td>
<td>RS</td>
<td>9</td>
<td>0.33**</td>
<td>7 (28)</td>
</tr>
<tr>
<td>CH</td>
<td>ZSU</td>
<td>12</td>
<td>0.37**</td>
<td>10 (36)</td>
</tr>
<tr>
<td>CH</td>
<td>ZSO</td>
<td>8</td>
<td>0.31*</td>
<td>0 (36)</td>
</tr>
<tr>
<td>CH</td>
<td>VWSA</td>
<td>5</td>
<td>0.20</td>
<td>0 (28)</td>
</tr>
<tr>
<td>CH</td>
<td>VWSB</td>
<td>12</td>
<td>0.19*</td>
<td>3 (36)</td>
</tr>
<tr>
<td>CH</td>
<td>VWSC</td>
<td>9</td>
<td>-0.02</td>
<td>0 (36)</td>
</tr>
<tr>
<td>NL</td>
<td>FV</td>
<td>1</td>
<td>Na</td>
<td>Na</td>
</tr>
<tr>
<td>NL</td>
<td>MSV</td>
<td>9</td>
<td>-0.10</td>
<td>0 (28)</td>
</tr>
<tr>
<td>NL</td>
<td>VKV</td>
<td>8</td>
<td>0.26*</td>
<td>1 (36)</td>
</tr>
<tr>
<td>NL</td>
<td>Z</td>
<td>1</td>
<td>Na</td>
<td>Na</td>
</tr>
</tbody>
</table>

*P < 0.05, **P < 0.01, ***P < 0.001

Linkage disequilibrium (LD): number of pairwise combinations of loci that were in significant genotypic disequilibrium after sequential Bonferroni correction (the number of tested combinations are in parentheses, for VWSA there were too many missing data for locus 04 therefore only 28 combinations are tested, and some loci were monomorphic which explains the other cases of reduced number of combinations)

Table 5: Analysis of molecular variance (AMOVA), including repeated MLGs (All MLGs) and only unique MLGs (CC)

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>Sum of squares</th>
<th>Variance components</th>
<th>Percentage of variation</th>
<th>Fixation indices</th>
<th>Pvalue</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All MLGs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among countries</td>
<td>1</td>
<td>61.906</td>
<td>0.23551</td>
<td>8.01</td>
<td>0.080 F&lt;sub&gt;CT&lt;/sub&gt;</td>
<td>P = 0.004</td>
</tr>
<tr>
<td>Among populations within countries</td>
<td>11</td>
<td>259.514</td>
<td>0.61959</td>
<td>22.28</td>
<td>0.242 F&lt;sub&gt;SC&lt;/sub&gt;</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Within populations</td>
<td>435</td>
<td>851.894</td>
<td>1.96289</td>
<td>69.71</td>
<td>0.376 F&lt;sub&gt;ST&lt;/sub&gt;</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Total</td>
<td>447</td>
<td>1173.27</td>
<td>2.82533</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among countries</td>
<td>1</td>
<td>16.448</td>
<td>0.09248</td>
<td>3.06</td>
<td>0.031 F&lt;sub&gt;CT&lt;/sub&gt;</td>
<td>P = 0.053</td>
</tr>
<tr>
<td>Among populations within countries</td>
<td>11</td>
<td>110.507</td>
<td>0.37994</td>
<td>13.85</td>
<td>0.130 F&lt;sub&gt;SC&lt;/sub&gt;</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Within populations</td>
<td>233</td>
<td>582.386</td>
<td>2.49951</td>
<td>83.09</td>
<td>0.248 F&lt;sub&gt;ST&lt;/sub&gt;</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Total</td>
<td>245</td>
<td>709.341</td>
<td>3.0069</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The UPGMA dendrograms reflect the results of AMOVA. In the dendrogram including all MLG’s the two Dutch lakes NL-Ford Vechten and NL-Zwemlust cluster together on a separate branch. NL-Lake Maarsseveen and NL-Vinkeveen also cluster together but are positioned within the cluster of Swiss lakes (Fig. 3A). In the dendrogram based on the clone corrected data set NL-Lake Maarsseveen and NL-Vinkeveen are positioned on separated branches, with NL-Lake Vinkeveen more closely related to Swiss lakes (Fig. 3B). The connected lakes CH-Lake Baldegg – CH-Lake Halwill and CH-lower Lake
Zurich - CH-upper Lake Zurich cluster together as well as the three different basins of Lake Luzern, indicating that lakes that are connected to each other are also genetically more similar (Fig. 3B).

**Fig. 3:** UPGMA cluster analysis of *Asterionella formosa* populations using the allele sharing distance (D<sub>AS</sub>). Numbers indicate bootstrap support values. UPGMA dendrograms are based on A) all repeated MLGs and B) unique MLGs. Populations are coded according to Table 1

When repeated MLGs were included, pairwise F<sub>ST</sub> values ranged from 0 to 0.549 and most were significant among populations (63 out of 78). F<sub>ST</sub> values decreased when only unique MLGs were included, ranging from 0 to 0.299 with only 15 out of 78 significant (Table 6). The significant F<sub>ST</sub> values were between CH-Lake Rot and NL-Lake Maarsseveen and the rest of the lakes. This pattern is again in agreement with the UPGMA dendrogram where the two lakes also were the most differentiated, being positioned at separate branches (Fig. 3B). D<sub>EST</sub> gave a similar pattern with only slightly higher estimates of differentiation (data not shown).
Table 6: Pairwise multi-locus $F_{ST}$ values between Asterionella formosa population pairs, including repeated MLGs (All MLGs) and only unique MLGs (CC)

**All MLGs**

<table>
<thead>
<tr>
<th></th>
<th>BS</th>
<th>HW</th>
<th>GS</th>
<th>RS</th>
<th>ZSU</th>
<th>ZSO</th>
<th>VWSA</th>
<th>VWSB</th>
<th>VWSC</th>
<th>FV</th>
<th>MSV</th>
<th>VKV</th>
<th>Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HW</td>
<td>0.154</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GS</td>
<td>0.184</td>
<td>0.088</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RS</td>
<td>0.346</td>
<td>0.327</td>
<td>0.304</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZSU</td>
<td>0.177</td>
<td>0.123</td>
<td>0.218</td>
<td>0.396</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZSO</td>
<td>0.148</td>
<td>0.090</td>
<td>0.067</td>
<td>0.283</td>
<td>0.193</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VWSA</td>
<td>0.223</td>
<td>0.088</td>
<td>0.184</td>
<td>0.386</td>
<td>0.082</td>
<td>0.197</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VWSB</td>
<td>0.130</td>
<td>0.060</td>
<td>0.042</td>
<td>0.273</td>
<td>0.125</td>
<td>0.051</td>
<td>0.090</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VWSC</td>
<td>0.205</td>
<td>0.141</td>
<td>0.066</td>
<td>0.309</td>
<td>0.270</td>
<td>0.096</td>
<td>0.249</td>
<td>0.037</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FV</td>
<td>0.440</td>
<td>0.358</td>
<td>0.347</td>
<td>0.490</td>
<td>0.441</td>
<td>0.396</td>
<td>0.443</td>
<td>0.339</td>
<td>0.333</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSV</td>
<td>0.200</td>
<td>0.199</td>
<td>0.152</td>
<td>0.340</td>
<td>0.319</td>
<td>0.161</td>
<td>0.338</td>
<td>0.176</td>
<td>0.178</td>
<td>0.397</td>
<td>0.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VKV</td>
<td>0.229</td>
<td>0.181</td>
<td>0.128</td>
<td>0.295</td>
<td>0.299</td>
<td>0.158</td>
<td>0.299</td>
<td>0.157</td>
<td>0.156</td>
<td>0.371</td>
<td>0.102</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Z</td>
<td>0.460</td>
<td>0.429</td>
<td>0.407</td>
<td>0.505</td>
<td>0.526</td>
<td>0.445</td>
<td>0.537</td>
<td>0.415</td>
<td>0.379</td>
<td>0.549</td>
<td>0.403</td>
<td>0.436</td>
<td>0.000</td>
</tr>
</tbody>
</table>

**CC**

<table>
<thead>
<tr>
<th></th>
<th>BS</th>
<th>HW</th>
<th>GS</th>
<th>RS</th>
<th>ZSU</th>
<th>ZSO</th>
<th>VWSA</th>
<th>VWSB</th>
<th>VWSC</th>
<th>FV*</th>
<th>MSV</th>
<th>VKV</th>
<th>Z*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HW</td>
<td>0.053</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GS</td>
<td>0.147</td>
<td>0.098</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RS</td>
<td>0.247</td>
<td>0.233</td>
<td>0.246</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZSU</td>
<td>0.117</td>
<td>0.095</td>
<td>0.203</td>
<td>0.299</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZSO</td>
<td>0.105</td>
<td>0.082</td>
<td>0.098</td>
<td>0.178</td>
<td>0.117</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VWSA</td>
<td>0.174</td>
<td>0.136</td>
<td>0.033</td>
<td>0.271</td>
<td>0.215</td>
<td>0.087</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VWSB</td>
<td>0.054</td>
<td>0.057</td>
<td>0.069</td>
<td>0.201</td>
<td>0.076</td>
<td>0.053</td>
<td>0.073</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VWSC</td>
<td>0.179</td>
<td>0.169</td>
<td>0.063</td>
<td>0.256</td>
<td>0.244</td>
<td>0.127</td>
<td>-0.025</td>
<td>0.106</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FV*</td>
<td>0.216</td>
<td>0.173</td>
<td>0.181</td>
<td>0.295</td>
<td>0.289</td>
<td>0.223</td>
<td>0.200</td>
<td>0.188</td>
<td>0.145</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSV</td>
<td>0.170</td>
<td>0.181</td>
<td>0.137</td>
<td>0.232</td>
<td>0.261</td>
<td>0.176</td>
<td>0.184</td>
<td>0.162</td>
<td>0.153</td>
<td>0.182</td>
<td>0.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VKV</td>
<td>0.103</td>
<td>0.096</td>
<td>0.100</td>
<td>0.186</td>
<td>0.186</td>
<td>0.104</td>
<td>0.095</td>
<td>0.096</td>
<td>0.091</td>
<td>0.138</td>
<td>0.072</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Z*</td>
<td>0.298</td>
<td>0.256</td>
<td>0.256</td>
<td>0.326</td>
<td>0.396</td>
<td>0.283</td>
<td>0.288</td>
<td>0.308</td>
<td>0.253</td>
<td>0.120</td>
<td>0.195</td>
<td>0.218</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Significant $F_{ST}$ values in bold ($P < 0.0005$ after sequential Bonferroni correction), *only one MLG; Lake Baldegg (BS), Lake Halwill (HW), Lake Greifen (GS), Lake Rot (RS), lower Lake Zurich (ZSU), upper Lake Zurich (ZSO), Lake Lucerne basin A (VWSA), Lake Lucerne basin B (VWSB), Lake Lucerne basin C (VWS C), Ford Vechten (FV), Lake Maarsseveen (MSV), Lake Vinkeveen (VKV), Lake Zwemlust (Z)
**Cryptic structure**

FCA performed on the clone corrected dataset resulted in the partitioning of individuals into three distinct genetic clusters which we termed A, B and C (Fig. 4). The largest cluster A represented a mix of individuals from all sampled lakes and basins, cluster B contained only individuals from Swiss lakes except CH-Lake Rot and CH-Lake Lucerne basin C and the smallest cluster C was only represented by CH-Lake Rot individuals. The Principle Coordinate Analysis (PcoA), performed with the subset of isolates analyzed with AFLP markers showed a similar clustering pattern. Clusters A and B were less strongly separated than in the FCA based on microsatellites. Cluster C, however, was also clearly distinct from cluster A and B (Fig. S1).

![Factorial Correspondence Analysis (FCA) including only unique MLGs of all Asterionella formosa populations. Only the first two axes are shown.](image)

**Fig. 4:** Factorial Correspondence Analysis (FCA) including only unique MLGs of all *Asterionella formosa* populations. Only the first two axes are shown.

\( F_{ST} \) values between the three clusters were high and each cluster contained private alleles with private allelic richness corrected for sample size, being highest for the largest cluster A (Table 7).
Table 7: Pairwise multi-locus $F_{ST}$ values between the three clusters A, B and C from the FCA cluster analysis

<table>
<thead>
<tr>
<th></th>
<th>cluster A</th>
<th>cluster B</th>
<th>cluster C</th>
<th>PAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>cluster A</td>
<td>-</td>
<td>3.84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cluster B</td>
<td>0.333</td>
<td>-</td>
<td>1.33</td>
<td></td>
</tr>
<tr>
<td>cluster C</td>
<td>0.321</td>
<td>0.593</td>
<td>-</td>
<td>0.56</td>
</tr>
</tbody>
</table>

Private allelic richness (PAR), Significant $F_{ST}$ values in bold ($P < 0.017$ after sequential Bonferroni correction)

The UPGMA dendrograms constructed for the populations within the major cluster A showed a similar pattern as the UPGMA analysis on the entire clone corrected dataset, i.e. no geographic clustering according to country was detected (Fig. 5A). Within cluster A, also no consistent clustering according to lake connectivity was found, whereas within cluster B lakes clustered together according to their connectedness (Fig. 5B).

Fig. 5: UPGMA cluster analysis of *Asterionella formosa* populations within cluster A (A) and cluster B (B) using the allele sharing distance ($D_{AS}$). UPGMA dendrograms are based on unique MLGs. Numbers indicate bootstrap support values. Populations are coded according to Table 1. For cluster B the three VWS basins are pooled together because of the very small sample sizes.
Discriminant Analysis of Principal Components (DAPC) analysis also showed a similar clustering pattern with the 3 major clusters as defined by FCA (Fig. S2). In addition it revealed even more substructure within the two major clusters. Because BIC values from the discriminant analysis of principal components (DAPC) decreased continuously with increasing number of K’s it was not straightforward to determine the optimal number of K (Fig. S3). Therefore the results are presented for different numbers of K, starting from K=3 until K=11 (the optimal K as determined by ‘goodfit’ criterion) (Fig. 6). With K set as 3, DAPC discriminates between the two major clusters A and B that were also found with FCA. The third cluster C which was comprised of a subset of CH-Lake Rot isolates was also differentiated from K=4 on (Fig. 6). With increasing number of K additional subclusters were found within both major clusters A and B. At the highest number of K (K=11), the larger cluster A contained 8 subclusters and cluster B only two.

Within the larger cluster A, individuals of most lakes were scattered over several clusters. The only lakes for which individuals grouped together in one cluster were NL-Lake Maarsseveen, CH-Lake Halwill and CH-lower and upper Lake Zurich (with the exception of one CH-lower Lake Zurich strain). Within cluster B CH-lower and upper Lake Zurich formed a separate cluster whereas the other lakes were not differentiated and clustered as one group together (Fig. 6). Similar results were found with InStruct (data not shown).
When individuals of each lake were separated according to their assignment to the three major clusters A, B and C, found by all clustering methods, $F_{IS}$ values decreased and became insignificant (Table 8). Linkage disequilibrium also decreased, except for the pooled CH-VWS basins (Table 8). However this may be due to the pooling of the basins since DAPC analysis showed that individuals of CH-VWS were scattered over several clusters.
Table 8: Wright’s inbreeding coefficient ($F_{IS}$) and linkage disequilibrium for each population separated according to the major clusters A, B and C.

<table>
<thead>
<tr>
<th>Population</th>
<th>N</th>
<th>$F_{IS}$</th>
<th>LD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cluster A</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BS</td>
<td>5</td>
<td>-0.238</td>
<td>0 (36)</td>
</tr>
<tr>
<td>HW</td>
<td>4</td>
<td>0.008</td>
<td>0 (36)</td>
</tr>
<tr>
<td>GS</td>
<td>22</td>
<td>0.037</td>
<td>8 (28)</td>
</tr>
<tr>
<td>RS</td>
<td>5</td>
<td>0.062</td>
<td>0 (36)</td>
</tr>
<tr>
<td>ZSU+ZSO</td>
<td>10</td>
<td>-0.095</td>
<td>4 (28)</td>
</tr>
<tr>
<td>VWSA+B+C</td>
<td>18</td>
<td>0.073</td>
<td>21 (36)</td>
</tr>
<tr>
<td>MSV</td>
<td>9</td>
<td>-0.103</td>
<td>0 (36)</td>
</tr>
<tr>
<td>VKV</td>
<td>9</td>
<td>0.259*</td>
<td>1 (36)</td>
</tr>
<tr>
<td><strong>Cluster B</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BS</td>
<td>5</td>
<td>0.04</td>
<td>0 (21)</td>
</tr>
<tr>
<td>HW</td>
<td>3</td>
<td>-0.429</td>
<td>Na</td>
</tr>
<tr>
<td>GS</td>
<td>6</td>
<td>-0.161</td>
<td>0 (13)</td>
</tr>
<tr>
<td>ZSU+ZSO</td>
<td>12</td>
<td>-0.172</td>
<td>0 (15)</td>
</tr>
<tr>
<td>VWSA+B</td>
<td>5</td>
<td>-0.391</td>
<td>Na</td>
</tr>
<tr>
<td><strong>Cluster C</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RS</td>
<td>6</td>
<td>-0.481</td>
<td>Na</td>
</tr>
</tbody>
</table>

Linkage disequilibrium (LD): number of pairwise combinations of loci that were in significant genotypic disequilibrium after sequential Bonferroni correction (the number of tested combinations are in parentheses. For HW, VWSA+B and RS the combination of low sample size, missing data and monomorphic loci did not allow the analysis of LD. The reduced number of tested combinations for the other lakes was mainly due to the presence of monomorphic loci.

*P < 0.05,

Discussion

Strong within lake population subdivision

The initial objective of our study was to determine genetic diversity and the scale of geographical population structure in the planktonic diatom *A. formosa*. We started with the assumption that each lake represented a single population. An unexpected pattern, however, revealed much higher genetic differentiation within lakes than among them. Cluster analyses without *a priori* assumptions of grouping detected three major genetically distinct clusters. Most intriguing was the dichotomous split up of all the Swiss lake populations into two clusters, whereas the Dutch populations were only present in the largest cluster and a subset of isolate of CH-Lake Rot formed a distinct cluster. The subset of strains that were analysed by AFLP (Amplified Fragment Length Polymorphism) revealed an identical pattern suggesting that these clusters were not a methodological artefact of the microsatellite markers (Fig. S1). Moreover, the clusters were robust towards removing loci that showed evidence for null alleles (data not shown). These results provided strong evidence for the presence of a Wahlund effect which may also explain the strong
deficiency of heterozygotes in almost all populations and a high proportion of loci in significant linkage disequilibrium in some of the populations.

Linkage across loci could be a sign for a dominant clonal reproductive mode. However, under such conditions one would also expect negative $F_{IS}$ values, indicating an excess of heterozygotes. This occurs due to the divergence of alleles within loci as the two copies will accumulate different mutations over time known as “Meselson effect” (Balloux et al. 2003; Halkett et al. 2005). Possible explanations for the observed positive $F_{IS}$ values are i) presence of null alleles, ii) inbreeding or iii) a Wahlund effect, i.e. population subdivision.

Our analysis with MICRO-CHECKER indeed revealed evidence for the presence of null alleles at some of the loci. However, despite exclusion of these loci from the analysis, $F_{IS}$ values remained positive. When null alleles would play an important role we would also expect that null homozygotes appear as missing genotypes (since homozygotes for a null allele will produce no PCR amplification). In general our dataset contained few missing values and we did not observe any pattern between $F_{IS}$ and the number of missing values across loci.

Inbreeding would be another mechanism which leads to an excess of homozygosity. In diatoms an immense variation of sexual reproductive patterns exist (Mann 1993) and there is evidence that some species are capable of intraclonal reproduction, i.e. selfing (Mann et al. 2004). When sexual reproduction occurs after a bloom consisting of few dominant genotypes, the chance of mating with a close relative could indeed be very high. Unfortunately there is no information on the mating system of *A. formosa* since sexual reproduction was never observed and could never be induced under laboratory conditions at present. However, when inbreeding/selfing would occur, one expects that it affects all loci equally (Conner and Hartl 2004), which was not the case.

When the individuals of each lake population were separated according to their assignment to the main clusters A, B and C, $F_{IS}$ values and LD decreased and most became insignificant. We are aware that in most of the cases where populations were split up, sample sizes became too low in order to properly test for Hardy Weinberg deviations and linkage. However, in the case of CH-Lake Greifen (cluster A) and CH-Lake Zurich (cluster A and B), sample sizes were still acceptable. Moreover, NL-Lake Maarsseveen, the only population which clustered as a single group together
showed no deviations of HWE and linkage. Taken all this, we conclude that subpopulation structure (i.e. Wahlund effect) was the main cause for deviations from Hardy Weinberg.

The unexpected presence of this strong population subdivision within lakes clearly influenced measures of genetic differentiation between lakes, since high structuring within populations leads consequently to an underestimation of between population structuring. This was visible from both the low Fst values between lakes and from the UPGMA dendrogram where Dutch lakes clustered together with Swiss lake and AMOVA analysis which did no gave evidence for significant regional structuring. In this respect, our study contrast greatly from the previous population genetic study with allozyme markers by (Soudek and Robinson 1983) which did not find genetic variation within populations but found considerable genetic differentiation among populations of different lakes.

Hidden diversity?
The results of our study raise an important question: are these subpopulations highly differentiated populations of a single species or do they represent different (cryptic) species? The present data is insufficient to provide a clear answer to this question. We can only argue that the observed pattern of genetically similar coexisting subpopulation pairs across most of the Swiss lakes (connected as well as unconnected) can only be achieved when gene flow between lakes is much higher than within lakes. Reproductive isolation between the coexisting subpopulations could lead to such a pattern, i.e. when they represent cryptic species.

Sympatric reproductive isolation has already been observed among several cryptic diatom species (Mann 1999, Amato et al. 2007). Reproductive barriers can occur on a spatial and/or temporal scale when populations for example differ in their breeding time. Genetically differentiated subgroups within a single geographical population of the marine dinoflagellate *Alexandrium tamarense* were attributed to the annual recruitment of the population from a heterogeneous cyst bed consisting of different year classes (Alpermann et al. 2009), similar to the recruitment of Daphnia from a heterogeneous dormant egg bank (Thielsch et al. 2009). Resting stages of *A. formosa* have never been found (Lund 1949) but cells have the ability to overwinter in a vegetative state (Lund 1949). Characteristic for diatom life history is the progressive cell size reduction during asexual growth with a reconstitution of cell size through sexual reproduction when a certain minimum cell size is attained (Lewis 1984). The simultaneous occurrence of different size classes within a lake has been observed in many freshwater diatoms, including *A.*
formosa (Mann 1988). Genotypes with different cell sizes may attain their sexual phase at different time points, thereby exchanging genes only with a certain subset of genotypes. Such asynchronous reproduction periods could generate coexisting populations with strong genetic differentiation. We, therefore, measured the frustule length of a subset of our isolates (from -80°C backups) that occurred in both clusters A and B. In Lower and Upper Lake Zurich we found two different size classes that corresponded to the two clusters A and B (Fig. S4). This suggests that such a temporal mechanism could be a potential explanation for the observed population subdivision. However, this clear dichotomy in size was not found in the other lakes which also had representatives in both clusters (Fig. S2).

Finally, a combination of mating experiments, multiple molecular markers and detailed morphological analysis is needed to determine to which extent these subpopulations are differentiated and may represent “cryptic species” within A. formosa (Mann 1999).

Local and regional diversity

Local (within lake) genotypic diversity of A. formosa, assessed in this study with microsatellite markers, was in between the two extreme findings of Soudek and Robinson (1983) (no diversity within lakes) and De Bruin et al. (2004) (each isolate genetically unique). The discrepancy between the levels of clonal diversity between both studies and ours is most probably due to the different markers used (Nybom 2004, Pang et al. 2010). In all lakes, also NL-Lake Maarseveen (studied by De Bruin et al. (2004)), we detected identical MLGs that belonged to the same clonal lineage with high probability. However, the proportion of clones varied among lakes. Two Dutch A. formosa populations showed extreme low diversity, i.e. they were represented by a single clone. These populations reflect the strong clonality of this species as observed by Soudek and Robinson (1983). The reasons for the extreme low genotypic richness in Lake Zwemlust and Fort Vechten could be due to both historical and morphological characteristics of these lakes. Both are artificial, isolated, small and shallow lakes. In the parthenogenetic water flea Daphnia it has been shown that populations inhabiting smaller water bodies had lower clonal diversity, mainly due to smaller population sizes causing increased genetic drift (Vanoverbeke et al. 2007). Moreover Lake Zwemlust was subjected to a recent biomanipulation event in 1989 where the whole lake was drained and allowed to refill with groundwater (Gulati and van Donk 2002). Founder effects could additionally play a role in the very low genetic diversity within this lake. The smaller differences in clonal richness between the other lakes may be related to different successional stages of the A.
Asterionella formosa bloom. Although we tried to minimize the time between samplings we observed during the isolation process that populations within lakes were at different stages of bloom development, i.e. *A. formosa* differed in abundance. Populations which were potentially in the early stage of a bloom (low cell abundances) showed the tendency to have higher clonal diversity than populations at the peak or end of a bloom (high abundances) (see Table 1). In addition, higher abundance was usually accompanied with higher prevalence of chytrid parasitism (personal observation, not quantified). Mechanisms like directional clonal selection where some genotypes are favoured under certain environmental conditions and profit from rapid asexual growth could lead to a decrease in clonal diversity. This was shown in a study on the cyanobacterium *Microcystis aeruginosa* where one dominant ITS (internal transcribed spacer) genotype was progressively selected during the development of the bloom (Briand et al. 2009). A microsatellite based study of Rynearson and Armbrust (2005) on the other hand did not observe a reduction of clonal diversity in the marine diatom *Ditylum brightwellii* during the entire spring bloom, suggesting balancing selection of genotypes caused by frequent environmental changes.

Overall clonal diversity of the freshwater diatom *A. formosa* was rather low (R = 0.51 averaged over all populations) compared to the majority of studies on marine protists (Evans et al. 2004; Iglesias-Rodriguez et al. 2006; Lowe et al. 2010; Masseret et al. 2009; Rynearson and Armbrust 2000) using microsatellite markers. We do not think that the quality of our microsatellite markers caused these different results since all loci showed moderate (5 alleles) to high (18 alleles) polymorphism. Since only few microsatellite studies exist on genotypic diversity within freshwater protists (Evans et al. 2009) it is difficult to estimate if the lower clonal diversity observed in *A. formosa* is species dependent or results from inherent differences between freshwater lake and marine habitats (as discussed above small vs. large habitats). When we consider the three distinct genetic groups as the unit of diversity we can say that this diversity was geographically unevenly distributed. Group A was present in all lakes whereas group B only occurred in the sampled Swiss lakes and group C was found in a single lake CH-Lake Rot. The presence of multiple genetic groups in the analysed Swiss lakes compared to a single genetic group in the Dutch lakes may be due to both historical and/or environmental factors (Vyverman et al. 2007). The Swiss lakes in this study were all natural lakes and of much older origin (retreat of Pleistocene glaciers ca. 10 000-16 000 years ago, Wetzel 2001) compared to the relatively young and artificial Dutch lakes that were sampled (oldest one from the 18th century). Asterionella in Swiss lakes may thus have had more time and opportunities to diverge. Swiss lakes had also a higher degree of connectedness than the
Dutch lakes which were all strongly isolated water bodies. Patterns of diatom richness has been shown to be highest in regions with numerous, highly connected water bodies where chances of successful colonization increase and local extinction rates are reduced, in agreement with theory of island biogeography and metacommunity concepts (Vyverman et al. 2007). In general the Swiss lakes also differed from the Dutch lakes in morphology, i.e. larger and deeper lakes. Larger habitats and spatial heterogeneity often correlates with higher diversity because of increased opportunities for diversifying selection (Vellend and Geber 2005). Interestingly, an early study by Ruttner (1937) described already the presence of two “ecotypes” of A. formosa in alpine lakes on the basis of their frustules length and vertical distribution. The smaller form hypolimnetica (33 to 54 µm) was found to be dominant in the deeper colder layers (10 m-40 m) of alpine lakes and described as stenotherm. The larger form epilimnetica (63 to 90 µm) was more dominant in the upper layers (0 m-22 m) and described as eurytherm. Beside cell size differences no other morphological differences were found between the two varieties. A hypothetical scenario for the distribution of the genetic groups can be formulated as Group B representing an “ecotype” that has diverged and spread within Swiss lakes, occupying similar ecological niches. This “ecological niche” may not be present in Dutch lakes (i.e. everything is everywhere but the environment selects) Another alternative is that group B only diverged recently and did not arrived yet in the sampled Dutch lakes. Or group A may have colonized the Dutch lakes first and benefits from this priority effect (monopolisation theory, see De Meester et al. 2002), preventing cluster B from establishing itself.

**Conclusion and Outlook:**

Our results on the genetic diversity and population structure patterns in the common freshwater diatom Asterionella formosa showed surprising but interesting patterns. However we are still in a very preliminary stage and elucidating these patterns will require much more sampling effort, additional molecular analyses and research on several aspects of diatom biology and ecology. It is crucial to obtain a better characterization of the three genetically distinct clusters in order to determine if we are dealing with a species complex within A. formosa. This will require a combination of several tools. (1) Molecular analysis with mitochondrial markers such as cox1 and cob genes have been successfully used in other protist studies for discerning cryptic species (Alverson 2008). (2) Fine-grained morphological investigations may reveal subtle differences between the groups. Pappas & Stoermer (2003) have put the taxonomic status of A. formosa earlier already into question and provide evidence for at least seven distinct shape groups of A.
formosa from the Great Lakes based on morphometric data. (3) Physiological studies may identify if the observed neutral genetic divergence also reflects adaptive divergence since ecological differences may influence genetic differences when it changes patterns of gene flow. (4) Ideally mating experiments should be performed to detect reproductive isolation between members of the different genetic groups (Mann 1999)

Over the past twenty years, an increasing number of cryptic and/or pseudo-cryptic microalgae species have been discovered within traditionally described cosmopolitan morphospecies (Amato et al. 2007; Beszteri et al. 2007; Poullickova et al. 2010; Smayda 2011; Trobajo et al. 2009). Moreover, the question whether dealing with large intraspecific genetic differentiation or presence of cryptic species is becoming a recurrent issue in population genetic studies with diatoms. This study again underlines that population genetic studies on protists are not trivial tasks since the potential presence of cryptic species can generate patterns which are difficult to interpret (Evans et al. 2009).

Asterionella formosa is ubiquitous and one of the dominant spring bloom phytoplankton species in many temperate lakes. It constitutes an important component of the food web and functioning of temperate lakes and is often used as a bioindicator. The potential hidden diversity found in this study may have implications for the further use of A. formosa in biogeographical studies and ecological monitoring programs (Mann 2010).

Acknowledgements

We thank Riet Schocher and Jean-Claude Bernegger from the Environment and Energy Competence Centre (uwe), Canton of Lucerne (Switzerland) for offering the facilities to sample Lake Lucerne, Lake Rot and Lake Baldegg. We also thank Jukka Jokela, Kirstin Kopp and Luc De Meester for helpful input and discussions. This research was funded by the ETH Board (CCES7 GEDIHAP).

References


de Wit R, Bouvier T (2006) 'Everything is everywhere, but, the environment selects'; what did Baas Becking and Beijerinck really say? Environ. Microbiol. 8:755-758


Heller R, Siegismund HR (2009) Relationship between three measures of genetic differentiation $G(ST)$, $D(EST)$ and $G'(ST)$: how wrong have we been? Mol. Ecol. 18:2080-2083


Mann DG (2010) Discovering diatom species: is a long history of disagreements about species-level taxonomy now at an end? Plant Ecology and Evolution 143:251-264


Pappas JL, Stoermer F (2003) Morphometric comparison of the neotype of *Asterionella formosa* Hassall (Heterokontophyta, Bacillariophyceae) with *Asterionella edlundii* sp. nov. from Lake Hovsgol, Mongolia. Diatom 19:55-65


Appendix

Table S1: Number of alleles observed on each locus in each population (Na), sample sizes (N), expected (He) and observed (Ho) heterozygosities, F<sub>s</sub>, probabilities of the Hardy-Weinberg test for a heterozygote deficiency (p-val), standard error of F<sub>s</sub> from a locus to the other in each population (SE)

<table>
<thead>
<tr>
<th>Locus</th>
<th>Pop</th>
<th>BS</th>
<th>HW</th>
<th>GS</th>
<th>RS</th>
<th>ZSU</th>
<th>ZSO</th>
<th>VWSA</th>
<th>VWSB</th>
<th>VWSC</th>
<th>FV</th>
<th>MSV</th>
<th>VKV</th>
<th>Z</th>
<th>Σ pop</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ast01</td>
<td>Na</td>
<td>6</td>
<td>4</td>
<td>7</td>
<td>2</td>
<td>5</td>
<td>6</td>
<td>3</td>
<td>5</td>
<td>5</td>
<td>2</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>He</td>
<td>0.79</td>
<td>0.75</td>
<td>0.75</td>
<td>0.42</td>
<td>0.53</td>
<td>0.85</td>
<td>0.62</td>
<td>0.75</td>
<td>0.58</td>
<td>1.00</td>
<td>0.76</td>
<td>0.53</td>
<td>0.00</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>Hobs.</td>
<td>0.60</td>
<td>0.29</td>
<td>0.64</td>
<td>0.36</td>
<td>0.13</td>
<td>0.71</td>
<td>0.40</td>
<td>0.58</td>
<td>0.43</td>
<td>1.00</td>
<td>0.78</td>
<td>0.38</td>
<td>0.00</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>F&lt;sub&gt;s&lt;/sub&gt;</td>
<td>0.25</td>
<td>0.64</td>
<td>0.15</td>
<td>0.13</td>
<td>0.75</td>
<td>0.17</td>
<td>0.38</td>
<td>0.23</td>
<td>0.28</td>
<td>NA</td>
<td>-0.02</td>
<td>0.30</td>
<td>NA</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>p-val</td>
<td>NS</td>
<td>*</td>
<td>NS</td>
<td>NS</td>
<td>***</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NA</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NA ***</td>
</tr>
<tr>
<td>Ast02</td>
<td>Na</td>
<td>5</td>
<td>4</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>7</td>
<td>4</td>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>He</td>
<td>0.75</td>
<td>0.67</td>
<td>0.64</td>
<td>0.71</td>
<td>0.51</td>
<td>0.30</td>
<td>0.36</td>
<td>0.59</td>
<td>0.49</td>
<td>1.00</td>
<td>0.84</td>
<td>0.70</td>
<td>1.00</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>Hobs.</td>
<td>0.40</td>
<td>0.29</td>
<td>0.21</td>
<td>0.36</td>
<td>0.07</td>
<td>0.00</td>
<td>0.00</td>
<td>0.18</td>
<td>0.13</td>
<td>1.00</td>
<td>0.89</td>
<td>0.25</td>
<td>1.00</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>F&lt;sub&gt;s&lt;/sub&gt;</td>
<td>0.48</td>
<td>0.59</td>
<td>0.67</td>
<td>0.50</td>
<td>0.87</td>
<td>1.00</td>
<td>1.00</td>
<td>0.70</td>
<td>0.76</td>
<td>NA</td>
<td>-0.07</td>
<td>0.66</td>
<td>NA</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>p-val</td>
<td>*</td>
<td>*</td>
<td>***</td>
<td>*</td>
<td>***</td>
<td>NS</td>
<td>NS</td>
<td>*</td>
<td>NS</td>
<td>NA</td>
<td>NS</td>
<td>**</td>
<td>NA</td>
<td>***</td>
</tr>
<tr>
<td>Ast04</td>
<td>Na</td>
<td>4</td>
<td>5</td>
<td>9</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>2</td>
<td>7</td>
<td>3</td>
<td>2</td>
<td>6</td>
<td>5</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>He</td>
<td>0.69</td>
<td>0.87</td>
<td>0.72</td>
<td>0.73</td>
<td>0.78</td>
<td>0.65</td>
<td>0.43</td>
<td>0.82</td>
<td>0.44</td>
<td>1.00</td>
<td>0.82</td>
<td>0.73</td>
<td>0.00</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>Hobs.</td>
<td>0.69</td>
<td>0.87</td>
<td>0.71</td>
<td>0.68</td>
<td>0.76</td>
<td>0.65</td>
<td>0.43</td>
<td>0.77</td>
<td>0.43</td>
<td>1.00</td>
<td>0.82</td>
<td>0.73</td>
<td>0.69</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>F&lt;sub&gt;s&lt;/sub&gt;</td>
<td>0.40</td>
<td>0.60</td>
<td>0.61</td>
<td>0.18</td>
<td>0.47</td>
<td>0.57</td>
<td>0.50</td>
<td>0.67</td>
<td>0.50</td>
<td>1.00</td>
<td>0.67</td>
<td>0.56</td>
<td>0.40</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>p-val</td>
<td>**</td>
<td>*</td>
<td>*</td>
<td>**</td>
<td>*</td>
<td>NS</td>
<td>NS</td>
<td>**</td>
<td>NS</td>
<td>NA</td>
<td>NS</td>
<td>NS</td>
<td>NA</td>
<td>***</td>
</tr>
<tr>
<td>Ast08</td>
<td>Na</td>
<td>5</td>
<td>4</td>
<td>7</td>
<td>5</td>
<td>6</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>1</td>
<td>5</td>
<td>6</td>
<td>1</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>He</td>
<td>0.73</td>
<td>0.49</td>
<td>0.75</td>
<td>0.77</td>
<td>0.41</td>
<td>0.78</td>
<td>0.80</td>
<td>0.79</td>
<td>0.81</td>
<td>0.00</td>
<td>0.77</td>
<td>0.84</td>
<td>0.73</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>Hobs.</td>
<td>0.70</td>
<td>0.43</td>
<td>0.46</td>
<td>0.55</td>
<td>0.33</td>
<td>0.71</td>
<td>0.80</td>
<td>0.67</td>
<td>1.00</td>
<td>0.00</td>
<td>0.56</td>
<td>0.89</td>
<td>0.70</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>F&lt;sub&gt;s&lt;/sub&gt;</td>
<td>0.04</td>
<td>0.14</td>
<td>0.39</td>
<td>0.30</td>
<td>0.20</td>
<td>0.09</td>
<td>0.00</td>
<td>0.16</td>
<td>-0.30</td>
<td>NA</td>
<td>0.29</td>
<td>-0.06</td>
<td>NA</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>p-val</td>
<td>NS</td>
<td>NS</td>
<td>**</td>
<td>*</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NA</td>
<td>NS</td>
<td>NS</td>
<td>NA</td>
<td>**</td>
</tr>
<tr>
<td>Ast03</td>
<td>Na</td>
<td>6</td>
<td>6</td>
<td>5</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>8</td>
<td>6</td>
<td>2</td>
<td>5</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>He</td>
<td>0.65</td>
<td>0.63</td>
<td>0.70</td>
<td>0.69</td>
<td>0.72</td>
<td>0.60</td>
<td>0.72</td>
<td>0.86</td>
<td>0.80</td>
<td>0.50</td>
<td>0.71</td>
<td>0.77</td>
<td>0.50</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>Hobs.</td>
<td>0.68</td>
<td>0.68</td>
<td>0.72</td>
<td>0.73</td>
<td>0.74</td>
<td>0.65</td>
<td>0.80</td>
<td>0.90</td>
<td>0.86</td>
<td>1.00</td>
<td>0.77</td>
<td>0.81</td>
<td>1.00</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>F&lt;sub&gt;s&lt;/sub&gt;</td>
<td>0.13</td>
<td>0.17</td>
<td>-0.22</td>
<td>0.60</td>
<td>0.11</td>
<td>0.13</td>
<td>0.00</td>
<td>-0.02</td>
<td>-0.18</td>
<td>NA</td>
<td>-0.13</td>
<td>-0.10</td>
<td>NA</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>p-val</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NA</td>
<td>NS</td>
<td>NS</td>
<td>NA</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>Ast05</td>
<td>Na</td>
<td>7</td>
<td>4</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>8</td>
<td>6</td>
<td>1</td>
<td>6</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>---</td>
<td>-------</td>
<td>-----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td></td>
<td>He</td>
<td>0.84</td>
<td>0.80</td>
<td>0.78</td>
<td>0.75</td>
<td>0.54</td>
<td>0.63</td>
<td>0.78</td>
<td>0.88</td>
<td>0.78</td>
<td>0.00</td>
<td>0.86</td>
<td>0.89</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hobs.</td>
<td>0.90</td>
<td>0.86</td>
<td>0.75</td>
<td>0.33</td>
<td>0.27</td>
<td>0.29</td>
<td>0.60</td>
<td>0.83</td>
<td>0.63</td>
<td>0.00</td>
<td>0.78</td>
<td>0.78</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$F_{IS}$</td>
<td>-0.08</td>
<td>-0.07</td>
<td>0.04</td>
<td>0.57</td>
<td>0.52</td>
<td>0.56</td>
<td>0.25</td>
<td>0.06</td>
<td>0.21</td>
<td>NA</td>
<td>0.10</td>
<td>0.13</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p-val</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>*</td>
<td>*</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NA</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Ast10</th>
<th>Na</th>
<th>2</th>
<th>3</th>
<th>2</th>
<th>1</th>
<th>2</th>
<th>2</th>
<th>2</th>
<th>2</th>
<th>3</th>
<th>2</th>
<th>2</th>
<th>4</th>
<th>2</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>He</td>
<td>0.10</td>
<td>0.54</td>
<td>0.49</td>
<td>0.00</td>
<td>0.34</td>
<td>0.44</td>
<td>0.47</td>
<td>0.39</td>
<td>0.51</td>
<td>1.00</td>
<td>0.53</td>
<td>0.66</td>
<td>1.00</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hobs.</td>
<td>0.10</td>
<td>0.57</td>
<td>0.14</td>
<td>0.00</td>
<td>0.42</td>
<td>0.57</td>
<td>0.20</td>
<td>0.33</td>
<td>0.63</td>
<td>1.00</td>
<td>1.00</td>
<td>0.33</td>
<td>1.00</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$F_{IS}$</td>
<td>NA</td>
<td>-0.07</td>
<td>0.71</td>
<td>NA</td>
<td>-0.22</td>
<td>-0.33</td>
<td>0.60</td>
<td>0.15</td>
<td>-0.25</td>
<td>NA</td>
<td>-1.00</td>
<td>0.51</td>
<td>NA</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p-val</td>
<td>NA</td>
<td>NS</td>
<td>***</td>
<td>NA</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NA</td>
<td>NS</td>
<td>NS</td>
<td>NA</td>
<td>**</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Ast13</th>
<th>Na</th>
<th>5</th>
<th>3</th>
<th>3</th>
<th>4</th>
<th>4</th>
<th>5</th>
<th>5</th>
<th>5</th>
<th>5</th>
<th>2</th>
<th>3</th>
<th>5</th>
<th>2</th>
<th>8.00</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>He</td>
<td>0.72</td>
<td>0.67</td>
<td>0.46</td>
<td>0.55</td>
<td>0.69</td>
<td>0.79</td>
<td>0.87</td>
<td>0.71</td>
<td>0.81</td>
<td>1.00</td>
<td>0.31</td>
<td>0.76</td>
<td>1.00</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hobs.</td>
<td>0.50</td>
<td>0.43</td>
<td>0.29</td>
<td>0.50</td>
<td>0.36</td>
<td>0.43</td>
<td>1.00</td>
<td>0.75</td>
<td>1.00</td>
<td>1.00</td>
<td>0.33</td>
<td>0.56</td>
<td>1.00</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$F_{IS}$</td>
<td>0.31</td>
<td>0.38</td>
<td>0.38</td>
<td>0.10</td>
<td>0.49</td>
<td>0.48</td>
<td>-0.18</td>
<td>-0.05</td>
<td>-0.26</td>
<td>NA</td>
<td>-0.09</td>
<td>0.28</td>
<td>NA</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p-val</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>**</td>
<td>*</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NA</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Ast14</th>
<th>Na</th>
<th>3</th>
<th>4</th>
<th>4</th>
<th>3</th>
<th>3</th>
<th>3</th>
<th>4</th>
<th>4</th>
<th>4</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>2</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>He</td>
<td>0.61</td>
<td>0.71</td>
<td>0.64</td>
<td>0.48</td>
<td>0.60</td>
<td>0.67</td>
<td>0.73</td>
<td>0.72</td>
<td>0.65</td>
<td>1.00</td>
<td>0.58</td>
<td>0.76</td>
<td>1.00</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hobs.</td>
<td>0.20</td>
<td>0.29</td>
<td>0.29</td>
<td>0.50</td>
<td>0.73</td>
<td>0.29</td>
<td>0.60</td>
<td>0.50</td>
<td>0.75</td>
<td>1.00</td>
<td>1.00</td>
<td>0.56</td>
<td>1.00</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$F_{IS}$</td>
<td>0.68</td>
<td>0.62</td>
<td>0.55</td>
<td>-0.03</td>
<td>-0.22</td>
<td>0.59</td>
<td>0.20</td>
<td>0.31</td>
<td>-0.17</td>
<td>NA</td>
<td>-0.80</td>
<td>0.29</td>
<td>NA</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p-val</td>
<td>*</td>
<td>*</td>
<td>NS</td>
<td>NS</td>
<td>*</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NA</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NA</td>
<td>***</td>
</tr>
</tbody>
</table>

|   | Σ loci | Na  | 43 | 37 | 44 | 35 | 38 | 35 | 49 | 46 | 16 | 42 | 43 | 15 | 102 |
|---|-------|-----|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|   |        | He  | 0.66 | 0.69 | 0.67 | 0.58 | 0.60 | 0.64 | 0.65 | 0.69 | 0.68 | 0.78 | 0.69 | 0.74 | 0.67 | 0.76 |
|   |        | Hobs.| 0.49 | 0.48 | 0.51 | 0.36 | 0.41 | 0.46 | 0.54 | 0.53 | 0.69 | 0.78 | 0.76 | 0.58 | 0.67 | 0.51 |
|   |        | $F_{IS}$ | 0.27 | 0.32 | 0.28 | 0.41 | 0.38 | 0.30 | 0.18 | 0.17 | -0.02 | NA | -0.11 | 0.24 | NA | 0.34 |
|   |        | p-val | ** | * | *** | ** | *** | * | NS | * | NS | NA | NS | * | NA | *** |
|   |        | SE $F_{IS}$ | 0.09 | 0.09 | 0.10 | 0.13 | 0.13 | 0.13 | 0.13 | 0.12 | 0.09 | NA | 0.15 | 0.08 | NA | 0.03 |
Fig. S1: Principle Coordinate Analysis (PcoA), performed with a subset of *A. formosa* isolates analyzed with AFLP markers based on Jaccard’s similarity coefficient.

Fig. S2: Scatterplot of Discriminant analysis of principal components (DAPC) for K = 11, including unique MLGs of all *Asterionella formosa* populations. The scatterplot shows the first two principal components of the DAPC. The eleven clusters are shown by different colours and inertia ellipses, while dots represent individual isolates.
**Fig. S3:** BIC values of the DAPC analysis for increasing number of K (from K=1 until K=20).

**Fig. S4:** Box plot of frustules cell length of upper and Lower Lake Zurich (ZSU and ZSO), Lake Baldegg (BS), Lake Greifen late sampling (GSL), Lake Lucerne basin B (VWSB) and Lake Halwill (HW) within each of the clusters A and B. The horizontal line represents the median and whiskers indicate the 5th and 95th percentiles.
Summary discussion and outlook

Human activities have a major impact on ecosystems worldwide. The building blocks of ecosystems are organisms that interact with each other and their environment. It is therefore important to assess how anthropogenic factors in the environment potentially destabilize species interactions since this can have far reaching consequences for whole ecosystem functioning. Species interactions vary from competition to predator prey or host parasite interactions. Environmental change, including eutrophication, climate warming and pollution may change the course of all these interaction types. In this thesis I focused on the potential for toxicants in the environment to change host parasite interactions in lake plankton. Anthropogenic stressors such as herbicides / toxicants are commonly expected to weaken the host resistance due to repair – resistance trade-offs and therefore make the host more vulnerable to parasite infection. However, decreased host abundance due to decreased host population growth under impact of environmental stress might also disrupt parasite transmission. Moreover other toxicants, e.g. fungicides affect the parasite, rather than the host. In these ways, pesticides might in fact decrease the burden of disease. This unexpected twist may have far reaching consequences for host and parasite abundances, their interaction and persistence in ecosystems.

In this thesis I used a diatom host - chytrid parasite system to explore how an anthropogenic pollutant (herbicide) affects the strength and direction of host-parasite interactions.

The diatom host Asterionella formosa is often one of the dominating phytoplankton species during the phytoplankton spring bloom in temperate lakes. The chytrid parasite Zygorhizidium plankticum is an obligate, host-specific parasite of A. formosa. Hence, the parasite is completely dependent on its host for reproduction and survival. The parasite’s free swimming motile stages (zoospores) have a limited time to find and infect their host. After zoospore encystment a germ tube is formed which enters the host cell through the girdle zone. Via the germ tube nutrients are extracted from the host cell and used for the development of the sporangium. New zoospores are formed either asexually or sexually and are released from the sporangium by dehiscence (Doggett and Porter 1996; Ibelings et al. 2004).

The first study evaluated how the herbicide affected the prevalence and spread of the parasite infection in the host population (chapter 1). The second study concentrated on the effects of the
herbicide on parasite transmission and infectiveness, a pivotal step in the life history of the parasite (chapter 2). A third study explored the genetic diversity and spatial structure of the host A. formosa (chapter 3). I will first discuss the two chapters dealing with the host-parasite-herbicide interaction and thereafter discuss the results of the third study with an outlook on its possible implications for the host-parasite interaction described in chapters 1 and 2.

In chapter 1 we showed that one-time exposure to the herbicide diuron as well as the chytrid parasite reduced the fitness of all host populations. However, the extent to which host fitness was reduced was related to the mean cell size of the host genotypes. The host population with the smallest cell size experienced the highest fitness reduction under herbicide exposure whereas host populations with larger mean cell size suffered more from parasites. Smaller phytoplankton species have been found to be more sensitive to toxicants because they accumulate higher amounts of the toxicant relative to larger species (Lockert et al. 2006; Tang et al. 1998). The increased impact of parasites on host populations with larger cell volume is mainly driven by a higher parasite reproductive output on larger host cells (chapter 2). Characteristic for diatom life history is the progressive cell size reduction during asexual growth with a reconstitution of cell size through sexual reproduction when a certain minimum cell size is attained. The same genotype can thus produce different phenotypes (i.e. cell sizes), depending on its stage of cell-size reduction. The fact that phenotype plays an important role in diatom host ecology does not mean that a genetic basis between A. formosa and Z. planktonicum is absent. Earlier studies by De Bruin et al. (2008; 2004) have repeatedly demonstrated that genetically different A. formosa isolates varied in susceptibility to a number of parasite isolates, with several – genetically distinct – host isolates showing resistance to some parasite strains (never to all). In addition, different Z. planktonicum parasite isolates differed in their infectivity on a single host isolate. Our results however show that phenotypic traits (host cell volume) may obscure clear genotypic differences and therefore have to be considered when studying strain sensitivity to toxicants or host-parasite genotype interactions.

In chapter 1 we also show that herbicides modify the spread of disease. This modification was twofold in the sense that parasite transmission was first inhibited and later enhanced compared to control conditions. This resulted at first in an antagonistic interaction between herbicide and parasite on exponential host growth rate, since herbicide exposed hosts profited from the lower parasite transmission. From this we could conclude that herbicide stress decreased the impact of disease on host population growth. However, after this initial phase, the parasite showed accelerated transmission rates, therefore still managed to infect more hosts, in the presence of
the herbicide and consequently reduced the final density of uninfected hosts. Thus herbicide exposure in the end increased the severity of epidemics. However the different patterns of early vs. late parasite transmission indicate that herbicides can affect host-parasite interactions in opposite ways. While experimental epidemiology gives insight in secondary order effects at the population level, it has its drawbacks for elucidating mechanistic cause and effect relationships because the host density and stressor environment cannot be kept constant.

In order to better understand and predict disease dynamics we designed experiments and an agent based simulation model (ABM) to test specific hypotheses about the mechanisms that we hypothesized could underlie the observed dynamics (chapter 2). We hypothesized that acute herbicide exposure initially constrains parasite transmission either through a direct host density effect (because diuron reduces host population growth through inhibition of photosynthesis) or by interfering with host finding mechanisms based on chemotactic cues. Earlier studies proposed that zoospores depend on photosynthesis exudates of the host to recognize and localize host cells because severe light limitation decreases zoospore infectivity and dark conditions even completely inhibit infections. Light limitation or photosynthesis inhibition by the herbicide diuron could have analogous effects on parasite transmission. Further we hypothesized that the subsequent increase in prevalence of infection results from the combination of several mechanisms. First, higher host density increases contact rates and make chemotaxis processes less relevant for transmission success (Kuhn 1997). Second, longer exposure to herbicides reduces the energy budget of the host and makes them more susceptible for infection (Rohr et al. 2008a; Saarinen and Taskinen 2005). Third, a herbicide exposed environment could affect the parasite reproduction and/or maturation time. The experimental results showed that increased host exposure time to the stressor did not lead to increased parasite infectivity (parasite transmission). Neither did we find large effects of herbicide exposure on the reproductive output and/or development time of the parasite. However, we did find that herbicide exposure negatively interfered with parasite transmission and this, as hypothesized, only at low host density. At high host density, herbicide exposure even enhanced transmission. The ABM model that simulated herbicide exposure by the simple absence of chemotaxis was able to produce patterns similar to those observed in our experiment. However, it could not completely reproduce the significant higher infection success in the presence of the herbicide at high host density. Still our experimental and simulation results support the hypothesis that chemotaxis is an important process in this host-parasite system but also that the importance of chemotaxis in locating hosts is host density dependent. Thus in
chapter 2 we could reproduce the infection dynamic observed in chapter 1 solely through the interaction effect between herbicide exposure and host density on parasite transmission success. Interference of the herbicide with host finding mechanisms explained the initial parasite transmission inhibition. Why parasite transmission success is enhanced by herbicide exposure at high host density remains unresolved at present. An interesting hypothesis is ‘density-dependent immune prophylaxes’. For example, it has been shown that grasshoppers in dense populations up-regulate their immune defences to prepare for possible epidemics which are more likely to occur under these conditions (Wilson et al. 2002). A. formosa could have a similar strategy that it only up regulates immune defences at high host densities. Interference of the herbicide with mounting up this immune defence could then perhaps explain the increased infection success of the parasite. However, longer pre exposure to the herbicide alone, without additional herbicide exposure, did not result in increased infections. How A. formosa defends itself against chytrid attacks is still a black box. (Canter and Jaworski 1979) have observed that some clonal strains of A. formosa show hypersensitive death reaction to infection by Rhizophydiun planktonicum resulting in growth arrestment and finally death of the parasite. However, in all the infection experiments we conducted no signs of a hypersensitive reaction were observed.

To return to the main question of this thesis: does anthropogenic pollution increase or decrease the impact of disease in host populations?

The experimental results of both chapter 1 and 2 indicate that herbicide exposure will only increase the spread of disease when sufficient high host densities are present to outweigh the negative effects of herbicide exposure on host finding. Peak concentrations of herbicides are commonly found in spring after field application and rain events (Rabiet et al. 2010). This is also the time where the phytoplankton spring bloom develops (including A. formosa bloom) and chytrid epidemics occur. The “low” host density conditions in our experiments were already very high and comparable with peak bloom densities in the field (Ibelings et al. 2011). It may therefore be expected that the negative effect of herbicide exposure on chytrid host finding mechanisms (transmission efficiency) is even more pronounced in the field where densities of A. formosa are relatively low compared to our experimental host densities.

Combinations of experiments and a long term field data study on A. formosa- chytrid epidemics in Lake Maarsseveen (The Netherlands) have shown that climate change reduces epidemics. During cold winters, temperatures below 3°C offer A. formosa a disease-free window of opportunity for
growth allowing blooms of high densities that later facilitate chytrid transmission. Climate change has reduced the occurrence of such disease free growth periods. In warmer winters, the host is therefore denied a bloom and consequently the parasite cannot form an epidemic because insufficient hosts are available for efficient transmission (Ibelings et al., 2011; Gsell et al, in press). The negative effects of climate change on A. formosa bloom formation in combination with herbicide exposure interfering with host finding mechanisms could become critical for the survival of chytrids, but this is a matter of speculation since no work has been done to investigate the 3-way interaction between climate, pollution and disease.

It is clear that testing only one herbicide with one specific mode of action at only one concentration cannot tell the whole story about the impact of anthropogenic pollution on disease. However, in microalgae, many abiotic and biotic stress factors are likely to affect photosynthesis, so if decreased photosynthesis negatively affects parasite transmission, one could generalize that increased stress conditions in the environment will probably lead to a decreased spread of disease in phytoplankton populations. We are aware however that caution is required when extrapolating results from batch experiments to the field. Under natural conditions, in the pelagic water, water turbulence can also influence zoospore movement and host finding efficiency (Bertrand et al. 2004; Llaveria et al. 2010). We also used only one chytrid strain in our experiments. There might be genetic variation in chytrids for host-finding ability. Reduction in chemical host cues (through photosynthesis inhibition) could select for chytrids with the most efficient host finding mechanisms and mitigate the negative effect on parasite transmission.

An increasing number of studies have been carried out examining the effects of different pollutants on a variety of host-parasite systems but showed contrasting results. This may not be so surprising since parasites are a heterogeneous group of organisms with a huge variety in life history and host exploitation strategies, making it difficult to formulate general predictions on how pollution affects disease dynamics. However, a way to improve general predictions about the outcome of the combined effects of pollution and parasites is by classifying host-parasite interactions into functional groups and try to detect general patterns associated with specific host-parasite characteristics. Some scientists have started to relate the effect of pollution to specific life history traits of parasites. For example, a meta-analysis study on pollution and parasitism in aquatic animals by (Blanar et al. 2009) could show that monoxenous parasite taxa (1 obligatory host in life cycle) were more susceptible to pollution than heteroxenous (life cycle requiring more
than one host). Similarly, directly exposed (ectoparasites and parasites with free living stages) and freshwater taxa were more susceptible to a wider range of pollutants than indirectly exposed (internal parasites) and marine taxa. Theoretical models by (Lafferty and Holt 2003) on the impact of stress on disease spread in host populations suggest that the impact of host-specific diseases (parasite specialists) generally decline with stress while the impact of non-specific disease (parasite generalists) increases with stress. This thesis together with earlier work on a host specific chytrid with density dependent transmission, support these theoretical expectations.

In chapter 3 we studied the genetic diversity and population structure of the host Asterionella formosa in different lakes and on different spatial scales with microsatellite markers, and partly also with AFLP markers. Many freshwater and marine algal species (including A. formosa) are described as having cosmopolitan distributions which seems to be in accordance with the “everything is everywhere, the environment selects” metaphor Beijerinck (1919). However, an increasing body of research contrasts this idea and show evidence that microorganisms exhibit a biogeography (Vyverman et al. 2007). Whether these widely distributed morphologically similar algal species also share a similar gene pool remains often unclear. However, this is an important question since genetic variability forms the basis for adaptation of species to a changing world, whether these changes are due to natural factors like coevolving parasites or human caused pollution. There is increasing evidence that populations become more susceptible to disease as their genetic diversity decreases. At the same time parasites can be important drivers behind species and genetic diversity in natural populations (Altizer et al. 2003, Spielman et al. 2004).

In the context of island biogeography theory, relatively strong spatial isolation of populations in freshwater lakes should restrict gene flow and lead to higher genetic differentiation among lakes. The initial objective was to determine the geographical scale at which gene pools of A. formosa populations are structured. An unexpected pattern, however, revealed much higher genetic differentiation within lakes than among them. Three major, genetically very distinct, clusters were detected. Most intriguing was the dichotomous split up of all the Swiss lake populations over two clusters, whereas the Dutch populations were only present in the largest cluster. This raises an interesting question: do we have to resolve another “identity crisis” of the presumed cosmopolitan diatom A. formosa? Over the last decade, an increasing number of cryptic microalgae species have been discovered within traditionally described cosmopolitan
morphospecies, thus it would not be surprising to find similar results for *A. formosa* (Poulickova et al. 2010; Smayda 2011; Trobajo et al. 2009).

Further work should determine how far diverged these subpopulations are and if they can still be considered a single species. A combination of molecular, morphological and physiological techniques is required. More conservative markers such as ribosomal DNA or internal transcribed spacer regions (ITS) and detailed morphological characterisation with scanning electronic microscopy should be applied. In addition physiological studies should be carried out to see to which extent ecophysiological traits are diverged between isolates of the two subpopulations.

Interestingly, an early study by Ruttner (1937) described already the presence of two “ecotypes” of *A. formosa* in alpine lakes on the basis of their frustules length and vertical distribution. The smaller form hypolimnetica (33 to 54 um) was found to be dominant in the deeper colder layers (10m-40m) of alpine lakes and described as stenotherm. The larger form epilimnetica (63 to 90 um) was more dominant in the upper layers (0m-22m) and described as eurytherm. Beside cell size differences no other morphological differences were found between the two varieties. The different size classes found between the two clusters of Lower and Upper Lake Zurich could correspond to Ruttner’s “ecotypes”.

Abiotic factors such as light, temperature, nutrients etc. can all be important drivers of population subdivision. However, another intriguing hypothesis to test is whether chytrid parasites could be the drivers of the observed subpopulation division in the Swiss *A. formosa* populations. An interesting recent study by (Sonstebo and Rohrlack 2011) found preliminary evidence that chytrids could be responsible for the population subdivision and succession of genetically and biochemically distinct ecotypes in the cyanobacterium *Planktothrix*. Experimental studies, where isolates from the two genetically distinct subpopulations within one lake are confronted with chytrid parasite isolates from the same lake, could asses if chytrids show distinct preferences. When this would be the case, it may have implications for studying *A. formosa*-chytrid coevolution and local adaptation. When host subpopulations correspond to parasite subpopulations and one pools host and parasites from the different subpopulations together this will obscure patterns of local adaptation (Ebert and Hamilton).

Beside the subpopulation structure within lakes, we detected also considerable potential gene flow among lakes within the larger cluster. Metapopulation dynamics are thought to play an important role in the evolution and ecology of antagonistic host-parasite interactions and
dynamics of infectious disease (Gandon 2002; Thompson 2005). The relative migration rates of both host and parasite is likely to be the key factor (Gandon and Michalakis 2002). If hosts migrate much more than parasites, hosts will be locally adapted and vice versa when parasites migrate more than hosts. Population genetic studies on chytrids could clarify whether they are more (lower migration) or less structured (higher migration) than their hosts and how this may relate to host-parasite coevolution and infection dynamics in the field.

The results of this thesis show that there is considerable genotypic diversity in *A. formosa* populations within lakes and that individual genotypes differ remarkably in phenotype (i.e. cell size). As a consequence of this variability in phenotype of individual clones, the capacity to deal with anthropogenic and natural stressors gets further complicated. Therefore, the influence of herbicides and the interaction with parasites may show complex dynamics that demand further detailed insight into the system to be able to make any reliable predictions. Higher genetic diversity at local than at regional scales claims for strong local interactions, maintaining *A. formosa* diversity. Whereas the occurrence of three distinct genetic clusters on a larger scale in *A. formosa* population show that regional processes (including potential formation or coexistence of cryptic species) are at work, adding yet another layer of complexity.

References


Canter HM, Jaworski GHM (1979) Occurrence of a hypersensitive reaction in the planktonic diatom *Asterionella formosa* Hassall parasitized by the chytrid *Rhizophydium planktonicum* Canter Emend., in culture. New Phytol 82:187-


Curriculum vitae

Silke Van den Wyngaert
Date of birth: 23 November 1981
Nationality: Belgian
Languages: Dutch (native), French (fluent), English (fluent), German (fluent)

Address (privat)
Roswiesenstrasse 55
8309 Nürensdorf, Switzerland
phone: +41 76 471 65 30 8600
email: silke@uglylab.eu

Address (work)
Eawag, Aquatic Ecology
Überlandstrasse 133
Dübendorf, Switzerland
phone: +41 58 765 5616
email: silke.wyngaert@eawag.ch

University education

2008 – present  PhD thesis – Eawag (domain of ETH Zürich, Switzerland), department of Aquatic Ecology.
Thesis title: Host - Parasite - Herbicide Interactions in Lake Phytoplankton: an Eco-Evolutionary Approach
Supervisors: Dr. Piet Spaak and Dr. Bas Ibelings

2007 - 2008  Master of Science in Biology – Microbiology (University of Zürich)
Titel: Spatio-temporal dynamics of Planktothrix rubescens and heterotrophic bacteria in Lake Zurich. - Supervisor: Dr. Thomas Posch – Limnological station UZH

2003 - 2007  Bachelor of Science in Biology (University of Zürich)

2003  Licenciate in African Studies (University of Gent)

Teaching and supervision experience

2011  Supervision of a practicum student

2010  Co-supervisor of a master student:
Titel: Development of a 3D Agent-based simulation tool and its application to a host-parasite system.

2008  Teaching assistant for the aquatic microbial ecology course (Limnological station UZH): practical part on benthic an pelagic ciliates
Presentations at conferences

2011 August 12th Symposium on Aquatic Microbial Ecology (SAME), Rostock, Germany
Presentation: Parasite life history traits under environmental variation: implications for disease dynamics

2011 February 6th International Symposium on Eco-Evolutionary Dynamics, Leuven, Belgium
Presentation: “Impact of the herbicide diuron on a phytoplankton-parasite interaction and its potential to alter infection dynamics”

2010 June ASLO/NABS summer meeting, Santa Fe, New Mexico, USA
Presentation: “Effects of anthropogenic pollution on host-parasite interactions; An experimental and cellular automaton modeling approach”

2010 February Biology10 meeting, Neuchatel, Switzerland
Poster: “Effects of anthropogenic pollution on host-parasite interactions”

2009 January 3rd meeting Swiss microbial Ecology, Einsiedeln, Switzerland
Presentation: “Spatio-temporal dynamics of Planktothrix rubescens, picocyanobacteria and heterotrophic bacteria in Lake Zurich”

Scientific awards

2010 - ASLO Outstanding Student Presentation Award
2009 - Swiss Hydrobiology-Limnology Award

Others

2010 - Co-organizer of the special session “Role of Environmental Change and Other Stressors on Predation and Parasitism in Aquatic Systems”, ASLO/NABS summer meeting, Santa Fe, USA

List of publications


Website

www.uglylab.eu
Acknowledgements

The realisation of this thesis would not have been possible without the following persons crossing my path:

Bas Ibelings, who gave me the opportunity in the first place to start a PhD at Eawag. Thanks for the support during the course of my PhD.

Piet Spaak, who gave me shelter in his group and became my official Doctor-Vater. Thanks for always making sure that working conditions were as smooth and optimal as possible and for keeping your door always open for questions and advice.

Alena Gsell, my “senpai” during this PhD. She taught me all the tricks to handle Asterionella and the chytrids in the lab. Thanks for sharing the passion and frustration of science with me and for the many motivating words.

Christoph Tellenbach, the general problem solver in matters of statistics and computer issues but also a bright scientist with whom I could discuss many scientific ideas.

Claudia Buser, Markus Möst and Remo Freimann or “The G17”: It was great fun being part of this perfectly disordered chaotic office.

Maria Alp, Aline Frossard, Andi Bruder, Katja Leicht, Anja Westram, Matt Seymour, thanks for being great colleagues and friends. Your presence surely made work much more fun!

Esther Keller, for always taking time to answer any kind of theoretical and practical questions.

Jukka Jokela, who gave scientific inputs on many aspects of this thesis and Kirstin Kopp, who gave scientific inputs on population genetics.

ECO in general, a diverse and pleasant department with a very stimulating atmosphere.

I also want to thank the people from the NIOO (Nico Helmsing, Suzanne Nauz-Wieser, Roos Keijzer, Susanne Wilkens and many others) for making my two-month stay at the NIOO a very nice experience.

Thomas Posh, who introduced me in the first place into the wonderful world of aquatic microbial ecology and limnology and motivated me to continue a scientific research career.

Regula Illi, who took care for of the algae and the chytrids when I was not around.

Caroline Baumgartner and Silvana Käser who did great job in algae shaking during my holidays.

Finally I want to thank my family for their continuous support, Olivia Buser for mental support and last but not least my husband Olivier for not letting me forget that “la vie est belle”.

119