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

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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 (Jokela *et al.*, 2003; Decaestecker *et al.*, 2007; Duncan and Little, 2007). 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, b) 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 and Lund, 1948; Canter, 1950; 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 rainfall (Rabiet *et al.*, 2010). This is also the time when phytoplankton spring blooms develop and chytrid epidemics occur (Ibelings *et al.*, 2004). We therefore believe that this stressor interaction may significantly affect phytoplankton population dynamics and disease in the field. 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: (i) Do host genotypes show variation for parasite resistance and herbicide resistance traits? If so, are these two traits correlated? (ii) How does herbicide exposure interact with parasite stress: is the nature of the interaction additive, synergistic or antagonistic? (iii) 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 × parasite and between both fixed factors and the covariate host population cell volume. Variation explained by the random factor host genotype compared with 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 with 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).

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 analysed 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). 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 (Fig. 2A and B). This trade-off

Table 1. Mixed-effects model to test for the effects of the single and combined stress treatments on average host population growth rate.

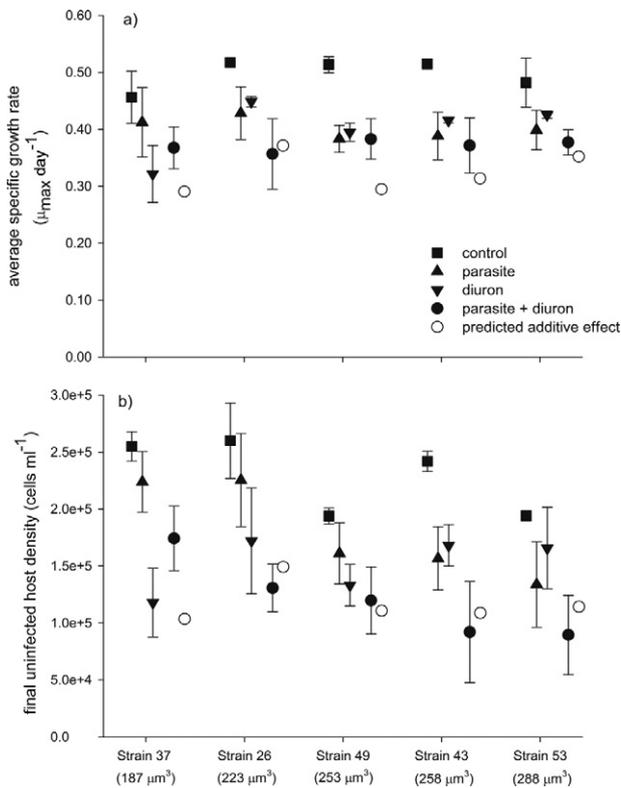| Host population growth rate	Estimate	Standard error	*t*-value	pMCMC
Intercept	0.4204	0.0593	7.093	0.0001
Diuron (D)	−0.2062	0.0569	−3.621	0.0014
Parasite (P)	0.0524	0.0621	0.843	0.4260
Cell volume (CV)	0.0003	0.0002	1.309	0.2686
D × P	0.0645	0.0173	3.731	0.0008
D × CV	0.0005	0.0002	2.010	0.0600
P × CV	−0.0006	0.0003	−2.414	0.0246

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.

pattern, mediated by host cell volume, was expressed strongest in the response variable percentage reduction in final uninfected host density (Fig. 2B).

Parasite fitness

Parasite population growth rate increased significantly in all host populations exposed to diuron (Table 3, Fig. 3) and was positively related to mean host population cell

Table 2. Mixed-effects model to test for the effects of the single and combined stress treatments on final uninfected host density.

Final uninfected host density:	Estimate	Standard error	*t*-value	pMCMC
Intercept	461844.0	121025.5	3.816	0.0004
Diuron (D)	−392947.7	104548.2	−3.759	0.0002
Parasite (P)	−36263.3	87471.3	−0.415	0.6589
Cell volume (CV)	−897.2	495.8	−1.810	0.0087
D × P	295654.3	123703.0	2.390	0.0130
D × CV	1225.7	428.3	2.862	0.0032
P × CV	−118.2	358.3	−0.330	0.7253
D × P × CV	−1066.9	506.8	−2.105	0.0278

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 *post hoc* analysis revealed a significant difference between parasite growth rate infecting host populations with the smallest and largest mean cell volume ($P < 0.011$).

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 with the non-diuron treatment (Fig. 4C).

Discussion

In this study we investigated how abiotic and biotic stressors interact, resulting in environment by environment (E × 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 × E and G × E × 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 × 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).
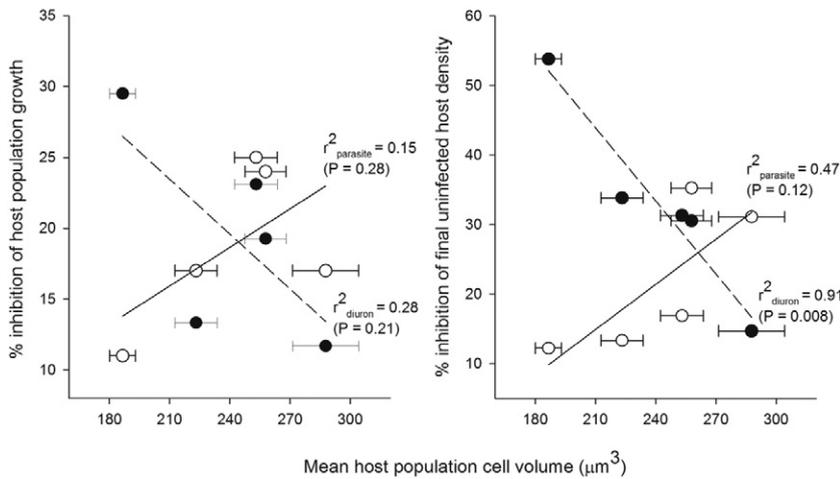
Fig. 2. Relationship between host population cell volume (mean ± SE) and host fitness inhibition.
A. % growth reduction.
B. % reduction in final uninfected host density. The broken line shows linear regression analysis for the stressor diuron (black circles) and the unbroken line shows linear regression analysis for the stressor parasite (white circles).

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

Table 3. Mixed-effects model to test for the effect of diuron exposure on parasite population growth rate.

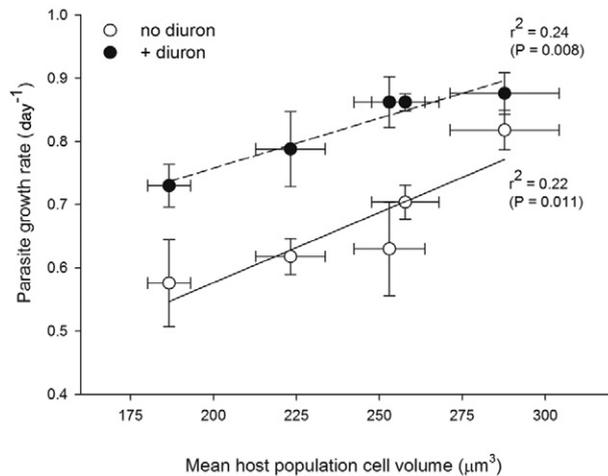| Parasite population growth rate:	Estimate	Standard error	*t*-value	pMCMC
Intercept	−0.8175	0.7452	−1.097	0.342
Diuron (D)	0.5656	0.0969	5.835	0.001
Cell volume (CV)	0.0124	0.0030	4.086	0.004

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).

genetically differentiated based on AFLP marker analysis – was presumably too low to detect differences in susceptibility to parasitism. The study of De Bruin and colleagues (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 × 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 per cent 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 (Tang *et al.*, 1998; Lockert *et al.*, 2006). 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
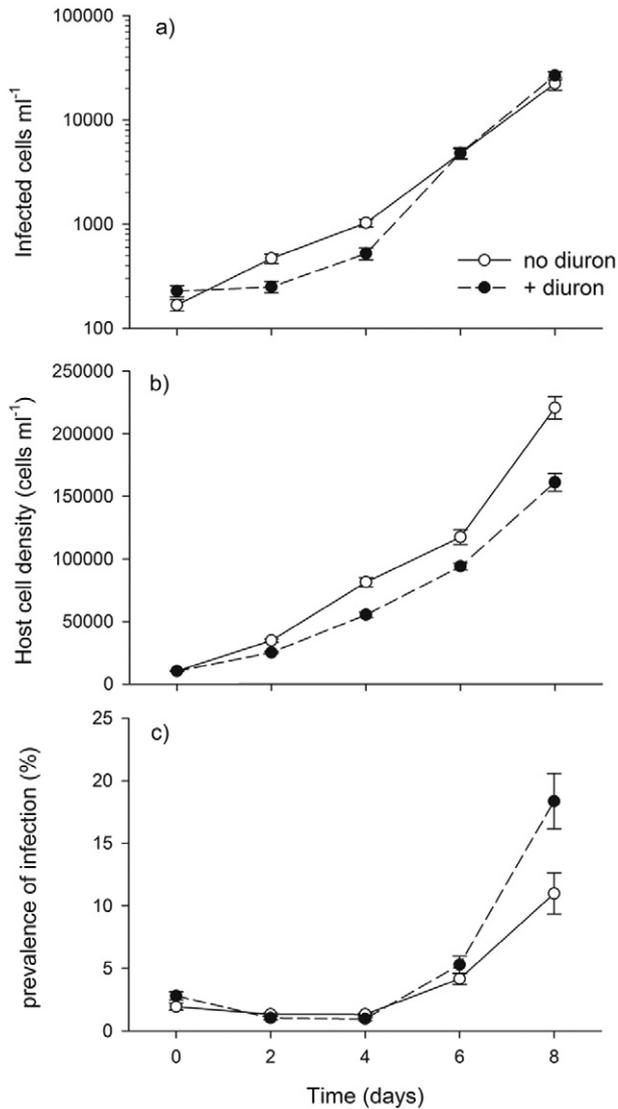
Fig. 4. Comparison of (A) the increase of infections (mean ± SE), (B) host density (mean ± SE) and (C) prevalence of infection (mean ± SE) between the parasite exposed treatments in the absence of diuron (white circles) and presence of diuron (black circles). Data pooled together for all five host populations.

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 *Rhizophydium 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 com-

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 (Hall *et al.*, 2007; Duffy *et al.*, 2011) 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

bined 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 with 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 (Kuhn, 1997; Gerardo *et al.*, 2006). Previous experiments have shown that zoospores are unable to infect *A. formosa* in darkness or at very low irradiance (Canter and Jaworski, 1981; Bruning, 1991b). 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 *c.* 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 (Davis *et al.*, 1976; Altman and Campbell, 1977) 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 with 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 *A. 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. *Zygorhizidium 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°14′28.28″N, 5°08′57.11″E, The Netherlands). We conducted a full factorial experiment with five different host genotypes (based upon AFLP fingerprinting, Gsell *et al.*, 2012), one parasite genotype and four treatments: (i) stress-free (positive control), (ii) diuron, (iii) parasite, and (iv) 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 c. 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 = $ length $\times$ (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 ml⁻¹ and sporangia ml⁻¹ respectively (Guillard, 1973):

$$K' = \ln(N_2/N_1)/(t_2 - t_1)$$

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 with 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 analysed by a linear mixed effect model (lmer 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 analyse 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.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Daily measurements of photosynthetic yield for all treatments in the three *A. formosa* strains over the experimental period (7 days).

Fig. S2. Fitted dose-response curves show the variation in (A) photosynthesis inhibition and (B) growth inhibition (mean $\pm$ SD) among three different *A. formosa* strains after seven days of diuron exposure. The EC_{50} values for each strain are back transformed.